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PRINCIPAL INVESTIGATOR: David E. Millhorn, Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45267-0553

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David E. Millhorn, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cincinnati
Cincinnati, Ohio 45267-0553
email - David.Millhorn@UC.Edu

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The primary objective of this research project is to determine the role of the mitogen-activated protein kinase (MAPK) pathways (specifically p38 kinase) in mediating the cellular response to hypoxia-stress. The overall scope of this project is to understand how neurons adapt to chronic hypoxia. The neural-like PC12 cell line is used as a model system to identify the molecular mechanisms that mediate tolerance to hypoxia. The inability to develop tolerance can lead to neurodegeneration and possibly cell death. Our work on this project resulted in the publication of 4 original papers, 1 review paper, and 1 book chapter. We found that exposure to prolonged hypoxia activates the p38α and p38γ isoforms, but not the p38β or p38δ isoforms of the p38 kinase pathway. We showed that the down-stream targets of these p38 kinase isoforms are cyclin D1 and a cyclin A-like molecule. We propose that activation of these cyclins during hypoxia stimulates cell proliferation and might protect neurons in the intact nervous system against damaging effects of hypoxia. We also discovered that the activation of the hypoxia-induced transcription factor, EPAS1, is regulated by the p42/p44 MAPK pathway, but in a manner that is independent of ras but dependent on calcium/calmodulin.

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5. INTRODUCTION

Neurons within the central nervous system are highly dependent upon aerobic metabolism. Reduced oxygen (hypoxia/ischemia) can lead to neuronal dysfunction, and if the exposure is prolonged and severe it can cause neurodegeneration. The long-range goal of our research is to identify and characterize the signal transduction pathways and genes that promote neuroprotection against hypoxia stress. We hypothesize that dysfunction of these pathways during hypoxia can lead to neuronal damage αvδ death. The specific objective of the current project is to determine the role of the mitogen- and stress-activated signal transduction pathways in regulating the cellular response to hypoxia. Our studies are performed on a clonal cell line that was derived from pheochromocytoma tumors. Because these cells (PC12) display many characteristics that common to neurons, we use them as a cellular model system for studying hypoxia-induced signal transduction and gene regulatory.

6. BODY

During the past year (year 2) we continue our research on the MAPK pathways (Objectives 1 and 2) as well as initiate work on the Kv1.2 potassium (Objective 3) in regulating the cellular response to hypoxia as described in the Statement of Work. A summary of the findings from this research is provided below. Detailed descriptions of this research are provided in the attached journal articles.

Study 1. One of the early events in O2 chemosensitivity in neurons is inhibition of an O2-sensitive K channel. Characterization of the molecular composition of the native O2-sensitive K channels in chemosensitive cells is important to understand the mechanisms that couple cellular function to O2 tension. To gain insights into how cells respond to reduced O2 tension, we studied the function of the Kv1.2 channel in the O2-sensitive PC12 cell line. Whole-cell patch clamp recordings showed that the O2-sensitive K current in PC12 cells is inhibited by charybdotoxin, a blocker of Kv1.2 channels. PC12 cells express the Kv1.2 α-subunit of K channels: Western blot analysis with affinity-purified anti-Kv1.2 antibody revealed a band a 80Kd. Specificity of this antibody was established in Western blot and immunohistochemical studies. Anti-Kv1.2 dialysed through the patch pipette completely blocked the KO2 current, while the anti-Kv2.1 and irrelevant antibodies had no effect. The O2 sensitivity of recombinant Kv1.2 and Kv2.1 channels was studied in Xenopus oocytes. Hypoxia inhibited the Kv1.2 current only. These findings show that the KO2 channel in PC12 cells belongs to the Kv1 subfamily of K channels and that the Kv1.2 α-subunit is important in conferring O2 sensitivity to this channel.

This study was published in the Journal of Physiology 524:783-793, 2000. This article is attached.

Study 2: The p38 signalling pathway is part of the MAPK superfamily and is activated by various stressors such as hypoxia. Our previous studies revealed that PC12 cells express two P38 isoforms that are activated by hypoxia. PC12 cells also synthesize and secrete catecholamines, including dopamine, in response to hypoxia. We have now used this system to study the interaction between D2-dopamine receptor signaling and the p38 stress-activated protein kinases. Our results show that two D2 receptor antagonists, butachamol and sulpiride, enhance hypoxia-induced phosphorylation of p38γ, but not p38α. This effect persists in protein kinase A (PKA)-deficient PC12 cells, demonstrating that p38γ modulation by the D2 receptor is independent of the cAMP/PKA pathway. We further whow that removal of extracellular calcium blocks the hypoxia-induced increase in p38γ activity. These results are the first to demonstrate that p38γ can be regulated by the D2 receptor and calcium following hypoxic exposure.

This study was published in Cellular Signalling 12: 463-467, 2000. This article is attached.

Study 3: The Effects of hypoxia on the stress- and mitogen-activated protein kinase (SAPK and MAPK) signaling pathways were studied in PC12 cells. Exposure to moderate hypoxia (5% O2) was found to progressively stimulate phosphorylation and activation of p38γ in particular, and also p38α, two isoforms of the p38 family of stress activated protein kinases. In contrast, hypoxia had no effect on enzyme activity of p38b, p38d or JNK, another stress-activated protein kinase. Prolonged hypoxia also induced phosphorylation and activation of p42/p44 MAPK, although this activation was modest when compared to NGF and UV-induced activation. We further showed that activation of p38g and MAPK during hypoxia requires calcium, as treatment with Ca free media or calmodulin antagonists blocked the activation of p38g and MAPK. These studies demonstrate that an extremely typical physiological stress (hypoxia) causes selective activation of specific elements of the SAPKs and MAPKs, and identifies Ca/Calmodulin as a critical upstream activator.

This study was published in Oxygen Sensing: Molecule to Man, edicted by S. Lahiri, Kluwer Academic Press/Plenum Publishers, 2000. This article is attached.

Study 4: Transplantation of dopamine-secreting cells harvested from fetal mesencephalon directly into the striatum has had limited success as a therapy for Parkinson's Disease. A major problem is that the majority of the cells dies during the first 3 weeks following transplantation. Hypoxia in the tissue surrounding the graft is a potential cause of the cell death. We have used subtractive cDNA libraries and microarray analysis to identify the gene expression profile that regulates tolerance to hypoxia. An improved understanding of the molecular basis of hypoxia-tolerance may allow investigators to engineer cells that can survive in the hypoxic environment of the brain parenchyma following transplantation.

This study was published in Parkinson's and Related Disorders 7: 273-281, 2000. This article is attached.

Study 5: We investigated the effect of hypoxia on glutamate metabolism and uptake in rat pheochromocytoma (PC12) cells. Various key enzymes relevant to glutamate production metabolism and transport were coordinately regulated by hypoxia. PC12 cells express two glutamate-metabolizing enzymes, glutamine synthetase (GS) and glutamate decarboxylase (GAD), as well as the glutamate-producing enzyme, phosphate-activated glutaminase (PAG). Exposure to hypoxia for 6h or longer increased expression of GS mRNA and protein and enhanced GS enzymatic activity. In contrast, hypoxia led to an increase in GAD65 and GAD67 protein levels and GAD enzymatic activity. PC12 cells express three Na-dependent glutamate transporters; EAAC1, GLT-1 and GLAST. Hypoxia increased EAAC1 and GLT-1 protein levels, but had no effect of GLAST. Chronic hypoxia significantly enhanced the Na-dependent component of glutamate transport. Furthermore, chronic hypoxia decreased cellular content of glutamate, and increased cellular glutamine. Taken together, the hypoxia-induced changes in enzymes related to glutamate metabolism and transport are consistent with a decrease in extracellular concentration of glutamate. This may have a role in protecting PC12 cells from the cytotoxic effects of glutamate during chronic hypoxia.

This study was published in the Journal of Neurochemistry 76: 1935-1948, 2001. This article is attached.

A detailed description of protocols, methods and experimental approaches is provided in the attached journal articles. Statistical application and data analysis is also provided in the attached articles.

7. KEY RESEARCH ACCOMPLISHMENTS:

- First evidence that the p38 kinase signal transduction pathway is activated by hypoxia.
- Activation of the p38 kinase pathway is isoform specific; only the α and γ isoforms of this enzyme are activated by hypoxia.
- Cyclin D1 is inhibited by hypoxia via the p38 kinase pathway.
- We were first to show that the mitogen-activated protein kinase (MAPK) is also activated by hypoxia in PC12 cells. (The MAPK system is a parallel system to the p38 kinase pathway).
- We were first to show that the hypoxia induced transcription factor EPAS1 is phosphorylated during hypoxia and that this phosphorylation is leads to transactivation of genes that contain the HRE sequences.
- We also showed that the calcium/calmodulin pathway interacts with the MAPK pathway to phosphorylate and activate EPAS 1

8. REPORTABLE OUTCOMES:

- Four full journal and two full review articles and a book chapter were published during the past year. These are provided in the appendix to this progress report.

9. CONCLUSIONS:

Significant progress was made on all objectives of the Statement of Work. We further characterized the roles of the stress-activated and mitogen-activated protein kinase pathways in regulating the cellular response to hypoxia. We also demonstrated that the glutamate synthesizing pathways and membrane transporters are regulated in PC12 cells during chronic hypoxia exposure. This regulation leads to decreased extracellular concentrations of glutamate, which is a cellular excito-toxin. We propose that the regulation of these pathways and glutamate synthesis and release protects cells against the harmful effects of hypoxia. In addition, we showed that the Kv1.2 potassium channel regulates membrane potential during hypoxia, and that the a-subunit is primarily responsible for this important function. Our research provides important insights concerning the molecular mechanisms involved in tolerance to hypoxia, which may play an important role in neurodegenerative diseases. Finally we have begun gene expression profile studies which will give additional insights concerning the broad spectrum of genes that regulate hypoxia tolerance.

10. REFERENCES (Journal of Biological Chemistry format)

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11. APPENDICES:

(see attached journal articles)

Journal of Neurochemistry, 2001, **76**, 1935–1948

Hypoxia regulates glutamate metabolism and membrane transport in rat PC12 cells

Shuichi Kobayashi and David E. Millhorn

Department of Molecular and Cellular Physiology, University of Cincinnati, USA

Abstract

We investigated the effect of hypoxia on glutamate metabolism and uptake in rat pheochromocytoma (PC12) cells. Various key enzymes relevant to glutamate production, metabolism and transport were coordinately regulated by hypoxia. PC12 cells express two glutamate-metabolizing enzymes, glutamine synthetase (GS) and glutamate decarboxylase (GAD), as well as the glutamate-producing enzyme, phosphate-activated glutaminase (PAG). Exposure to hypoxia (1% O_2) for 6 h or longer increased expression of GS mRNA and protein and enhanced GS enzymatic activity. In contrast, hypoxia caused a significant decrease in expression of PAG mRNA and protein, and also decreased PAG activity. In addition, hypoxia led to an increase in GAD65 and GAD67 protein levels and GAD enzymatic activity. PC12 cells express three Na^+-dependent glutamate transporters; EAAC1, GLT-1 and GLAST. Hypoxia increased EAAC1 and GLT-1 protein levels, but had no effect on GLAST. Chronic hypoxia significantly enhanced the Na^+-dependent component of glutamate transport. Furthermore, chronic hypoxia decreased cellular content of glutamate, but increased that of glutamine. Taken together, the hypoxia-induced changes in enzymes related to glutamate metabolism and transport are consistent with a decrease in the extracellular concentration of glutamate. This may have a role in protecting PC12 cells from the cytotoxic effects of glutamate during chronic hypoxia.

Keywords: glutamate decarboxylase, glutamate transporter, glutaminase, glutamine synthetase.

J. Neurochem. (2001) **76**, 1935–1948.

Glutamate is the principal excitatory amino acid (EAA) in the mammalian central nervous system (CNS) (Watkins and Evans 1981). Glutamatergic neurons and synapses are distributed widely throughout the CNS (Orrego and Villaneuva 1993). Glutamate has a key physiological role in various physiological and pathological processes, including learning and memory, excitotoxicity following trauma-induced brain injury, hypoxia/ischemia, and possibly neurodegenerative disorders. It is generally thought that the release of EAA such as glutamate and the subsequent activation of *N*-methyl-D-aspartate receptors play major roles in neuronal excitotoxicity and death caused by excessive accumulation of intracellular Ca^{2+} (Choi 1994; Szatkowski and Attwell 1994; Lipton 1999). If the extracellular glutamate concentration rises too high, neuronal death can result (Choi 1994; Szatkowski and Attwell 1994; Lipton 1999). Therefore, the resting concentration of extracellular glutamate must be kept very low to prevent neuronal damage.

Since high levels of extracellular glutamate have been implicated in neuronal damage in response to hypoxia, the status of glutamate metabolism during hypoxia is important. Aberrant metabolism prolongs and enhances the harmful effects of glutamate. In brain, the metabolism of glutamate is compartmentalized into two theoretical pools, a large neuronal pool and a small glial pool (Kvamme 1998; Hertz *et al.* 1999). In general, released glutamate is thought to be recaptured by nerve terminals or transported into glial cells. In glial cells, glutamine synthetase (GS) converts glutamate

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Address correspondence and reprint requests to D. E. Millhorn, Department of Molecular and Cellular Physiology, 231 Albert Sabin Way, University of Cincinnati, PO Box 670576, Cincinnati, OH 45267–0576, USA. E-mail: David.Millhorn@UC.Edu

Abbreviations used: CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; EAA, excitatory amino acid; EAAT, excitatory amino acid transporter; GABA, γ-aminobutyric acid; GAD: glutamate decarboxylase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid responsive element; GS: glutamine synthetase; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PAG, phosphate-activated glutaminase; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate.

to glutamine, which in turn enters specific neurons and serves as a precusor for the re-synthesis of glutamate via the action of phosphate-activated glutaminase (PAG). This pathway is termed the glutamate–glutamine cycle (Kvamme 1998; Hertz *et al.* 1999).

In neurons, glutamate can also be converted into γ-aminobutyric acid (GABA), by glutamate decarboxylase (GAD) (Erlander and Tobin 1991). GABA is the major inhibitory neurotransmitter in brain. There are two isoforms of GAD, GAD65 and GAD67, which are encoded by two different genes (Erlander *et al.* 1991; Michelsen *et al.* 1991). These two forms differ in their intraneural distribution, affinity for the coenzyme and in their function (Kaufman *et al.* 1991). Since GAD catalyzes the conversion of glutamate to GABA, the activity of GAD plays a role in controlling the intracellular levels of glutamate. However, virtually nothing is known about the effects of hypoxia on regulation of these enzymes.

Termination of glutaminergic neurotransmission is tightly controlled by the re-uptake of glutamate into both neurons and astrocytes (Takahashi *et al.* 1997; Palacin *et al.* 1998). Glutamate transporters help to maintain the extracellular glutamate concentration below neurotoxic levels and thereby help to prevent neuronal damage from excessive activation of glutamate receptors. To date, five members of the human family of excitatory amino acid transporters (EAAT) have been cloned (Palacin *et al.* 1998). The principal EAAT subtypes expressed in rat brain have been designated as EAAC1, GLT-1 and GLAST. The distribution of the various EAAT subtypes in brain reveals both discrete and overlapping localizations of the individual transporters (Palacin *et al.* 1998). GLT-1 and GLAST have been classified as astro-glial transporters due to their predominant and widespread expression in astrocytes. EAAC1, in contrast, is predominantly neuron-specific, with the exception of a small population of EAAC1-expressing cells that stain for glial cell fibrillary acidic protein (Palacin *et al.* 1998). Like the biosynthetic and metabolic enzymes in the glutaminergic pathway, very little is known about the effects of hypoxia on EAATs.

We have investigated the effects of hypoxia on various elements of the glutamate metabolic and reuptake systems in clonal rat pheochromocytoma (PC12) cells. PC12 cells, which are derived from chromaffin cells of the adrenal medulla, have been widely used as a model system for sympathetic ganglion-like cells (Green and Tischler 1976). We found that PC12 cells express many enzymes in the glutamate biosynthetic, metabolic and uptake pathways. Importantly, we found that exposure to hypoxia up-regulates the expression and function of GS and GADs, while concomitantly down-regulating those of PAG. Chronic hypoxia also up-regulates the expression of EAAC1 and GLT-1, but not GLAST. We further found that chronic hypoxia enhances the uptake of extracellular glutamate into PC12 cells. We propose that the regulation of glutamate metabolism and uptake play significant roles in the cellular adaptations of PC12 cells to long-term hypoxia.

Materials and methods

Cell culture

PC12 cells were purchased from American Tissue Type Collection and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) that contained 15 mM HEPES buffer, 2 mM L-glutamine, 10% fetal bovine serum, penicillin/streptomycin (100 μ/mL, 100 μg/mL). The cells were grown in an incubator with an environment of 21% O_2 and 5% CO_2 (remainder N_2) at 37°C. The medium was changed twice weekly. PC12 cells were exposed to hypoxia (1% O_2 with 5% CO_2) in an incubator (model 3159, Forma Scientific) in which the environment was maintained for the length of the experiment. One percent O_2 corresponds to the PO_2 value of 7.1 mmHg by calculation.

For a differentiation study, PC12 cells were incubated in DMEM medium which was supplemented with 50 ng/mL NGF. The supplemented medium was changed every other day.

Northern blot analysis

Northern blot analyses were performed as previously described (Kobayashi and Millhorn 1999). Briefly, total cellular RNA was extracted from PC12 cells using TRI-REAGENT (Molecular Research Center). A 20-μg aliquot of total RNA in 1 × MOPS, 0.4 M formaldehyde was heated up to 65°C for 15 min and then electrophoresed in a 1% formaldehyde gel (1 × MOPS buffer, 0.4 M formaldehyde, 1% agarose). Following electrophoresis, agarose gels were stained with SYBR Green (Molecular Probes) and the ribosomal bands were quantified using optimal density approach. The RNA was then transferred onto a nylon membrane (Hybond™ N+, Amersham) using 20 × SCC (3 mM sodium chloride/0.3 M sodium citrate) as the transfer buffer. The membranes were cross-linked using an UV cross-linker (Fisher). Membranes were stained with methylene blue to ensure quantitative transfer of the RNA to the membrane. Data from unevenly loaded membranes were discarded from further analyses. The membrane was prehybridized for 2 h in a solution (1% SDS and 0.1 M NaCl) and then hybridized overnight in a buffer (high efficiency hybridization system, Molecular Research Center) with 1.0×10^6 cpm/mL of ^{32}P-labeled probe. Following hybridization, the membranes were washed three times at 55°C in 2 × SCC/0.1% SDS, and then exposed to a storage phosphor screen (Molecular Dynamics) for 4–5 h. The screen was scanned by an optical scanner (Storm™, Molecular Dynamics), and the signals were quantified using digital image analyzing software (ImageQuaNT™, Molecular Dynamics).

The cDNA probes for GS and PAG were prepared by RT-PCR followed by ligation of the products into a plasmid vector, pCR™2.1. Two isoenzymes have been identified for PAG (Curthoys and Watford 1995; Kvamme 1998). These isoforms, corresponding to the kidney/brain type and liver type of PAG, are encoded from two different genes (Curthoys and Watford 1995; Kvamme 1998). Since PAG expressed in neuronal tissues is the kidney/brain type (Curthoys and Watford 1995), only this subtype of PAG was examined in this study. Primers were constructed

based on the reported cDNA sequence in a rat (Van de Zande *et al.* 1988; Shapiro *et al.* 1991). The sequences of primers were as follows, GS: 5′-ACC CGT ACT CTG GAC TGT GAC-3′ and 5′-GCC GAC GGT CTT CAA AGT AAC−3′ (predicted length of the amplified DNA fragment is 895 bp); PAG: 5′-TGA CCT GGG AAC TGA GTA TGT-3′ and 5′-CAG CAA ACA GGA GGT TTA TCA C-3′ (788 bp). Both PCR products were sequenced and confirmed to be identical to the reported cDNA sequences. The probe was labeled using a random-primed DNA labeling kit (Prime-A-Gene™, Promega) and 2-[α-^{32}P]-deoxycytidine 5′ triphosphate (Dupont NEN).

Reverse transcription-polymerase chain reaction

RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer Cetus). In these experiments, 3 μg of total RNA was reverse transcribed with 2.5 μM Oligo dT (16 mer) primer, 1 mM dNTPs, 1 unit/μL RNAse inhibitor, 2.5 unit/μL MuLV reverse transcriptase for 15 min at 42°C. Primers for PCR were constructed based on the reported cDNA sequence in rats (GAD65: Erlander *et al.* 1991; GAD67: Michelsen *et al.* 1991; EAAC1: Kanai *et al.* 1995; GLT-1: Pines *et al.* 1992; GLAST: Storck *et al.* 1992; glucocorticoid receptor: Miesfeld *et al.* 1986). The sequences and the predicted length of the amplified DNA products were as follows: GAD65: (5′-GCC ATC TCC AAC ATG TAC-3′) and (5′-CCA GCT CCA AAC ACT ACT TAT C-3′) (703 bp); GAD67: (5′-CAC CCG TGT TTG TTC TTA TG-3′) and (5′-GCT CCA GGC ATT TGT TGA TC-3′) (801 bp); EAAC1: (5′-TCC TGG GCC TGA TTA TCT TC-3′) and (5′-CTA AGG CCA GGC ATC TAG AAC-3′) (955 bp); GLT1: (5′-GTA TCG CCT GCT TGA TCT GTG-3′) and (5′-TGT GCG GCA TAG ACA CAC TG-3′) (754 bp); GLAST: (5′-GAA TGG CGG CCC TAG ATA G-3′) and (5′-CCG GGT TAC CAG GAA GTA GAG-3′) (707 bp); glucocorticoid receptor: (5′-CCT CTG GAG GAC AGA TGT AC-3′) and (5′-GGT TTC CGC TTG ATT GTC-3′) (876 bp).

DNA was amplified in the presence of 1.5 mM $MgCl_2$, 1 × reaction buffer, 2.5 U AmpliTaq DNA polymerase. The PCR conditions were as follows: 2 min denaturation at 94°C, followed by 35 cycles consisting of 90 s at 94°C, 1 min at 54°C (for GAD65 and glucocorticoid receptor), at 55°C (for GAD67) or at 57°C (for EAAC1, GLT1 and GLAST), and then 90 s at 72°C. The samples were then kept at 72°C for 7 min. The products of RT-PCR were analyzed by electrophoresis on 1% agarose gels and verified by sequence analysis. Amplification of G3PDH, a housekeeping gene, was also performed as a control. The primers for the rat G3PDH gene were as follows: 5′-ACC ACA GTC CAT GCC ATC AC-3′ (forward), 5′-TCC ACC ACC CTG TTG CTG TA-3′ (reverse) (452 bp).

Immunoblot analysis

Immunoblot analyses were performed as previously reported (Kobayashi and Millhorn 1999). Briefly, PC12 cells were plated on 35-mm culture dishes and exposed to hypoxia (1% O_2 with 5% CO_2) for various periods of time, as indicated. Cells were then washed with ice-cold phosphate-buffered saline (PBS) and harvested by scraping in 0.5 mL of a solution containing 0.25 M sucrose, 25 mM Tris pH 7.2, 25 mM NaCl and 5 mM $MgCl_2$. Cells were collected by centrifugation for 5 min at 3000 *g* at 4°C. The supernatant was removed by aspiration and the cells were briefly sonicated at 4°C with a microultrasonic cell disrupter (Kontes) in 0.2 mL of lysis buffer which contained 10 mM Tris (pH 7.4), 1% Triton X-100, 0.2 mM sodium vanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL leupeptin and 2 μg/mL aprotinin. The protein concentration in the sample was determined by the method of Bradford (1974).

The cellular homogenates were boiled for 2 min in sample buffer containing 50 mM Tris pH 6.7, 2% SDS, 2% β-mercaptoethanol and bromophenol blue as a marker. Samples containing 40 μg of protein were then run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schull). Membranes were blocked with 3% non-fat dry milk in a buffer containing 10 mM sodium phosphate (pH 7.2), 140 mM NaCl, and 0.1% Tween-20. Blots were then immunolabeled overnight at 4°C with antibodies against either GS (rabbit, polyclonal, 1 μg/mL), PAG (rabbit, polyclonal, 1 : 200), GAD65 (goat, polyclonal, 1 μg/mL), GAD67 (goat, polyclonal, 1 μg/mL), EAAC1 (mouse, monoclonal, 2 μg/mL), GLT-1 (rabbit, polyclonal, 1 : 1000) or GLAST (rabbit, polyclonal, 1 : 1000). Blots were then incubated with either anti-rabbit or anti-mouse secondary antibodies coupled to horseradish peroxidase at dilutions of 1 : 2000 or 1 : 5000, respectively. Immunolabeling was detected by enhanced chemiluminescence (Amersham). Immunoreactivity was quantified using densitometric analysis with an ImagePro digital analysis system (Media Cybernetics). At the dilutions of antibodies used, immunoreactivity for each protein was found to be a linear over a fivefold range of protein concentrations.

Enzyme activity assays

For enzyme activity assays, PC12 cells were plated on 35-mm culture dishes and exposed to hypoxia (1% O_2 with 5% CO_2) for various periods of time. Cellular protein was extracted in 0.4 mL of 25 mM Tris, pH 7.5, 20 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM PMSF, and 0.4 mg/mL Pefabloc SC (Boehringer Manheim), using a microultrasonic cell disrupter (Kontes). Cellular homogenates were then centrifuged at 20 000 *g* for 20 min at 4°C. Supernatants were used for GS, PAG and GAD enzymatic assays.

Enzyme activity GS was assayed as previously reported (Pishak and Phillips 1979). GS activity was evaluated as conversion of [^{14}C]-glutamate to [^{14}C]-glutamine. GS activity was assayed in a final volume of 25 μL containing 50 mM imidazole-HCl, pH 6.8, 15 mM $MgCl_2$, 10 mM ATP, 10 mM L-[U-^{14}C]glutamate (200 mCi/mmol), 4 mM NH_4Cl, 1 mM 2-mercapotethanol and 50 μg of cellular protein. The reaction proceeded for 1 h at 37°C in a shaking water bath. Reactions were stopped by adding 100 μL of ice-cold distilled water and placing the tubes immediately on ice for a period not exceeding 15 min 15 μL of supernatant was analyzed on Kodak cellulose-coated plastic TLC sheets (Kodak) in 2-propanol:formic acid:H_2O (40 : 2 : 10) (Jones and Heathcote 1972). The sheets were then exposed to a storage phosphor screen (Molecular Dynamics) for 48 h. The screen was scanned by a phosphorimager (Storm™, Molecular Dynamics), and the radioactive spots corresponding to glutamine and glutamate were quantified using digital image analysis software (ImageQuaNT™, Molecular Dynamics).

PAG activity was analyzed as previously reported (Collins *et al.* 1994). PAG activity was estimated as conversion of [^{14}C]-glutamine to [^{14}C]-glutamate. PAG activity was assayed in a final volume of 25 μL containing 37.5 mM potassium phosphate buffer, pH 8.2,

0.25 mM KCN and 15 mM L-[U-^{14}C]glutamine (182 mCi/mmol). The reaction proceeded for 60 min at 30°C in a shaking water bath. The blank contained extraction buffer in place of cell extract. The reaction was terminated and transferred to Kodak cellulose-coated plastic TLC sheets as above. The sheets were then quantified by phosphorimager, as described for the GS enzyme activity assay.

GAD activity was analyzed as previously reported (Tursky and Bandzuchova 1999). The amount of [^{14}C]GABA formed from U[^{14}C]-glutamate was determined. The reaction was performed in a volume of 70 μL in the following medium: 50 mM HEPES, pH 6.8, 20 μM pyridoxal 5′-phosphate, 5 mM L-glutamate, pH 6.8, with U[^{14}C]-glutamate (200 mCi/mmol), and 100 μg cell protein. The reaction was carried out under an atmosphere of nitrogen in a shaking water bath at 37°C for 60 min. Reactions were stopped by the addition of 50 μL of 2.5% trichloracetic acid. The reaction mixture was quantitatively transferred onto a Dowex 1 acetate column (3 × 50 mm). GABA was eluted with 2.5 mL of double-distilled water into glass scintillation vials with a filter paper circle at the bottom. After evaporation of the water at 70°C, [^{14}C]-GABA levels on the filter papers were determined by liquid scintillation counting. The reaction mixture, which was deproteinated prior to the addition of U[^{14}C]-glutamate, was used to estimate blank values.

As preliminary studies, we measured the enzyme activities for GS, PAG and GAD at three different incubation times (30, 60 and 90 min), and found that the activity for each enzyme was linear with time over these time points.

Measurement of Na^+-dependent glutamate transport experiments

The uptake of L-[^{3}H]-glutamate into PC12 cells was determined as previously reported (Dunlop *et al.* 1999). PC12 cells were initially grown in 35-mm dishes under normoxia. When cell confluency reached 70%, the dishes were either maintained in normoxia or transferred to an incubator set to maintain 1% O_2. At the end of the incubation period (12 or 24 h of either normoxia or hypoxia), the cells were washed twice with 2 mL of either prewarmed (37°C) sodium containing HEPES-buffered solution (140 mM NaCl, 1.2 mM K_2HPO_4, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 5 mM Tris base, pH 7.4) or choline containing solution (equimolar replacement of sodium with choline). PC12 cells were then incubated at 37°C with HEPES-buffered solution containing 50 μM L-[^{3}H]-glutamate (60 Ci/mmol) for 10 min under normoxia. Uptake was terminated by placing the culture dishes on ice and rapidly removing the radioactive medium, followed by three washes with ice-cold choline-buffer. The cells were removed from the dishes by scraping with a thin plastic sheet into 500 μL of 1 M NaOH solution, and then transferred to test tubes where they were disrupted by vortexing. Liquid scintillation counting was used to analyze levels of [^{3}H]-glutamate in 350 μL aliquots of lysate. Uptake rates were calculated from the uptake of L-[^{3}H]-glutamate into the cells and the specific activity of the medium. The results were expressed as nmol/mg protein/10 min. Na^+-dependent uptake was defined as the difference in radioactivity accumulated in Na^+-containing buffer and in choline-containing buffer.

As a preliminary study, we examined the glutamate transport activity in the presence and absence of Na^+ at three different incubation times (5, 10 and 15 min), and found that the activity was linear with time over this period.

Analysis of cellular glutamate and glutamine content in PC12 cells

PC12 cells were plated on 60-mm dishes and exposed to normoxia or 1% O_2 for 12 and 24 h. At the end of each incubation period, cells were mechanically harvested in 350 μL of ice-cold PBS, placed into separate tubes and homogenized at 4°C with a micro-ultrasonic cell disrupter. Aliquots were reserved for the measurement of protein by the method of Bradford. Immediately after sonication, 300 μL aliquots of the samples were deproteinated with 15 μL 100% trichloracetic acid. Samples were centrifuged at 25 000 *g* for 5 min, and 250 μL of the deproteinated supernatant were immediately neutralized with 30 μL of 3.3 N potassium hydroxide.

The amount of cellular glutamate and glutamine in PC12 cells was measured as previously reported (Lund 1986). All assay reagents were purchased as a kit (Sigma, glutamine/glutamate determination kit) and prepared according to the manufacture's instructions. Determination of L-glutamine is a two step reaction; first, L-glutamine is deaminated to L-glutamate. Second, L-glutamate is dehydrogenated to α-ketoglutarate, which is accompanied by the reduction of NAD^+ to NADH. Aliquots of 250 μL of samples were first incubated for 1 h at 37°C in a 0.1-M acetate buffer, pH 5.0, in the presence (sample GLN and GLU) or absence (sample GLU) of 1 U/mL glutaminase in a total volume of 1 mL. Aliquots of 500 μL of these samples were then incubated for 40 min at room temperature in a buffer containing 50 μM Tris, 1 μM EDTA, 1.6 μg/mL hydrazine, 1.5 mM NAD, 0.5 mM ADP and 12 U/mL glutamic dehydrogenase in a total volume of 1 mL. After this incubation, optical absorbance at 340 nm was measured spectrophotometrically to evaluate the conversion of NAD^+ to NADH, and thus the amount of glutamate that was oxidized. The endogenous L-glutamate concentration (sample GLU) was determined based on a standard curve using L-glutamine. The L-glutamine concentration was calculated by the difference between the endogenous L-glutamate concentration and the total L-glutamate concentration (sample GLN and GLU). The results were expressed as nmol/mg protein. Data were accumulated from five independent experiments.

Data analysis

The results were expressed as averages ± SEM (*n*: number of observations). Analysis of variance followed by Student's *t*-test was used for evaluating the significance of the obtained data. Statistical significance was accepted at the conventional $p < 0.05$ level by two-tail evaluation.

Materials

L-[^{14}C]-Glutamate (200 mCi/mmol), L-[^{14}C]-glutamine (182 mCi/mmol), [^{3}H]-glutamate (60 Ci/mmol) were purchased from Moravek Biochemicals. The primary antibody for rat EAAC1 was purchased from Chemicon International. Primary antibodies for GS, GAD65 and GAD67 were purchased from Santa-Cruz. Primary antibodies for GLT-1 and GLAST were purchased from Abcam. Primary antibody for PAG was a generous gift by Dr Curthoys (Department of Biochemistry and Molecular Biology, Colorado State University, CO).

Results

Exposure to hypoxia enhances the expression and function of glutamine synthetase in PC12 cells

Glutamine synthetase (GS) converts glutamate to glutamine. northern blot analysis was used to determine if hypoxia regulates gene expression for GS in PC12 cells. In Fig. 1(a), it can be seen that progressive exposure to hypoxia (1% O_2 for 3, 6, 12, 18 or 24 h) gradually increased GS mRNA expression in PC12 cells. This effect was statistically significant at exposure times of 6 h and longer periods of hypoxia, and was maximal at 12 h. Figure 1(b) shows that the amount of GS protein, as determined by immunoblot analysis, was also significantly increased after 6 h of exposure to 1% O_2.

We also tested the effect of hypoxia on GS enzyme activity in PC12 cells. Cells were incubated under normoxia or 1% O_2 for 6, 12 and 24 h, and the GS activity was measured. The GS activity was evaluated as conversion of [^{14}C]-glutamate to [^{14}C]-glutamine. As shown in Fig. 1(c), we found that hypoxia significantly increased the GS activity after hypoxic exposure lasting 12 h ($p < 0.01$). Thus, the mRNA and protein expression and enzymatic activity for GS were all increased in PC12 cells during hypoxia. This is consistent with an enhanced conversion of glutamate to glutamine, which in turn would decrease the intracellular levels of glutamate.

Chronic exposure to hypoxia down-regulates phosphate-activated glutaminase in PC12 cells

Phosphate-activated glutaminase (PAG) produces glutamate from glutamine. We performed northern blot and immunoblot analyses to determine the effect of hypoxia on PAG gene and protein expression in PC12 cells. The upper panel in Fig. 2(a) shows the effect of progressive exposure to hypoxia (1% O_2) on PAG mRNA in PC12 cells. It can be seen that the level of PAG mRNA gradually decreased with prolonged hypoxia. The lower panel shows that the mean

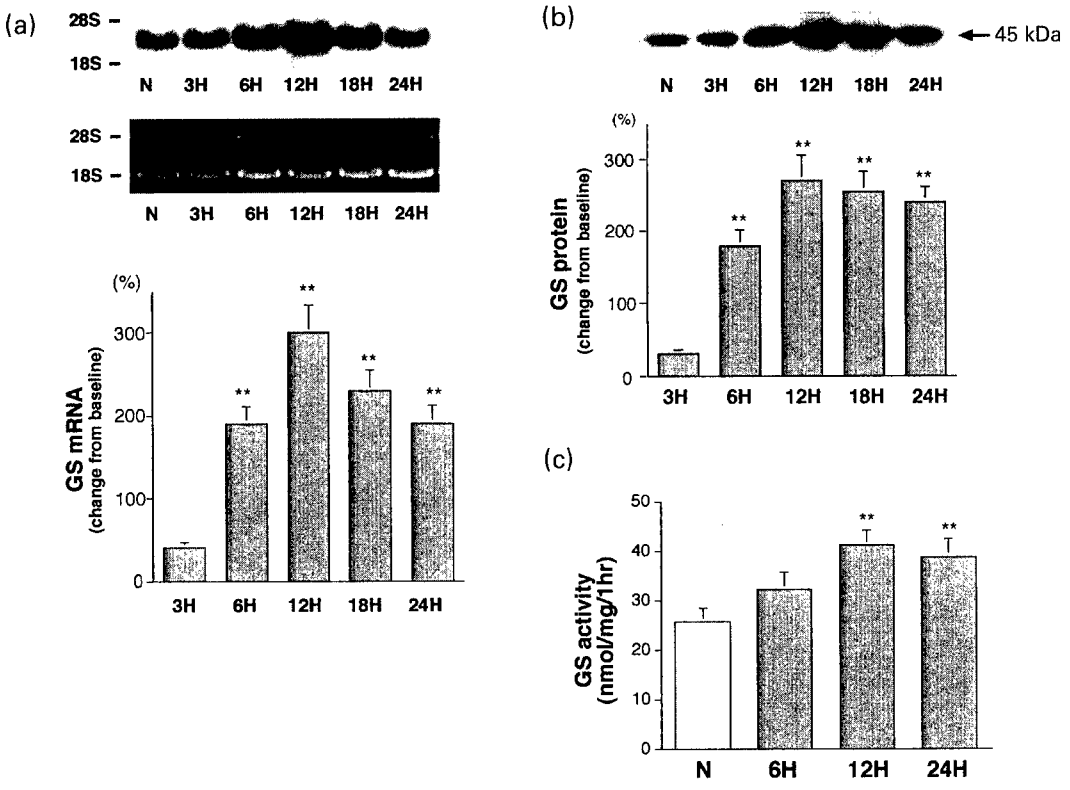

Fig. 1 Hypoxia enhances the expression and function of glutamine synthetase in PC12 cells. (a) The upper panel is a representative northern blot showing the effects of hypoxia on GS mRNA levels. PC12 cells were exposed to either normoxia (N) or 1% O_2 for various times, between 3 and 24 h, as indicated. The lower panel shows the average percentage change from baseline ± SEM (**$p < 0.01$, $n = 5$ for each group). (b) The upper panel is a representative immunoblot showing the effect of hypoxia on GS protein levels. PC12 cells were exposed to either normoxia (N) or 1% O_2 for various times, between 3 and 24 h, as indicated. The lower panel shows the average percentage change from baseline ± SEM (**$p < 0.01$). (c) The effect of hypoxia on GS enzyme activity in PC12 cells. PC12 cells were exposed to either normoxia (N) or 1% O_2 for 6, 12, or 24 h, as indicated. GS enzyme activity was measured as conversion of [^{14}C]-glutamate to [^{14}C]-glutamine. Data are expressed as average activity (nmol/mg/h) ± SEM, with $n = 5$ in each group (**$p < 0.01$, $n = 5$).

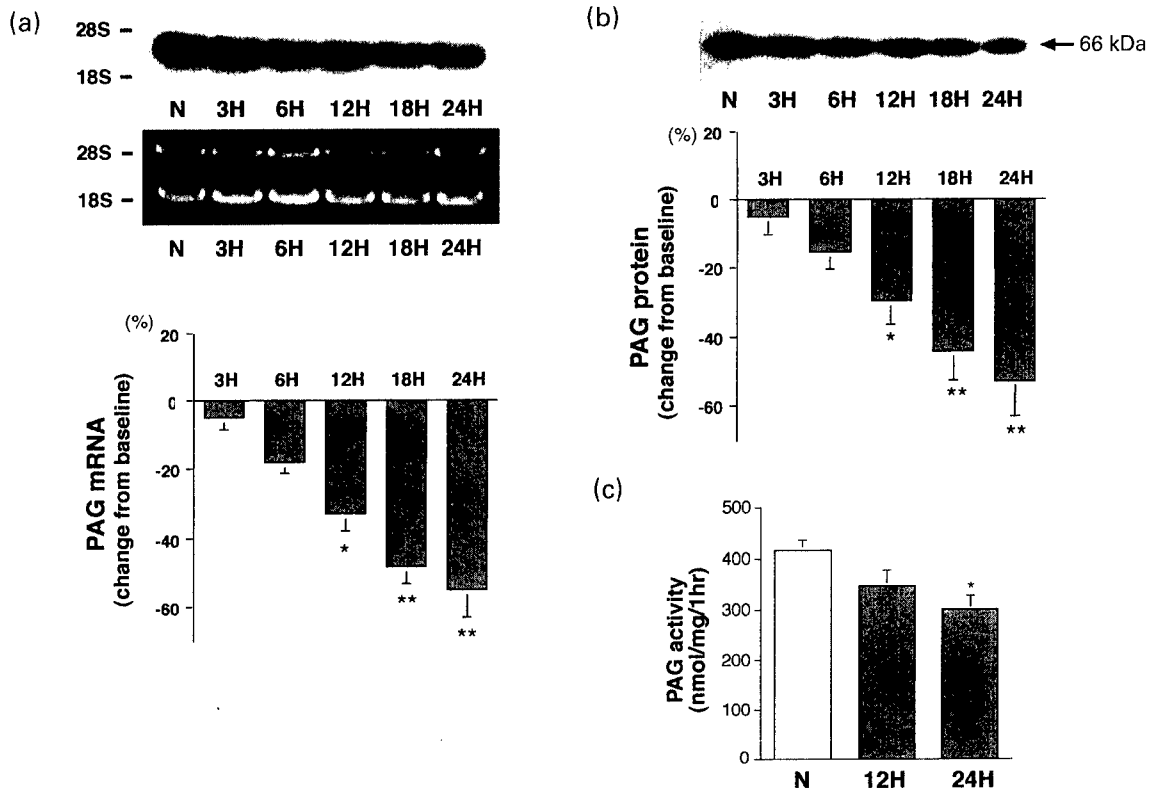

Fig. 2 Chronic hypoxia down-regulates phosphate-activated glutaminase in PC12 cells. (a) The upper panel is a representative northern blot showing the effects of hypoxia on PAG mRNA levels. PC12 cells were exposed to either normoxia (N) or 1% O_2 for various times, between 3 and 24 h, as indicated. The lower panel shows the average percentage change from baseline ± SEM (**$p < 0.01$, $n = 5$ for each group). (b) The upper panel is a representative immunoblot showing the effect of hypoxia on PAG protein levels. PC12 cells were exposed to either normoxia (N) or 1% O_2 for various times, between 3 and 24 h, as indicated. The lower panel shows the average percentage change from baseline ± SEM (*$p < 0.05$, **$p < 0.01$, $n = 5$). (c) Effect of hypoxia on PAG enzyme activity in PC12 cells. PC12 cells were incubated in either normoxia (N) or 1% O_2 for 12 or 24 h. PAG enzyme activity was measured as conversion of [^{14}C]-glutamine to [^{14}C]-glutamate. Data are expressed as average activity (nmol/mg/h) ± SEM, with $n = 5$ in each group (*$p < 0.05$).

PAG mRNA level was significantly decreased after exposure to hypoxia for 12 h or longer (*$p < 0.05$, **$p < 0.01$). We next performed immunoblot analyses to measure total cellular PAG protein levels during hypoxia. Figure 2(b) shows that the amount of PAG protein also gradually decreased on a similar time course during exposure to 1% O_2 (*$p < 0.05$, **$p < 0.01$).

We also tested the effect of hypoxia on PAG enzyme activity in PC12 cells. Cells were incubated in either normoxia or 1% O_2 for 12 or 24 h, and the resulting PAG activity was measured. The PAG activity was estimated as conversion of [^{14}C]-glutamine to [^{14}C]-glutamate. PAG enzyme activity was also significantly inhibited after 24 h hypoxic exposure, as shown in Fig. 2(c) (*$p < 0.05$). Thus, mRNA and protein expression as well as enzymatic activity for PAG was inhibited during hypoxia. This would reduce the conversion of glutamine to glutamate which would make less glutamate through this pathway during chronic hypoxia.

It is known that PAG activity is inhibited by acidic pH (Curthoys and Watford 1995). We measured media pH under normoxia and during 1% O_2 for 12 and 24 h. Results show that media pH became acidic with time during 1% O_2 exposure (control: 7.31 ± 0.02, 12 h: 7.20 ± 0.03 and 24 h: 7.04 ± 0.02, $n = 4$, respectively).

Chronic hypoxia up-regulates GAD65 and GAD67 expression in PC12 cells

Glutamate decarboxylase (GAD) catalyses the conversion of glutamate to GABA. There are two known isoforms, GAD65 and GAD67, which are encoded from two different genes (Erlander *et al.* 1991; Michelsen *et al.* 1991). We examined the effect of hypoxia on gene and protein expression for GAD65 and GAD67 (Fig. 3). Since preliminary studies revealed that neither GAD65 nor GAD67 mRNA was detectable by northern blot, RT-PCR analysis was used. It can be seen that both GAD65 and GAD67 mRNA were

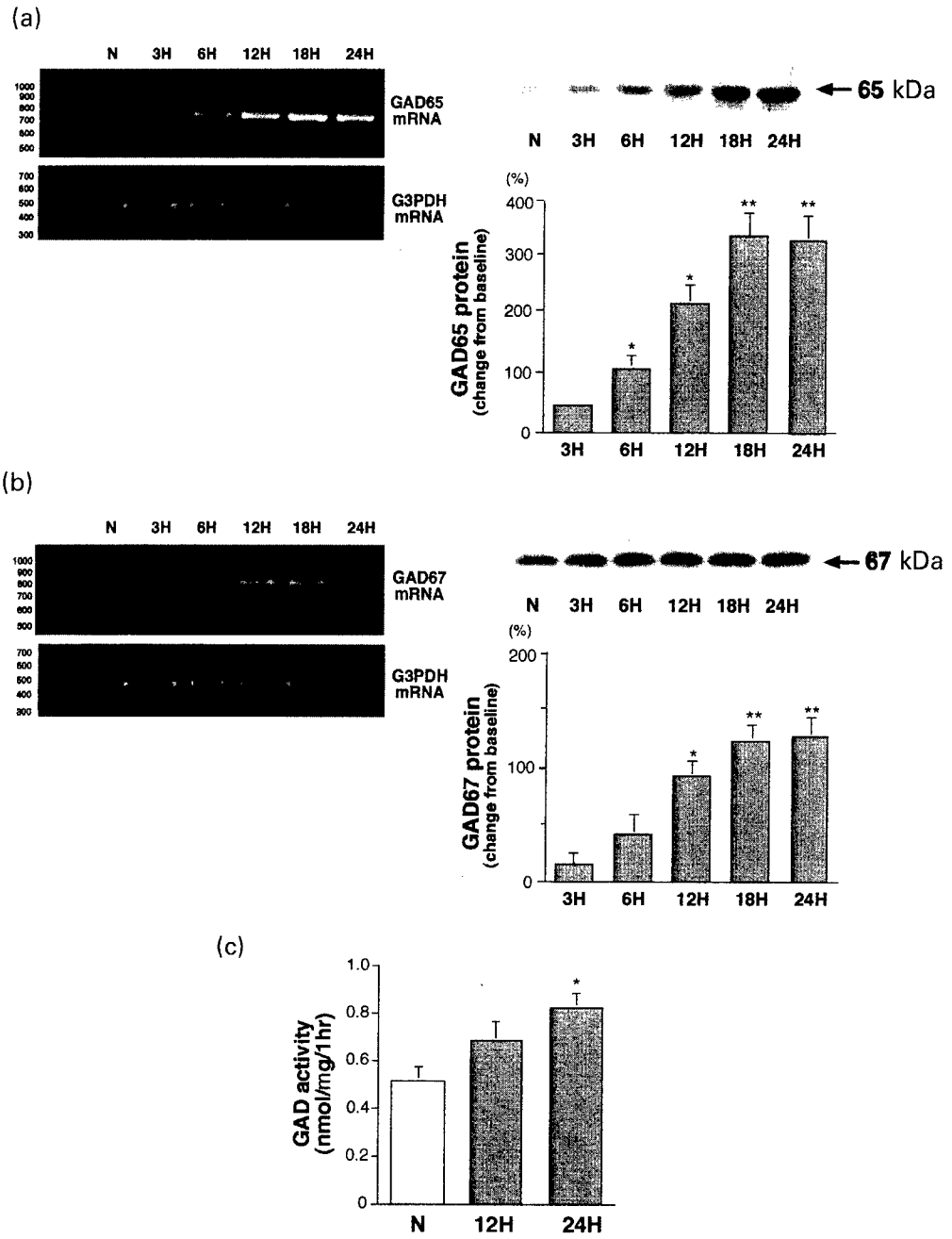

Fig. 3 Chronic hypoxia up-regulates GAD65 and GAD67 expression in PC12 cells. (a) The left panel shows the effect of hypoxia on GAD65 and G3PDH mRNA levels, as measured by RT-PCR. Ethidium bromide staining was used to visualize RT-PCR products on 1% agarose gels. The size of the marker DNA fragments in the 100 bp ladder (Promega) is shown in the left lanes. The predicted sizes of the GAD65 and G3PDH RT-PCR products were 703 bp and 452 bp, respectively. The right panel shows a representative immunoblot illustrating the effect of hypoxia on GAD65 protein levels. PC12 cells were exposed to either normoxia (N) or 1% O_2 for various times, between 3 and 24 h, as indicated. The lower panel shows the average percentage change from baseline ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, $n = 5$). (b) The left panel illustrates the effect of hypoxia on GAD67 and G3PDH mRNA levels, as measured by RT-PCR. A representative immunoblot is shown in the right panel, illustrating the effect of hypoxia on GAD67 protein levels. The lower panel shows the average percentage change from baseline ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, $n = 5$ for each group). (c) The effect of hypoxia on net GAD enzyme activity was evaluated as the conversion of [^{14}C]-glutamate to [^{14}C]-GABA. GAD activity was significantly increased after 24 h of exposure to 1% O_2 ($^{**}p < 0.01$, $n = 5$).

increased during exposure to 1% O_2 in a time-dependent manner (Figs 3a and b, left panels). In contrast, G3PDH mRNA levels remained constant for up to 24 h exposure to 1% O_2, as determined by RT-PCR.

We also performed immunoblot analysis to determine the levels of GAD65 and GAD67 proteins in total cellular homogenates. The right panel in Fig. 3(a) shows that the amount of GAD65 protein gradually increased in a time-dependent manner during exposure to 1% O_2. The effect of hypoxia on GAD65 protein was significant at 6 h and longer, and was maximally increased by 300% over basal levels at 18 h (right lower panel in Fig. 3a). GAD67 immunoreactivity was also gradually increased during hypoxia. Similar to the effect of hypoxia on GAD65, GAD67 immunoreactivity was significantly increased at 12 h and longer, and was maximally increased at 18 h (right lower panel in Fig. 3b). However, the hypoxia-induced increase in GAD65 was more marked than that of the GAD67 (about 120% increase over basal levels).

We next examined the effect of hypoxia on total GAD enzyme activity, which was evaluated as the conversion of [^{14}C]-glutamate to [^{14}C]-GABA. Figure 3(c) shows that the GAD activity was significantly increased after 24 h of exposure to 1% O_2 ($^*p < 0.05$). This is consistent with our finding that chronic hypoxia up-regulates the GAD65 and GAD67 protein levels. A reduction in GAD activity would increase the conversion of glutamate to GABA, which would reduce intracellular glutamate levels.

Hypoxia up-regulates expression for EAAC1 and GLT-1, but not that for GLAST

RT-PCR and immunoblot studies were performed to examine if hypoxia regulates glutamate transporter gene and protein expression in PC12 cells. RT-PCR studies showed that PC12 cells express EAAC1, GLT-1 and GLAST during normoxic conditions (Fig. 4). RT-PCR studies further show that hypoxia induced increases in EAAC1 and GLT-1 mRNAs, but had no effect on GLAST or G3PDH mRNA levels. The effects of hypoxia on glutamate transporter immunoreactivity were similar to those on mRNA levels. It can be seen in Fig. 4 that the protein levels for EAAC1 and GLT-1 increased gradually during hypoxia, with a maximal effect at 18 h, but that GLAST levels remained relatively stable (Figs 4a, b and c, respectively). Thus, the various glutamate transporters are differentially regulated by chronic hypoxia in PC12 cells.

We also evaluated glutamate uptake, to determine the effect of chronic hypoxia on the function of glutamate transporters. We measured the cellular uptake of [^{3}H]-glutamate from the extracellular media after 12 or 24 h of either normoxia or hypoxia. Experiments were performed in both the presence and absence of extracellular sodium in the media, to separate the Na^+-dependent and Na^+-independent components of glutamate transport. Exposure to hypoxia (1% O_2) significantly enhanced the total uptake (in the presence of sodium) of extracellular glutamate into PC12 cells at 12 and 24 h ($p < 0.05$, $p < 0.01$, respectively) (Fig. 5). However, Na^+-independent uptake (in the absence of sodium) in hypoxia-exposed cells remained at basal levels. Thus, Na^+-dependent uptake of glutamate was significantly enhanced when PC12 cells had been exposed to 1% O_2 for 12 h or longer, as shown in Fig. 5. These findings are consistent with our finding that EAAC1 and GLT-1 are up-regulated by chronic exposure to hypoxia.

The expression of glutamate transporter on PC12 cells has been previously studied (Ramachandran *et al.* 1993). They reported that glutamate transport activity and GLAST expression were found only in certain flattened cell mutants of PC12 cells. To rule out possible contamination of this flattened cells, we examined if our PC12 cells differentiate in the presence of NGF, which the flattened cells do not do. Our parent PC12 cells are of round or oval shape (Fig. 6a). In the presence of NGF, cells underwent morphologic differentiation starting on the 2nd day. The neuronal processes appeared and were further elongated day by day. Figure 6(b) shows differentiated PC12 cells which has been incubated with NGF for 7 days.

Cellular glutamate and glutamine during hypoxia in PC12 cells

PC12 cells were exposed to normoxia or 1% O_2 for 12 or 24 h and the cellular content of glutamate and glutamine were measured (Table 1). Glutamate levels were significantly reduced after 24 h exposure to hypoxia ($^{**}p < 0.01$, $n = 5$). In contrast, the amount of glutamine was significantly increased during the same period of hypoxia ($^{**}p < 0.01$, $n = 5$) (Table 1).

On the other hand, we failed to measure a significant amount of glutamate in the extracellular fluid not only under normoxia but also under 1% O_2 for 12 or 24 h (data not shown).

Table 1 Glutamate and glutamine content in PC12 cells (ng/mg protein)

	Normoxia	12 h hypoxia	24 h hypoxia
Glutamate	81.1 ± 6.3 (5)	73.3 ± 4.6 (5)	60.1 ± 4.7 (5)[a]
Glutamine	129.4 ± 6.0 (5)	144.0 ± 9.0 (5)	166.5 ± 9.6 (5)[a]

PC12 cells were plated on 60-mm dishes and exposed to normoxia or 1% O2 for 12 and 24 h. At the end of each incubation period, cells were collected and analyzed to measure the intracellular amount of glutamate and glutamine (see Materials and methods). The results were expressed as nmol/mg protein. Data were accumulated from five independent experiments. Means ± SEM are shown. [a]$p < 0.01$ from normoxia.

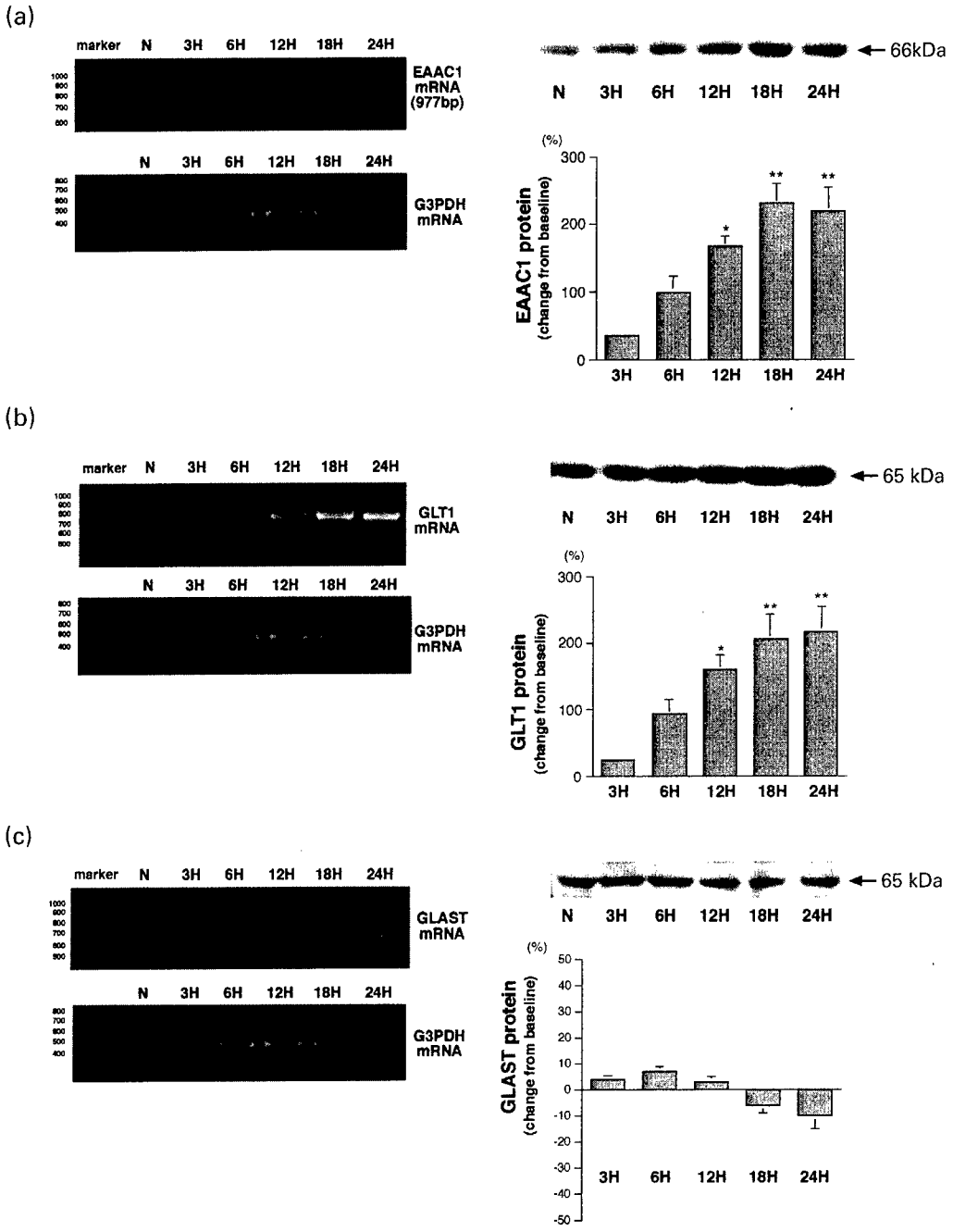

Fig. 4 Hypoxia up-regulates expression for EAAC1 and GLT1, but not that for GLAST. (a) PC12 cells were exposed to either normoxia (N) or 1% O_2 for various times, between 3 and 24 h, as indicated. Left panel: a representative RT-PCR experiment shows the effect of hypoxia on EAAC1 and G3PDH mRNA levels. The predicted product sizes for EAAC1 and G3PDH were 955 bp and 452 bp, respectively. Right panel: representative immunoblot analysis showing the effect of hypoxia on EAAC1 protein levels during hypoxia. The lower panel shows the average percentage change from baseline ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, $n = 5$). (b) Effect of hypoxia on GLT-1 mRNA and protein expression. Left panel: a representative RT-PCR experiment shows the effect of hypoxia on GLT1 and G3PDH mRNA levels. The predicted product sizes for EAAC1 and G3PDH were 754 bp and 452 bp, respectively. Right panel: representative immunoblot analysis showing the effect of hypoxia on GLT1 protein levels during hypoxia. The lower panel shows the average percentage change from baseline ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, $n = 5$). (c) Lack of effect of chronic hypoxia on GLAST mRNA and protein. Left panel: a representative RT-PCR experiment shows the effect of hypoxia on GLAST and G3PDH mRNA levels. The predicted product sizes for EAAC1 and G3PDH were 707 bp and 452 bp, respectively. Right panel: representative immunoblot analysis showing the effect of hypoxia on GLAST protein levels during hypoxia. The lower panel shows the average percentage change from baseline ± SEM ($n = 5$ at each time point).

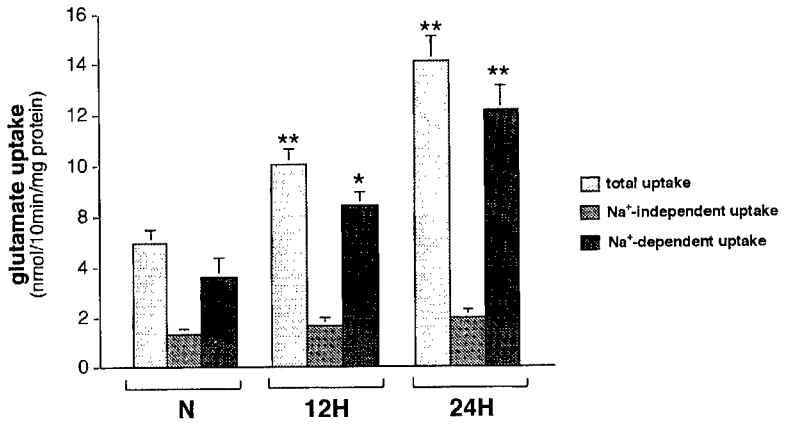

Fig. 5 Chronic hypoxia stimulates glutamate uptake into PC12 cells. [^{3}H]-Glutamate uptake was measured from the extracellular media in the presence or absence of extracellular sodium after exposure to either normoxia (N), 12 or 24 h of hypoxia (1% O_2). Exposure to hypoxia (1% O_2) significantly enhanced the total uptake (in the presence of sodium) of extracellular glutamate at 12 and 24 h ($^*p < 0.05$, $^{**}p < 0.01$, $n = 5$).

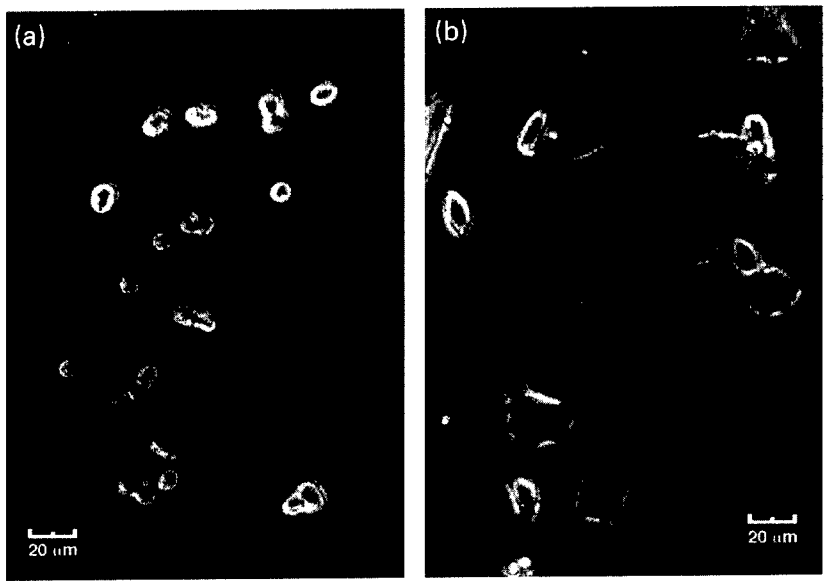

Fig. 6 NGF-induced differentiation of PC12 cells. Parental PC12 cells (a) were incubated in DMEM medium which was supplemented with 50 ng/mL NGF. (b) Differentiated PC12 cells after 7 days treatment with NGF, which shows appearance of neuronal processes and neuron-like morphology.

Expression of glucocorticoid receptor in PC12 cells

It is generally accepted that the expression of GS is specific to glial cells in the CNS (Norenbeg and Martinez-Hermandez 1979; Tansey *et al.* 1991). The promotor structure of rat GS includes a glucocorticoid-responsive element (GRE) and the expression of glucocorticoid receptors is a critical factor for cell type-specific expression of the GS gene (Vardimon *et al.* 1999). RT-PCR was performed to examine the expression of glucocorticoid receptor levels in PC12 cells. The results show that PC12 cells express glucocorticoid receptor mRNA during normoxia and hypoxia (Fig. 7). The mRNA level for this

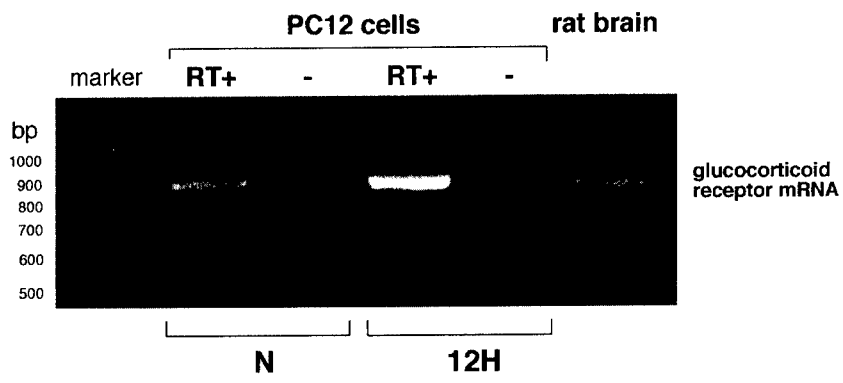

Fig. 7 Expression of glucocorticoid receptor in PC12 cells. RT-PCR products derived from rat glucocorticoid receptor mRNA were visualized by ethidium bromide staining on 1% agarose gels. PC12 cells were found to express glucocorticoid receptor mRNA during both normoxia and hypoxia (the predicted product: 876 bp). False amplification of the genomic DNA was ruled out by performing RT-PCR without reverse transcriptase as negative controls (shown as RT−). Total RNA from rat whole brain was used as positive control for glucocorticoid receptor.

receptor was not significantly altered during 12 h hypoxia ($n = 5$).

Discussion

One of the most important findings in our current study was that PC12 cells express major enzymes relevant to glutamate metabolism, GS, PAG, GAD65 and GAD67. This is the first report that shows colocalization of GS and PAG in the same cells. GS is a key enzyme in the recycling of glutamine and plays a critical role in the regulation of the concentration of glutamate in neural tissues (Kvamme 1998). GS catalyses the synthesis of glutamine, and is thereby an important precursor in various biosynthetic pathways (Kvamme 1998). It is generally accepted that the expression of high levels of GS in neural tissues is specifically confined to glial cells (astrocytes and oligodendrocytes), and is absent from neurons (Norenbeg and Martinez-Hermandez 1979; Tansey *et al.* 1991). Surprisingly, we found that PC12 cells express both GS and PAG, suggesting that PC12 cells have an intracellular glutamate–glutamine cycle.

We found that chronic exposure to hypoxia increased both GS mRNA and protein levels. It has been shown that GS gene expression can be regulated by agents such as glucocorticoids, cAMP, phorbol esters and growth factors (Lie-Venema *et al.* 1998). We found that there was a corresponding increase in GS enzyme activity in response to hypoxia. The effect of hypoxia on GS has been studied by many groups, but it is still of controversy. In *in vivo* studies, GS activity was shown to be enhanced in rat adult brain after 6 h of hypobaric hypoxia (Chandrasekaran *et al.* 1975), and in rat liver and muscles under intermittent hypobaric hypoxia (6 h per day for 7 days) (Vats *et al.* 1999). Another *in vivo* study showed that GS mRNA level, but not GS activity, was increased in rat brain during 3 h exposure to 8% O_2 and also during 6 h normoxic recovery period (Krajnc *et al.* 1996). One *in vitro* study showed that GS activity was increased in fetal mouse neuronal-glial mixed cell cultures not only during 24 h of 5% O_2 but also during 48 h posthypoxic recovery period (Sher and Hu 1990). More severe hypoxia (9 h anoxia) was found to decrease GS activity in primary culture of rat astro-glial cells, but to increase it above control during the posthypoxic period (Tholey *et al.* 1991). Therefore, it is likely that the effect of hypoxia on GS is influenced by many factors including materials (*in vivo* or *in vitro*), tissue or cell types, mRNA or enzyme activity, the level of hypoxia and also the period of hypoxia and posthypoxic recovery.

The regulatory elements of the rat GS gene have been characterized (Mill *et al.* 1991; Fahrner *et al.* 1993). The underlying cellular and/or molecular basis for the expression of GS in PC12 cells is uncertain. Several studies have shown that the induction of GS expression requires functional glucocorticoid receptors (Grossman *et al.* 1994). The 5′-flanking region of the GS gene includes a GRE (Chandrasekhar *et al.* 1999). It has been shown that glucocorticoids induce transcription of the GS gene in glial cells, but not in neurons, and that expression of the glucocorticoid receptor protein is predominantly restricted to glial cells (Grossman *et al.* 1994). Using RT-PCR, we report that PC12 cells do express glucocorticoid receptor mRNA. Since the glucocorticoid receptor is a principal factor in regulating the expression of GS, the presence of glucocorticoid receptor in PC12 cells could mediate the expression of GS in this cell line.

The phosphate-activated glutaminase (PAG) was inhibited by chronic hypoxia, at the levels of mRNA, protein, and enzyme activity. The decreased PAG enzyme activity may partly include inhibition by acidic pH (Curthoys and Watford 1995), since we showed that media pH became acidic with time during hypoxia exposure which was most likely induced by increased production of lactate. The PAG is the predominant glutamine-utilizing enzyme of the brain as well as an important contributor to transmitter pools of glutamate (Curthoys and Watford 1995). In brain, the enzyme is more abundant in neurons than in glia. Two PAG isoenzymes have been identified (Curthoys and Watford 1995; Kvamme 1998). These isoenzymes, designated as the kidney/brain type and the liver type, are the products of different genes and have different structural and kinetic properties that contribute to their function and short-term regulation (Curthoys and Watford 1995). Our current study shows that PC12 cells express the kidney/brain-type PAG and that hypoxia down-regulates the expression and function of this enzyme. Although the kidney/brain-type PAG is induced by other stimuli, such as metabolic acidosis (Curthoys and Watford 1995), regulation of this enzyme by hypoxia has never been reported. Interestingly, a previous report showed that activity of liver-type PAG enzyme is enhanced during long-term hypoxia (Vats *et al.* 1999). It has been proposed that the principal mechanism for the long-term regulation of the liver-type PAG is due to changes in the rate of gene transcription, although the regulation of the kidney/brain-type PAG is predominantly at a post-transcriptional level via changes in mRNA stability (Hwang *et al.* 1991; Curthoys and Watford 1995). Therefore, it is likely that different regulatory mechanisms are involved in the hypoxia-induced changes in the expression of the two PAG isoforms.

The glutamate decarboxylase isoforms, GAD65 and GAD67, were both up-regulated at the mRNA and protein levels during chronic hypoxia in PC12 cells. We also showed that the enzymatic activity of GAD was enhanced during hypoxia. The increased GAD enzyme activity may partly include activation by decreased cellular ATP (Martin and Rimvall 1993). Our previous study showed that cellular ATP content was significantly reduced in PC12 cells which had been exposed to 5% O_2 for up to 48 h (Kobayashi

and Millhorn 2000). Two brain isoenzymes, GAD65 and GAD67, are encoded by two different genes and differ in their intraneuronal distribution and in their function (Erlander *et al.* 1991). Although enzymatic GAD activity has previously been measured in PC12 cells (Matsuoka *et al.* 1989), we found that PC12 cells express both the GAD65 and GAD67 isoforms. Co-localization of TH and GAD has been described in cultured striatal neurons (Max *et al.* 1996). GABA is the major inhibitory neurotransmitter in brain and its formation is the main function of GAD in this tissue. Although a recent paper provides indirect evidence that hypoxia enhances the GAD activity in rat hippocampal slices (Madl and Royer 2000), our study clearly shows that the expression of GAD65 and GAD67 are increased during hypoxia. It should be noted that the effect of hypoxia on GAD65 was more dramatic than that for GAD67. A recent paper reported that in a model of temporal lobe epilepsy, both forms of GAD were increased but the increase was more marked for GAD65 (Esclapez and Houser 1999). GAD67 and GAD65 may play a role in tonic and phasic inhibition, respectively (Erlander and Tobin (1991). The functional significance of the up-regulation of GAD65 and GAD67 during hypoxia is not clear. Since GAD catalyzes the conversion of glutamate to GABA, increased activity of GAD is consistent with a decrease in intracellular glutamate levels. Interestingly, a recent paper reported that astrocytes stably transfected with GAD enhanced survival of co-cultured PC12 cells under hypoglycemic conditions (Bellier *et al.* 2000).

We also examined the expression of glutamate transporters in PC12 cells. Both RT-PCR and immunoblot analyses showed that PC12 cells express three major subtypes of glutamate transporters in the brain, EAAC1, GLT-1 and GLAST. We found that hypoxia up-regulates EAAC1 and GLT-1, but had no effect on the expression of GLAST. We also found that the glutamate uptake into PC12 cells was predominantly dependent on extracellular Na^+ under normoxic conditions. We further found that Na^+-dependent uptake of glutamate was significantly enhanced when cells had been pre-exposed to prolonged hypoxia. Thus, it is likely that the increased amounts of EAAC1 and GLT-1 were responsible for the enhanced uptake of glutamate during chronic hypoxia. It has been shown that the GLT-1 subtype accounts for the majority of glutamate transport activity in the brain and GLT-1 may represent as much as 1% of total brain protein (Danbolt *et al.* 1990). A previous study reported that glutamate transport activity and GLAST expression were found only in certain flattened cell mutants of PC12 cells (Ramachandran *et al.* 1993). The reason for the discrepancy between their results and ours is not clear. It may be possible that our PC12 cell strain is different from theirs and has a different pattern of gene expression. Although PC12 cells are clonal cell lines, many phenotypic variants of PC12 cells have been reported (Greene *et al.* 1991). The properties of PC12 cells depend on their sources. The consistency of cell lines is further compromised by spontaneous mutations and/or selection of subpopulations in different culturing conditions. These factors lead to production of phenotypic variants in clones used in different laboratories. Several previous studies have examined the control of glutamate transport during hypoxic/ischemic stress in neuronal tissues or cell cultures. Glutamate uptake activity was increased in rat cortical astrocytes during hypoxia (Sher and Hu 1990; Keheller *et al.* 1994; Stanimirovic *et al.* 1997), but these studies did not examine the expression of glutamate transporters. Our study reports that EAAC1 and GLT-1 levels are increased by hypoxia.

The effects of hypoxia on the intracellular content of glutamate and glutamine in PC12 cells were also evaluated. We found that hypoxia concomitantly *decreased* glutamate levels and *increased* glutamine levels. Our study indicates that PC12 cells possess the necessary elements for a glutamate-glutamine cycle. Chronic hypoxia induced a coordinate regulation in the activities of several key enzymes which are involved in the production and metabolism of glutamate in this cell type. Increased utilization of glutamate (enhanced metabolism by GS and GAD), accompanied by decreased production of glutamate (decreased generation by PAG), would result in an overall decrease in cellular capacity to form intracellular glutamate during chronic hypoxia. We also found that glutamate uptake activity was induced during chronic hypoxia. This would be expected to further decrease the extracellular glutamate levels. The functional significance of this coordinate pattern of regulation of glutamate metabolism and uptake during chronic hypoxia is not clear. However, the regulation of these cellular activities might be protective against hypoxia/ischemia-induced cell injuries. Many studies have shown that ischemic insults promote glutamate release and subsequent cellular degeneration (Choi 1994; Szatkowski and Attwell 1994; Lipton 1999). Several previous studies have reported that excess levels of extracellular glutamate are toxic in PC12 cells (Schubert *et al.* 1992; Froissrad and Duval 1994; Pereira *et al.* 1998). Therefore, altered enzyme activities and glutamate transport during chronic hypoxia may enhance the survival of PC12 cells by reducing extracellular concentration of glutamate. These regulatory mechanisms may have a protective role to reduce the susceptibility of PC12 cells to hypoxia. Further studies are required to investigate whether results obtained with PC12 cells are relevant to an understanding of neuronal/glial interactions with regard to glutamate metabolism and transport.

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Novel regulation of p38γ by dopamine D2 receptors during hypoxia

P. William Conrad, David E. Millhorn, Dana Beitner-Johnson*

College of Medicine, Department of Molecular and Cellular Physiology, University of Cincinnati, P.O. Box 67-0576, Cincinnati, OH 45267-0576, USA

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Abstract

The p38 signalling pathway is part of the MAPK superfamily and is activated by various stressors. Our previous results have shown that two p38 isoforms, p38α and p38γ, are activated by hypoxia in the neural-like PC12 cell line. PC12 cells also synthesize and secrete catecholamines, including dopamine, in response to hypoxia. We have now used this system to study the interaction between D2-dopamine receptor signalling and the p38 stress-activated protein kinases. Our results show that two D2 receptor antagonists, butaclamol and sulpiride, enhance hypoxia-induced phosphorylation of p38γ, but not p38. This effect persists in protein kinase A (PKA)-deficient PC12 cells, demonstrating that p38γ modulation by the D2 receptor is independent of the cAMP/PKA signalling system. We further show that removal of extracellular calcium blocks the hypoxia-induced increase in p38γ activity. These results are the first to demonstrate that p38γ can be regulated by the D2 receptor and calcium following hypoxic exposure.

Keywords: p38; MAPK; Dopamine; Hypoxia; Calcium

1. Introduction

Hypoxia is an extremely common physiological stress that is involved in a variety of pathological processes, including angiogenesis, tumour progression, and apoptosis. However, little is known about the specific intracellular pathways by which hypoxia triggers these events. The stress-activated protein kinases (SAPKs) regulate gene expression and cell function following exposure to various stressors [1,2]. The SAPKs consist of two homologous families of protein kinases, designated p38 (HOG/RK/SAPK2) and c-Jun N-terminal kinase (JNK/SAPK) [3]. Both the p38 and JNK pathways can be activated by osmotic stress, UV irradiation, and inflammatory cytokines [4–7]. However, JNK is unique in its ability to phosphorylate the transcription factor c-Jun [4]. In previous studies, we have shown that p38α, p38γ, and p42/p44 MAPK are activated by hypoxia in pheochromocytoma (PC12) cells [8,9]. In contrast, the SAPKs p38β, p38δ, and JNK are not regulated by exposure to low oxygen in this system [8].

Pheochromocytoma (PC12) cells are catecholaminergic cells derived from rat adrenal medullary tumours (for review see [10]). Upon stimulation with nerve growth factor (NGF), PC12 cells exit the cell cycle and extend neurites [10]. In the undifferentiated state, PC12 cells are exquisitely sensitive to changes in pO_2. Following exposure to hypoxia, PC12 cells depolarize and secrete various neurotransmitters, including dopamine [11,12]. In addition to neurosecretion, hypoxia stimulates various transcription factors [9,11,13] and regulates the expression of specific O_2-responsive genes in this cell type [9,14,15]. Thus, we have utilized this cell line in order to characterize further the intracellular signalling mechanism(s) by which cells respond and adapt to hypoxia.

The upstream signalling cascades that regulate p38 function have only partially been characterized. It has been shown that the p38 kinases are phosphorylated and activated by upstream MAP kinase kinases (MKKs), including MKK3, MKK6, and possibly MKK4 [16]. Further upstream of the MKKs lie a number of signalling molecules that can potentially activate p38, including the Rho family of GTPases as well as cell surface receptors [17–19]. For example, stimulation of either the insulin-like growth factor I (IGF-I) receptor or the TrkB

* Corresponding author. Tel.: 513 558 3690; fax: 513 558 5738.
E-mail address: dana.johnson@uc.edu (D. Beitner-Johnson)

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receptor can lead to p38 activation [18,19]. Recent reports have shown that dopamine (D1) receptors can also modulate the p38 signalling pathway [20]. Our laboratory has shown that upon exposure to hypoxia, PC12 cells secrete dopamine which feeds back through D2 receptors to modulate Ca^{2+} currents [21]. Thus, PC12 cells are a useful system in which to study the interaction of D2 receptors and the p38 signalling pathway.

In the current study, we demonstrate that two D2 antagonists, butaclamol and sulpiride, enhance p38γ phosphorylation during hypoxia and that the mechanism of this modulation is independent of PKA. We further show that hypoxia-induced phosphorylation of p38γ is dependent on intracellular Ca^{2+} levels. These results are, to our knowledge, the first to show that p38γ is regulated by Ca^{+2} and can be modulated by the endogenous release of dopamine.

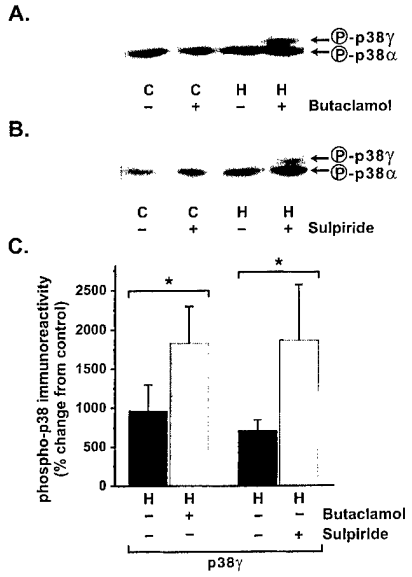

Fig. 1. The dopamine D2 receptor antagonists, butaclamol and sulpiride, enhance the hypoxia-induced phosphorylation of p38γ. PC12 cells were grown to approximately 80% confluence in 35-mm tissue culture dishes. Cells were pretreated for 1 h with either butaclamol (1 μM), sulpiride (10 μM), or vehicle (DMSO), followed by normoxic or hypoxic (5% O_2, 6 h) exposure. p38α and p38γ phosphorylation state was assayed using anti-phospho-p38 antibodies, as described in Materials and methods. (a) Representative immunoblot showing the effect of butaclamol on p38α (lower band) and p38γ (upper band) phosphorylation state. (b) Representative immunoblot showing the effect of sulpiride on p38α (lower band) and p38γ (upper band) phosphorylation state. (c) Immunoreactivity of phospho-p38γ in the absence (black bars) or presence (shaded bars) of D2 antagonists is expressed as average percent change from control ± S.E.M. from $n = 6$ dishes in each group performed in two separate experiments. Phospho-p38γ immunoreactivity was quantified by densitometry (*$p < .01$, by χ^2 test).

2. Materials and methods

2.1. Cell culture and materials

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Life Technologies, Gaithersburg, MD) supplemented with 20 mM HEPES pH 7.4, 10% foetal bovine serum (Gibco, Gaithersburg, MD), and with penicillin (100 U/ml) and streptomycin (100 μg/ml). Prior to experimentation, cells were grown to approximately 85% confluence in 35-mm or 60-mm tissue culture dishes (Corning) in an environment of 21% O_2, 5% CO_2, balanced with N_2. Hypoxia was achieved by exposing cells to 5% O_2, 5% CO_2, balanced with N_2 for various times in an O_2-regulated incubator (Forma Scientific, Marietta, OH). PKA-deficient PC12 cells (A123.7) were grown in Dulbecco's modified Eagle's medium with high glucose containing 20 mM HEPES pH 7.4, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% foetal bovine serum, 5% horse serum and gentamycin (100 μg/ml) in an environment of 21% O_2 and 10% CO_2 [22]. Butaclamol and sulpiride were obtained from RBI (Natick, MA).

2.2. Western blotting

Western blotting was performed as described previously [8]. For phospho-p38 blots, membranes were immunolabelled with antibodies recognizing phospho-tyr^{204} p38 (1:1000, New England Biolabs, Beverly, MA).

2.3. Immune complex kinase assay

p38γ kinase assays were performed as described previously [8]. Prior to hypoxic exposure, PC12 cells were switched to either Ca^{2+}-containing media, or Ca^{2+}-free media (supplemented with 1 mM EGTA). After 1 h, cells were exposed to normoxia or hypoxia (5% O_2, 6 h). Cells were then lysed and p38γ activity was assayed as the amount of ^{32}P incorporation into myelin basic protein, as quantified by Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

3. Results

There is increasing evidence that activation of cell surface receptors can modulate the p38 signalling pathway [18,19]. During hypoxia, PC12 cells secrete dopamine [11,12]. In previous studies, we have shown that dopamine feeds back though its receptors to modulate voltage-dependent K^+ and Ca^{2+} currents in PC12 cells [21] and that hypoxia activates p38α and p38γ in this cell type [8]. Thus, we have now investigated whether D2 receptors modulate p38α or p38γ during hypoxia. PC12 cells were pretreated for 1 h with two dopamine D2 receptor antagonists, either butaclamol (1 μM) or sulpiride (10 μM), prior to exposure to hypoxia (5% O_2, 6 h). As shown in Fig. 1a and b, pretreatment with either of these two antagonists had no effect on p38α phosphorylation (lower band), but increased the hypoxia-induced phosphorylation of p38γ (upper band). Using isoform-specific antibodies, in previous studies

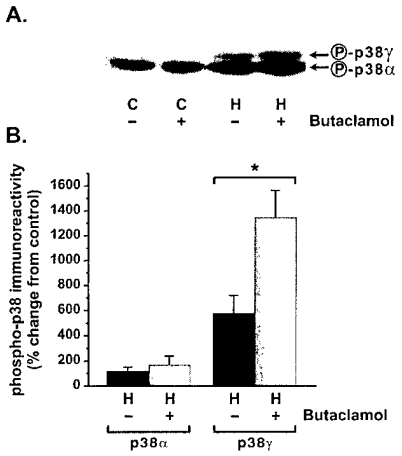

Fig. 2. D2 modulation of p38γ persists in PKA-deficient PC12 cells. PKA-deficient PC12 cells were grown to approximately 80% confluence in 35-mm tissue culture dishes. Cells were pretreated for 1 h with either butaclamol (1 μM), or vehicle, followed by normoxic or hypoxic (5% O_2, 6 h) exposure. p38α and p38γ phosphorylation state was assayed using anti-phospho-p38 antibodies. (a) Representative immunoblot showing the effect of D2 antagonists on p38α (lower band) and p38γ (upper band) phosphorylation state. (b) Immunoreactivity of p38α and p38γ in the absence (black bars) or presence (shaded bars) of butaclamol are expressed as average percent change from control ± S.E.M. and represents $n = 6$ dishes in each group performed in two separate experiments. Phospho-p38α/p38γ immunoreactivity was quantified by densitometry (*$p < .01$, by χ^2 test).

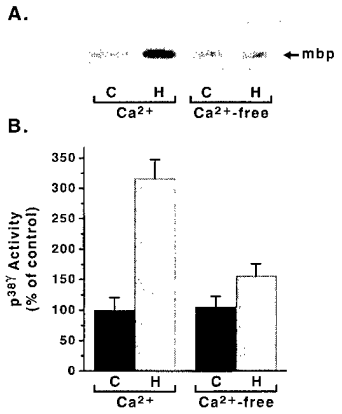

Fig. 3. p38γ activation by hypoxia is Ca^{2+}-dependent. PC12 cells were transfected with either FLAG-p38γ or the pcDNA3 vector, as performed previously [8]. After 48 h, cells were switched to either Ca^{2+}-supplemented or Ca^{2+}-free media for 1 h. Cells were then exposed to either normoxia (C, 21% O_2) or hypoxia (H, 5% O_2, 6 h) and assayed for p38γ activity, as described in Materials and methods. (a) p38γ activity was determined as the amount of ^{32}P incorporation into myelin basic protein (mbp). (b) p38γ kinase activity during normoxia (black bars) or hypoxia (shaded bars) in the presence or absence of extracellular Ca^{2+} are expressed as average percent of control ± S.E.M, and represent $n = 6$ dishes in each group, performed in two separate experiments.

we have identified this upper band as p38γ and not one of the other p38 isoforms (p38β, p38β2, p38δ) [8]. These results are shown quantitatively in Figure 1c, where it can be seen that butaclamol and sulpiride caused a significant increase in the level of p38γ phosphorylation during hypoxia.

Dopamine receptors are capable of mediating their effects through a number of different mechanisms. One of these is by coupling to andenylyl cyclase, which in turn regulates protein kinase A (PKA) [23]. In order to determine whether the D2-mediated effect on p38γ was mediated by PKA, we tested the ability of butaclamol to modulate p38γ in PKA-deficient PC12 cells (123.7 cells) [22]. We have previously confirmed that there is no detectable PKA enzyme activity in these cells [13]. Fig. 2a shows that the enhanced phosphorylation of p38γ persists in PKA-deficient PC12 cells. These results are shown quantitatively in Fig. 2b.

A second mechanism by which dopamine receptors mediate their effects is via the modulation of Ca^{2+} currents [21,24,25]. In previous studies, we have shown that D2 receptor agonists attenuate the hypoxia-induced increase in intracellular Ca^{2+} in PC12 cells [21]. These results demonstrate that the D2 receptor mediates at least some of its effects by the modulation of Ca^{2+} currents. To test whether modulation of Ca^{2+} current is the mechanism by which D2 receptors modulate p38γ phosphorylation, PC12 cells were pre-incubated for 1 h in either Ca^{2+}-free media (supplemented with 1 mM EGTA) or standard medium (including Ca^{2+}). Cells were then exposed to either normoxia or hypoxia (5% O_2) for 6 h. p38γ enzyme activity was assayed by immune complex kinase assays, as performed previously [8]. Fig. 3a shows that p38γ is activated by hypoxia and that removal of extracellular Ca^{2+} is able to abolish completely this hypoxia-induced activation. These results are shown quantitatively in Fig. 3b.

Our results demonstrate that p38γ phosphorylation is regulated by both the D2 receptor and intracellular Ca^{2+} levels. Fig. 4 summarizes these results. Following hypoxic exposure, an O_2-sensitive K^+ channel is inhibited [26]. This leads to membrane depolarization and influx of Ca^{2+} through voltage-dependent Ca^{2+} channels [26]. Increased intracellular Ca^{2+} initiates signalling cascades leading to dopamine (DA) release and p38γ phosphorylation. DA then binds to the D2 receptor where it negatively regulates both a voltage- dependent K^+ current (I_K) and a voltage-dependent Ca^{2+} current (I_{Ca}) [21]. The resulting decrease in intracellular free Ca^{2+} negatively regulates Ca^{2+}-activated signalling pathways, such as p38γ.

4. Discussion

The goal of this study was to determine whether p38α or p38γ phosphorylation is modulated by the dopamine D2 receptor. We found that D2 antagonists increase the hypoxia-induced phosphorylation of p38γ. We further show that the mechanism of this modulation occurs independently of PKA. Finally, we demonstrate that phos-

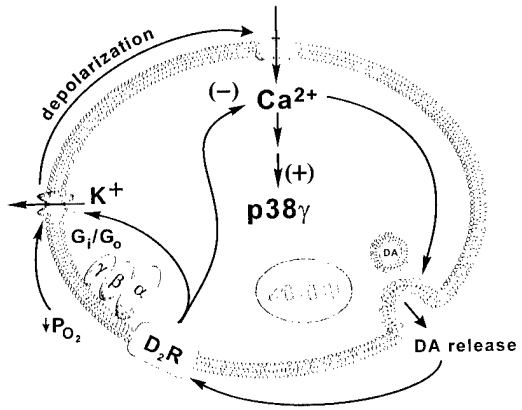

Fig. 4. Summary of the effects of hypoxia on D2 receptor, intracellular Ca^{2+}, and p38γ. Hypoxic exposure causes inhibition of an O_2-sensitive K^+ current, resulting in membrane depolarization and influx of Ca^{2+}. Increased intracellular Ca^{2+} initiates signalling cascades leading to dopamine (DA) release and p38γ phosphorylation. Dopamine then binds to the D2 receptor where it negatively regulates both a voltage-dependent K^+ current (I_K) and a voltage-dependent Ca^{2+} current (I_{Ca}). The resulting decrease in intracellular free Ca^{2+} negatively regulates Ca^{2+}-activated signalling pathways, such as p38γ.

phorylation of p38γ during hypoxia is dependent on intracellular Ca^{2+} levels.

The p38 family of protein kinases consists of five different isoforms and, like other signalling cascades, serves to integrate changes in the environment with changes in gene expression. Our results provide the first evidence showing that p38γ can be modulated by the dopamine D2 receptor. While others have shown that exogenous addition of D1 agonists can activate p38α [20], our results are the first to show that endogenous release of dopamine during hypoxia acts in an autocrine/paracrine manner to modulate p38γ phosphorylation. It is interesting to note that p38α phosphorylation is not modulated by D2 antagonists. As reported previously, p38α phosphorylation by hypoxia is relatively modest when compared to that of p38γ. Thus, any modulation of p38α by D2 antagonists probably falls within a range that is undetectable. Alternatively, it is conceivable that p38α is regulated via a different (possibly Ca^{2+}-independent) mechanism than p38γ.

Previous results from our lab have shown that D2 agonists inhibit the hypoxia-induced increase in intracellular Ca^{2+} levels [21]. Thus, the presence of D2 antagonists, such as butaclamol, are likely to relieve this inhibition, resulting in greater Ca^{2+} influx and hence, greater activation of p38γ. We attempted to test this directly, by assessing Ca^{2+}-imaging with Fura-2 in the presence or absence of D2 antagonists. However, we were unable to detect an increase in intracellular Ca^{2+} upon the addition of D2 antagonists. The lack of an effect may be due to the fact that dopamine levels fail to reach a concentration capable of eliciting an effect. This is probably the result of dopamine washout, as the Ca^{2+} imaging is done in a constant perfusion apparatus. In contrast, the hypoxia-induced Ca^{2+} influx is easily measured because the perfusate can be continuously bubbled with N_2 to maintain hypoxic conditions [21].

The fact that p38γ activation in the dopamine secreting PC12 cells is Ca^{2+}-dependent suggests that a similar mechanism may be at work in the excitable cells of the brain. Extensive studies have demonstrated that inhibition of calcium entry following an ischemic event is able to prevent subsequent neuronal loss [27,28]. Interestingly, D2 agonists have recently been shown to have neuroprotective effects [29,30]. Although the mechanism of this protection is unclear, inhibition of Ca^{2+} currents, such as occurs in PC12 cells, may be a critical component. Interestingly, chronic exposure of PC12 cells to moderate hypoxia (10% O_2, 24 h) abolishes the D2-mediated inhibition of Ca^{2+} current [31]. Thus, the D2 signalling system is responsive to both acute and chronic hypoxia in this cell type. Future experiments are aimed at more closely elucidating the mechanism of p38γ activation, as well as further characterizing the role of D2 receptors in modulating the molecular and cellular response to hypoxia.

Acknowledgments

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O_2-sensitive K^+ channels: role of the Kv1.2 α-subunit in mediating the hypoxic response

Laura Conforti*, Ilona Bodi†, John W. Nisbet and David E. Millhorn

*Department of Molecular and Cellular Physiology, *Department of Internal Medicine, Division of Nephrology and Hypertension and †Institute of Molecular Pharmacology and Biophysics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267, USA*

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1. One of the early events in O_2 chemoreception is inhibition of O_2-sensitive K^+ (K_{O_2}) channels. Characterization of the molecular composition of the native K_{O_2} channels in chemosensitive cells is important to understand the mechanism(s) that couple O_2 to the K_{O_2} channels.

2. The rat phaeochromocytoma PC12 clonal cell line expresses an O_2-sensitive voltage-dependent K^+ channel similar to that recorded in other chemosensitive cells. Here we examine the possibility that the Kv1.2 α-subunit comprises the K_{O_2} channel in PC12 cells.

3. Whole-cell voltage-clamp experiments showed that the K_{O_2} current in PC12 cells is inhibited by charybdotoxin, a blocker of Kv1.2 channels.

4. PC12 cells express the Kv1.2 α-subunit of K^+ channels: Western blot analysis with affinity-purified anti-Kv1.2 antibody revealed a band at ~80 kDa. Specificity of this antibody was established in Western blot and immunohystochemical studies. Anti-Kv1.2 antibody selectively blocked Kv1.2 current expressed in the *Xenopus* oocyte, but had no effect on Kv2.1 current.

5. Anti-Kv1.2 antibody dialysed through the patch pipette completely blocked the K_{O_2} current, while the anti-Kv2.1 and irrelevant antibodies had no effect.

6. The O_2 sensitivity of recombinant Kv1.2 and Kv2.1 channels was studied in *Xenopus* oocytes. Hypoxia inhibited the Kv1.2 current only.

7. These findings show that the K_{O_2} channel in PC12 cells belongs to the Kv1 subfamily of K^+ channels and that the Kv1.2 α-subunit is important in conferring O_2 sensitivity to this channel.

The ability to sense and respond to reduced oxygen (O_2) tension (hypoxia) is essential for the survival of mammalian cells. Specialized cells in the body (O_2-sensitive or chemoreceptor cells) can quickly sense and respond to O_2 deprivation. These O_2-sensitive cells are present in a variety of tissues including the carotid body (a small organ located near the bifurcation of the common carotid artery), the pulmonary vasculature, and pulmonary neuroepithelial bodies (small organs distributed widely throughout the airway mucosa). Stimulation of these cells results in cardiovascular and pulmonary responses that optimize the delivery of O_2 to vital organs, thereby preventing global or localized O_2 deficits that can produce irreversible cellular damage (Weir & Archer, 1995; Lahiri, 1997). Despite their critical homeostatic role, the mechanisms by which O_2-sensitive cells detect a change in O_2 tension (P_{O_2}) and transduce this signal into the appropriate biological response remain unknown.

The presence of O_2-sensitive K^+ (K_{O_2}) channels has been shown in different chemosensitive cells (Lopez-Barneo, 1996). Inhibition of the K_{O_2} channel activity is an important early event in the process of O_2 chemoreception, which leads eventually to cell depolarization, Ca^{2+} influx, neurotransmitter release, muscle contraction, regulation of protein kinases, and alterations in gene expression (Czyzyk-Krzeska *et al.* 1992; Bunn & Poyton, 1996; Lopez-Barneo, 1996; Beitner-Johnson & Millhorn, 1998). Therefore, K_{O_2} channels have been proposed as key elements in the detection of changes in O_2 availability by chemosensitive cells.

Although K_{O_2} channels in chemosensitive cells have been investigated extensively using electrophysiological techniques, there is relatively little information about their molecular identity. In most O_2-sensitive cells the K_{O_2} channels are voltage dependent: slow-inactivating voltage-dependent K^+ (K_V) channels in pulmonary artery smooth muscle cells, rabbit carotid body type I cells and pulmonary neuroepithelial body cells and Ca^{2+}-activated K^+ channels (K_{Ca}) in rat type I cells (Peers, 1990; Archer *et al.* 1996; Osipenko *et al.* 1997). However, in rat carotid body type I

cells a background K^+ current (K_{leak}) is also inhibited by hypoxia (Buckler, 1997). The rat phaeochromocytoma (PC12) cell line has been used as a model system for studying O_2-chemosensory mechanisms (Czyzyk-Krzeska *et al.* 1994; Norris & Millhorn, 1995; Bright *et al.* 1996; Zhu *et al.* 1997; Taylor & Peers, 1998). Importantly, PC12 cells express a slow-inactivating voltage-dependent K_{O_2} channel that is inhibited by hypoxia (Zhu *et al.* 1996; Conforti & Millhorn, 1997). Hence, this cell line provides a unique and useful model for combining electrophysiological studies with molecular biological experiments designed to clarify the molecular nature of voltage-dependent K_{O_2} channels and basic O_2-sensing mechanisms (Conforti *et al.* 1998).

Voltage-dependent K^+ (K_V) channels are complex hetero-oligomeric proteins formed by four α pore-forming subunits and auxiliary β-subunits (Jan & Jan, 1997). The genes that encode the K_V α-subunits have been classified into at least six subfamilies: *Shaker* (Kv1.1–1.7), *Shab* (Kv2.1–2.2), *Shaw* (Kv3.1–3.4), *Shal* (Kv4.1–4.3), Kv5.1 and Kv6.1 (Pongs, 1992). In addition, a novel family of electrically silent K_V α-subunits was recently identified (Patel *et al.* 1997). A K^+ channel composed of a silent *Shab*-like α-subunit (Kv9.3) cloned from rat pulmonary artery and Kv2.1 has been proposed as a possible K_{O_2} channel in pulmonary artery smooth muscle cells (Patel *et al.* 1997). Others have suggested that pulmonary artery smooth muscle cells express different K_{O_2} channels formed by either Kv2.1 or Kv1.5 α-subunits, which display different functional roles in the cellular response to hypoxia (Archer *et al.* 1998). In addition, other K_V channel subtypes have been implicated in the cellular response to hypoxia in pulmonary artery smooth muscle cells and other O_2-sensitive cell types (Vega-Saenz de Miera & Rudy, 1992; Conforti & Millhorn, 1997; Wang *et al.* 1997; Hulme *et al.* 1999; Perez-Garcia *et al.* 1999). Thus, the identity of the O_2-sensitive K^+ channels remains unclear.

The present study was undertaken to elucidate the molecular identity of the K_{O_2} channel in PC12 cells. The current findings provide evidence that the O_2-sensitive K^+ channel present in PC12 cells belongs to the Kv1 subfamily of K_V channels and that the Kv1.2 α-subunit is an important O_2-sensitive component of this channel.

METHODS

PC12 clonal cell line

PC12 cells, obtained from American Type Culture Collection, were grown in Dulbecco's Modified Eagle's–Ham's F-12 medium (DMEM–F-12) supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells were maintained in an incubator in which the environment (21% O_2, 5% CO_2, remainder N_2; 37 °C) was strictly maintained. Cells used for electrophysiological experiments were dissociated with 0·25% trypsin plus 1 mM EDTA and plated at low density (*ca* 100 000 ml^{-1}) on glass coverslips and were used 1–3 days after plating.

Expression of K_V channels in *Xenopus* oocytes

Xenopus oocytes were injected with cRNAs obtained as run-off transcripts of Kv1.2 (HBK5 cloned in pcDNA3 plasmid vector) and Kv2.1 cDNAs (DRK1 cloned in pBluescript-SK$^-$ plasmid vector). The double-stranded DNA templates were linearized and *in vitro* transcribed to cRNAs with mMessage mMachine kits (for T7 or SP6 promoter; from Ambion), according to the manufacture's protocol. After the transcription reaction was complete, the template DNA was degraded and cRNA was recovered by phenol–chloroform extraction followed by ethanol precipitation. The size of the *in vitro* transcription product, its quantity, and its quality were evaluated by denaturing agarose gel electrophoresis. The cRNAs were stored in RNase-free water at −80 °C.

Stage IV–V oocytes were isolated as follows. Frogs were anaesthetized with 0·2% tricaine methanesulphonate (MS 222). Clumps of oocytes were removed and washed in Ca^{2+}-free ND-96 solution containing (mM): 82·5 NaCl, 2·0 KCl, 1·0 $MgCl_2$, and 5·0 Hepes; pH 7·5. After removal of the oocytes, the frogs were allowed to recover and returned to their tanks. Single oocytes were dissociated with 3 mg ml^{-1} type II collagenase in Ca^{2+}-free ND-96 solution at 20 °C. After digestion, the follicular layer was removed mechanically with a fire-polished Pasteur pipette. cRNA (50 nl; 0·2 μg μl^{-1}) was injected into the oocyte with a Drummond 510 microdispenser via a sterile glass pipette with a tip of 20–30 μm. After injection the oocytes were maintained in a solution of the following composition (mM): 96 NaCl, 2·0 KCl, 1·0 $MgCl_2$, 1·8 $CaCl_2$, 5 Hepes, 2·5 sodium pyruvate, and 0·5 theophylline, with 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin; pH 7·5. Injected oocytes were stored in an incubator at 19 °C and were used for electrophysiological experiments after 24 h. We followed the methods previously described by Stuhmer & Parekh (1995).

Electrophysiology

Details of our patch-clamp station and the whole-cell and single-channel methods were published previously (Zhu *et al.* 1996; Conforti & Millhorn, 1997). Experiments were performed using Axopatch 200A (for whole-cell and single-channel voltage-clamp) and Axoclamp 2A (for two-electrode voltage-clamp) amplifiers (Axon Instruments). The digitized signals were stored and analysed on a personal computer using pCLAMP 5.5.1 and 6.0.3 software (Axon Instruments). Experiments were conducted at room temperature (25 °C).

Whole-cell voltage-clamp experiments were performed according to standard procedures (Hamill *et al.* 1981). The composition of the external solution was (mM): 140 NaCl, 2·8 KCl, 2·0 $CaCl_2$, 2·0 $MgCl_2$, 10 Hepes, and 10 glucose; pH 7·4. The composition of the pipette solution was (mM): 140 potassium gluconate, 1 $CaCl_2$, 11 EGTA, 2 $MgCl_2$, 3 ATP-sodium, and 10 Hepes; pH 7·2. ATP was included in the pipette solution to exclude the contribution of ATP-sensitive K^+ channels and to compensate for reduced cellular energy metabolism during hypoxia. K^+ currents were recorded by depolarizing voltage steps to +50 mV (800 ms duration) from a holding potential of −70 mV. Steady-state current amplitude was measured at the end of the test pulse. Current inhibition is reported as relative changes in current amplitude from the control (normoxia) values. For whole-cell experiments using antibodies, electrodes were dipped in an antibody-free solution and then back filled with the pipette solution containing the antibody of interest. Anti-Kv1.2 antibody was used at 0·03 μg ml^{-1}. This concentration was calculated as required for a 1:1 interaction with the number of K^+ channels in a single PC12 cell (calculated by dividing the single-

channel conductance into the maximal K_V conductance). Higher concentrations of anti-Kv1.2 antibody (0·3 μg ml^{-1}) induced nearly complete inhibition of the K^+ current. Anti-Kv2.1 antibody was used at 1:125 dilution. This concentration was previously used to block Kv2.1 channels in pulmonary artery smooth muscle cells (Archer *et al.* 1998). Electrodes had a resistance of 1–3 MΩ, which permits dialysis of the antibody into the cell (Vassilev *et al.* 1988; Naciff *et al.* 1996).

Whole-cell current from injected *Xenopus* oocytes was recorded using the two-electrode voltage-clamp technique, as previously described (Stuhmer & Parekh, 1995). The composition of the external solution was (mM): 115 NaCl, 2 KCl, 1·8 $CaCl_2$, and 10 Hepes; pH 7·2 (Stuhmer *et al.* 1989). The two electrodes had a resistance of 1–2 MΩ and were filled with 3 mM KCl. Whole-cell leak and capacitative currents were subtracted using currents elicited by small hyperpolarizing pulses ($P/4$). Currents were digitized between 0·5 and 5 kHz after being filtered between 0·2 and 1 kHz. For experiments using anti-Kv1.2 antibody, oocytes were injected with 0·01 μg anti-Kv1.2 antibody (in 50 nl) 2 h before recording. This amount of anti-Kv1.2 antibody was calculated to result in an intracellular concentration similar to that obtained in PC12 cells, assuming a cell volume 10^6 times higher in oocytes compared to PC12 cells.

Single-channel (cell-attached) voltage-clamp experiments were performed in *Xenopus* oocytes from which the vitelline membrane had been manually removed after shrinkage in a hyperosmotic medium (mM): 200 potassium aspartate, 20 KCl, 1·0 $MgCl_2$, 5 EGTA, and 10 Hepes; pH 7·3. Microelectrodes with resistances of 3–5 MΩ were prepared, fire-polished, and coated with Sylgard (Dow Corning). The external solution composition was (mM): 140 KCl, 2·0 $MgCl_2$, 10 Hepes, and 5 EGTA; pH 7·3. The pipette solution composition was (mM): 140 NaCl, 2·8 KCl, 5 Hepes, and 1 EGTA; pH 7·3. Ensemble-averaged currents and open channel probability (NP_o) were calculated using pCLAMP 6.0.3 software, as previously described (Conforti & Millhorn, 1997). Single-channel conductance was measured with ramp pulse depolarization from −60 mV (holding potential) to +50 mV (0·14 mV ms^{-1}), as previously described (Conforti & Millhorn, 1997).

Exposure of cells to hypoxia

During electrophysiological experiments the effect of hypoxia was studied by switching from a perfusion medium bubbled with air (21% O_2) to a medium equilibrated with 10% O_2 (balanced N_2) or 100% N_2 with 5 mM sodium dithionite ($Na_2S_2O_4$; an O_2 chelator). The corresponding mean O_2 partial pressures (P_{O_2}) in the chamber, measured with an O_2-sensitive electrode, were 150 mmHg (21% O_2), 80 mmHg (10% O_2) and 0 mmHg (N_2 + $Na_2S_2O_4$) (Zhu *et al.* 1996).

Western blotting

PC12 cell total lysate was prepared according to standard procedures. PC12 cells were harvested by resuspending them in lysis buffer containing (mM): 50 Hepes, 10 EDTA, 100 NaCl, and 1 PMSF with 1% Triton X-100, 2 μg ml^{-1} leupeptin, and 2 μg ml^{-1} aprotinin. After sonication and centrifugation, the protein content was measured using the Bio-Rad method. Aliquots of cell proteins (40 μg) were fractionated on 6% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific protein-binding sites were blocked by incubation in PBST (phosphate-buffered saline (PBS) with 0·1% Tween-20) with 3% non-fat dry milk for 1 h at room temperature. The blots were incubated with the first antibodies (1:200 dilution) overnight, at 4 °C. After washing 3–4 times, the strips were incubated for 1 h at room temperature with affinity-purified horseradish peroxidase-conjugated goat anti-rabbit antibodies at 1:2000 (DAKO, Denmark). Bands were visualized using Enhanced Chemiluminescence (ECL, Amersham Life Science Inc.) exposed to X-ray film. Prestained molecular mass standards were used to assess the apparent molecular mass.

Immunohistochemistry

PC12 cells were grown on slides for 3 days and fixed with 2% paraformaldehyde for 20 min. After washing with PBS, cells were permeabilized and blocked with PBS containing 0·2% Triton X-100 and 10% normal goat serum (NGS). Slides were incubated with anti-Kv1.2 antibody (2 μg ml^{-1} in 1% NGS in PBS) overnight at room temperature. Immunoreactivity was visualized with a fluorescein-conjugated goat anti-rabbit secondary antibody (ICN/Cappel). The fluorescence background was assessed by applying the same protocol to cells that were incubated overnight in 1% NGS in PBS.

Source and specificity of anti-Kv1.2 and anti-Kv2.1 antibodies

Affinity purified anti-Kv1.2 antibody (Alomone Labs) was prepared against the C-terminal part of the rat Kv1.2 protein, specifically amino acids 417–498 (Stuhmer *et al.* 1989). This sequence is specific for Kv1.2 except for 11 amino acids, which are similar to those of Kv1.1. The company specification indicates that there is no cross-reactivity with Kv1.1. Cross-reactivity with another member of the Kv1 subfamily, Kv1.3, was tested in Western blot experiments with Kv1.3 antigen, a glutathion-S-transferase (GST) fusion protein with the C-terminal 471–523 amino acids of the Kv1.3 protein. Anti-Kv1.3 antibody and its corresponding antigen were obtained from Alomone Labs. Specificity of anti-Kv1.2 antibody was also established in an immunohistochemical study using Kv1.2 antibody pre-incubated with a ten-fold molar excess of the antigen at room temperature for 1 h. The antigen for Kv1.2, a GST fusion protein containing the epitope against which the antibody was raised, was also obtained by Alomone Labs. The specific ability of anti-Kv1.2 antibody to block Kv1.2 current was established in *Xenopus* oocytes.

Polyclonal antibody against Kv2.1 was obtained from Upstate Biotechnology Incorporated. It is prepared against the C-terminal part of rat Kv2.1, amino acids 837–853 (Sharma *et al.* 1993).

Data analysis

All data are presented as means ± S.E.M. Statistical analyses were performed using Student's t test (paired or unpaired); $P < 0·05$ was defined as significant.

Chemicals

Sodium dithionite and charybdotoxin were obtained from Sigma Chemical Co.

RESULTS

The K_{O_2} channel in PC12 cells belongs to the Kv1 subfamily of K_V channels

PC12 cells express a slow-inactivating K_V current (I_K) that is inhibited by hypoxia (Fig. 1*A*). Potassium currents recorded in a normoxic environment (N; 21% O_2) were inhibited by 22 ± 3% ($n = 6$) by hypoxia (H = 10% O_2), as previously shown (Zhu *et al.* 1996). Charydbotoxin (CTX), a

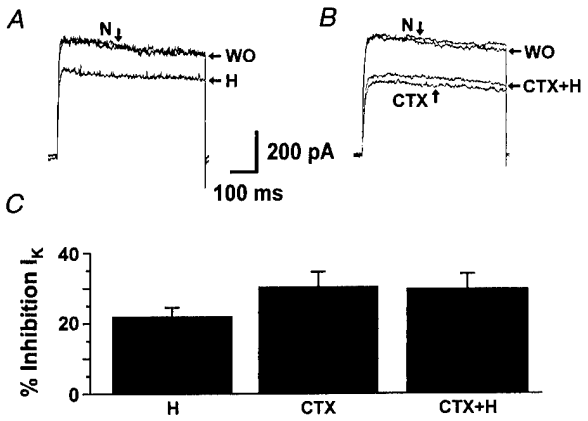

Figure 1. Sensitivity to charybdotoxin (CTX) of the K_{O_2} current recorded in PC12 cells

K^+ currents (I_K) were elicited by depolarizing voltage steps to +50 mV from a holding potential of −70 mV (every 5 s) in the whole-cell configuration. *A*, effect of hypoxia (H; 10 % O_2) in absence of CTX. Control currents were recorded in normoxia (N; 21 % O_2). WO indicates currents recorded after returning to normoxia. *B*, effect of hypoxia in the presence of CTX (20 nM). After steady-state inhibition of the K^+ current by CTX was reached, cells were exposed to H in the presence of CTX. WO indicates currents recorded after returning to normoxia without CTX. *C*, mean K^+ current inhibition by hypoxia alone (H, $n = 6$), CTX alone ($n = 7$) and hypoxia in the presence of CTX (CTX+H, $n = 7$).

potent blocker of Kv1.2 and Kv1.3, was used to ascertain the molecular nature of the K_{O_2} channel in PC12 cells (Grissmer *et al.* 1994; Russell *et al.* 1994). The effect of CTX on the K^+ current and the hypoxic inhibition of the K^+ current in the presence of CTX were studied in whole-cell configuration (Fig. 1*B*). Potassium currents recorded in a normoxic environment (N) were inhibited by 31 ± 6 % ($n = 7$) by CTX (20 nM). This amount of K^+ current inhibition by CTX is not statistically different from that induced by hypoxia only. Subsequent exposure of these cells to hypoxia in the presence of CTX (CTX+H) did not induce further inhibition ($n = 7$). These responses were reversible

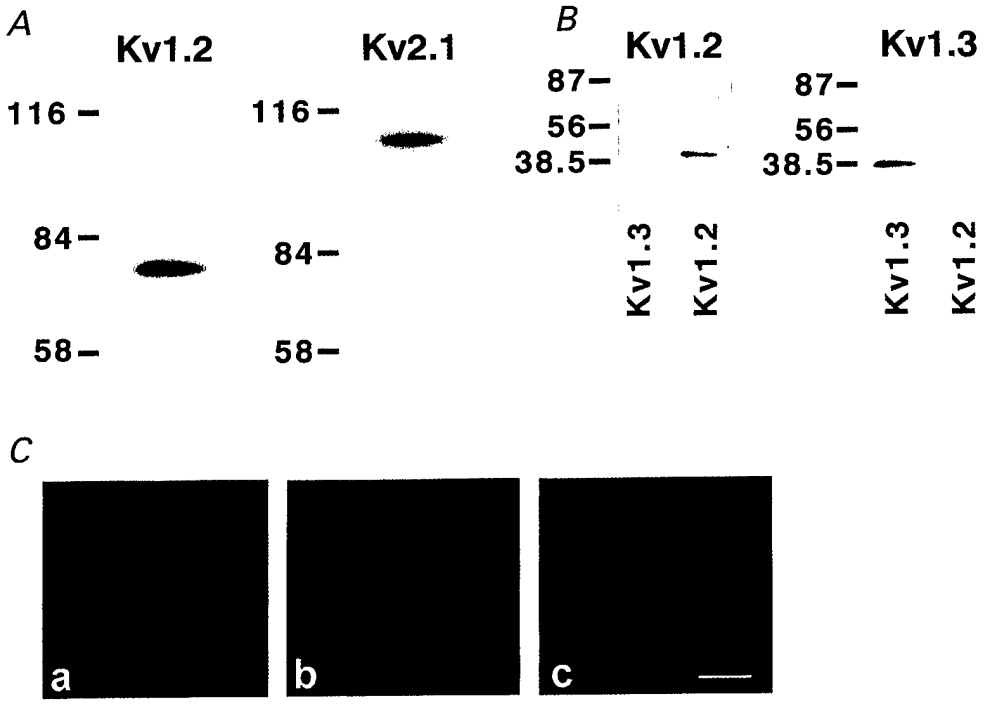

Figure 2. Expression of the Kv1.2 and Kv2.1 α-subunits of K^+ channels in PC12 cells and specificity of anti-Kv1.2 antibody

A, Kv1.2 and Kv2.1 polypeptide expression in PC12 cell total lysate. Immunoblots of rat PC12 cell protein (40 μg) were incubated with affinity-purified anti-Kv1.2 or anti-Kv2.1 antibodies. Molecular mass markers are indicated on the left in kilodaltons (kDa). *B*, immunoblots of GST-fusion proteins (50 ng) for Kv1.3 and Kv1.2 (indicated at the bottom of the blot) were incubated with anti-Kv1.2 antibody (left panel) or anti-Kv1.3 antibody (right panel). *C*, immunostaining of PC12 cells with anti-Kv1.2 antibody. Panel *a*, background staining of PC12 cells that were subjected to all steps in the staining protocol, except that the primary antibody was omitted. Panel *b*, labelling of PC12 cell membranes with anti-Kv1.2 antibody. Panel *c*, immunostaining of PC12 cell with anti-Kv1.2 antibody pre-incubated with the antigen against which the antibody is directed. The intensity of the fluorescent signal is comparable to the background fluorescence observed in panel *a*. Scale bar = 20 μm and applies to all panels in *C*.

upon returning to toxin-free normoxic conditions. These data show that CTX is able to inhibit O_2-sensitive K^+ channels, implying that the O_2-sensitive K^+ current in PC12 cells is carried by *Shaker*-type K_V channels.

Importance of the Kv1.2 α-subunit in the response to hypoxia in PC12 cells

We showed previously that exposure of PC12 cells to prolonged hypoxia increased the expression of the Kv1.2 gene, which in turn correlated with an enhanced O_2 sensitivity of the K_V current (Conforti & Millhorn, 1997). PC12 cells also express the Kv2.1 α-subunit which according to previous data is not O_2 sensitive in these cells (Conforti & Millhorn, 1997). Expression of Kv1.2 and Kv2.1 α-proteins was determined by immunoblot analysis (Fig. 2*A*). Western blot analysis with an affinity-purified antibody against Kv1.2 revealed a single band of ~80 kDa. Antibodies against Kv2.1 detected a single band of ~110 kDa. A similar band has been previously identified as the Kv2.1 α-subunit in PC12 cells (Sharma *et al.* 1993). The specificity of anti-Kv2.1 antibody has been previously demonstrated (Archer *et al.* 1998). The specificity of Kv1.2 antibody was established by immunoblot and immunohistochemical analyses. Anti-Kv1.2 antibody recognizes a single band of the predicted molecular mass in PC12 cell total lysate (Fig. 2*A*). This antibody did not cross-react with Kv1.3, another member of the Kv1 subfamily of K_V channels (Fig. 2*B*). Anti-Kv1.2 antibody recognized the Kv1.2 fusion protein, and did not cross-react with Kv1.3 antigen. The Kv1.3 antigen was recognized only by the anti-Kv1.3 antibody. The specificity of anti-Kv1.2 antibody was confirmed in immunohistochemical experiments. The intense, uniform labelling of the PC12 cell with anti-Kv1.2 antibody is shown in Fig. 2*Cb*. Background fluorescence in the absence of Kv1.2 antibody is shown in panel *a*. Comparable background fluorescence was observed when the anti-Kv1.2 antibody was pre-incubated with an excess of the matching Kv1.2 fusion protein (panel *c*), indicating the specificity of the antibody for Kv1.2. The ability of anti-Kv1.2 antibody to selectively block Kv1.2 channels was assessed in *Xenopus* oocytes (Fig. 3). Recombinant Kv1.2 current amplitude was significantly decreased in oocytes injected with anti-Kv1.2 antibody. A significant Kv1.2 current inhibition ranging from 44 to 82% was observed at different voltages (−10, 0, 20 and 50 mV) in a total of 13 oocytes. The same concentration of anti-Kv1.2 antibody did not reduce K^+ current amplitude measured at 0 and 20 mV in oocytes expressing Kv2.1 channels ($n = 14$). Figure 3 compares the effect of anti-Kv1.2 antibody on the K^+ current measured in oocytes expressing Kv1.2 channels with oocytes from the same batch expressing Kv2.1 channels.

We next tested the hypothesis that the K_{O_2} channel in PC12 cells is composed of Kv1.2 α-subunit(s) by comparing the efficiency of anti-Kv1.2 and anti-Kv2.1 antibodies in blocking the K_{O_2} current. Whole-cell voltage-clamp experiments were performed with anti-Kv1.2 or anti-Kv2.1 antibodies delivered to the cell by dialysis through the patch pipette. Figure 4 shows representative experiments performed in the presence (*A*) of anti-Kv1.2 antibody in the pipette. The left panel shows K^+ currents recorded in normoxia upon breaking into the whole-cell configuration (N_0). Within 8–10 min after breaking into the whole-cell configuration, dialysis of anti-Kv1.2 antibody (Kv1.2 Ab) through the patch pipette resulted in a $32 \pm 6\%$ ($n = 6$) decrease in K^+ current amplitude. Subsequent exposure to hypoxia (H, 10% O_2) did not inhibit the K^+ current. The averaged inhibition of the K^+ current by hypoxia in cells dialysed with antibody against Kv1.2 was $4 \pm 3\%$ ($n = 6$; Fig. 4*C*). Identical experiments were performed with anti-Kv2.1 antibody in the patch pipette (Fig. 4*B*). Within 8–10 min after breaking into whole-cell configuration dialysis of anti-Kv2.1 antibody (Kv2.1 Ab) through the patch pipette resulted in a $39 \pm 3\%$ ($n = 3$) decrease in K^+ current amplitude. Subsequent exposure to hypoxia (H, 10% O_2) inhibited the K^+ current by $24 \pm 2\%$ ($n = 3$). This amount of inhibition is significantly different from that observed in cells dialysed with anti-Kv1.2 antibody ($P < 0{\cdot}01$). Control experiments using an irrelevant antibody (rabbit anti-sheep IgG) in the pipette are shown in Fig. 4*C*. Ten minutes after breaking into the whole-cell configuration, no decrease in K^+ current amplitude was observed, but application of hypoxia caused a reversible inhibition of the K^+ current ($26 \pm 1\%$, $n = 3$). This level of K^+ current inhibition is not statistically different from the hypoxic inhibition in the presence of anti-Kv2.1 antibody and is also comparable to that induced by hypoxia in the absence of irrelevant antibody in the patch pipette (Fig. 1*A*; Zhu *et al.* 1996). These data suggest that the Kv1.2 α-subunit, but not Kv2.1, is critical in the response of PC12 cells to hypoxia.

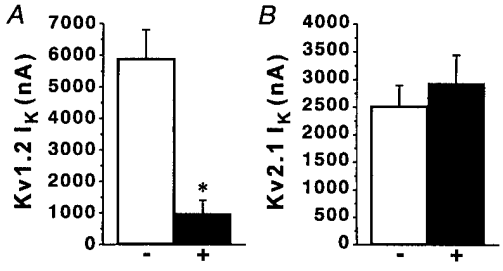

Figure 3. Effect of anti-Kv1.2 antibody on recombinant Kv1.2 and Kv2.1 channels

A, anti-Kv1.2 antibody blocks K^+ current (I_K) in oocytes expressing Kv1.2 channels. Kv1.2 currents were recorded in control oocytes (−, $n = 6$) and oocytes injected with anti-Kv1.2 antibody (0·01 μg in 50 nl) 2 h before recording (+, $n = 4$). * $P < 0{\cdot}01$ using Student's unpaired t test. *B*, lack of effect of anti-Kv1.2 antibody on K^+ currents in oocytes expressing Kv2.1 channels. Kv2.1 currents were recorded in control oocytes (−, $n = 5$) and anti-Kv1.2-injected oocytes (+, $n = 6$). I_K were elicited with voltage steps from a holding potential of −80 mV to between −10 and 0 mV in two-electrode voltage-clamp experiments.

O_2 sensitivity of Kv1.2 channels expressed in *Xenopus* oocytes

Because of the complex heteromeric structure of native K^+ channels, we studied the Kv1.2 channel responses to changes in P_{O_2} in the *Xenopus* oocytes. This expression system provides a means of expressing K_V channels of known composition. The sensitivity of Kv1.2 channels to hypoxia was compared to that of Kv2.1 channels, which are also expressed in PC12 cells (Sharma *et al.* 1993; Conforti & Millhorn, 1997). Run-off transcripts of cRNA were prepared and microinjected into *Xenopus* oocytes. Control oocytes were injected with the same volume (50 nl) of water. Electrophysiological experiments were performed 1–2 days after injection. Application of depolarizing voltage steps elicited outward K^+ currents only in oocytes injected with K^+ channel cRNAs (data not shown). The effect of hypoxia on the expressed K_V channels was studied by exposing the injected oocytes to an anoxic recording medium (100% N_2 and 5 mM sodium dithionite, $N_2S_2O_4$, an O_2 chelator; Fig. 5). Anoxia inhibited the K^+ current carried by Kv1.2 channels by $11 \pm 4\%$ ($n = 8$), and had no effect or slightly increased the K^+ current carried by Kv2.1 channels ($n = 8$). The hypoxic inhibition of Kv1.2 current was reversed upon returning to normoxia (Fig. 5*A* and *B*). The time course of the hypoxic response of Kv1.2 channels is shown in Fig. 5*B* (representative of 4 separate experiments). Inhibition of the K^+ current occurs at the onset of the anoxic medium and K^+ current returns to control values upon re-introduction of the normoxic medium. The effect of anoxia on the current–voltage relationships for K^+ current in Kv1.2 and Kv2.1

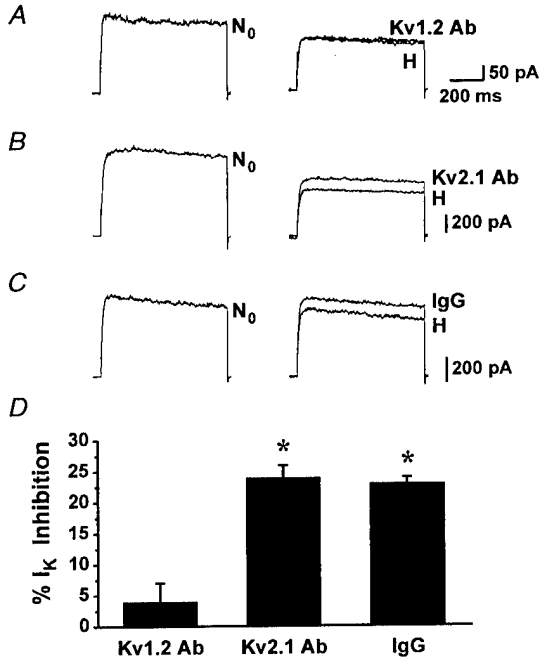

Figure 4. Effect of hypoxia on the K^+ current after selective block of the Kv1.2 and Kv2.1 channels by their corresponding specific antibodies

K^+ currents (I_K) were elicited with voltage steps from a holding potential of −70 mV to +50 mV (every 5 s) in experiments performed in presence of anti-Kv1.2 antibody (*A*), anti-Kv2.1 antibody (*B*) or irrelevant antibody (*C*) in the pipette. The representative K^+ current traces were recorded in normoxia (21 % O_2) upon breaking into the whole-cell configuration (left panel, N_0), in normoxia 8–10 min into the whole-cell configuration (right panel, labelled with the name of the antibody used in each experiment), and after exposure to hypoxia (10 % O_2, H). *D*, mean current inhibition by hypoxia in the presence of each antibody. * $P \leqslant 0{\cdot}001$.

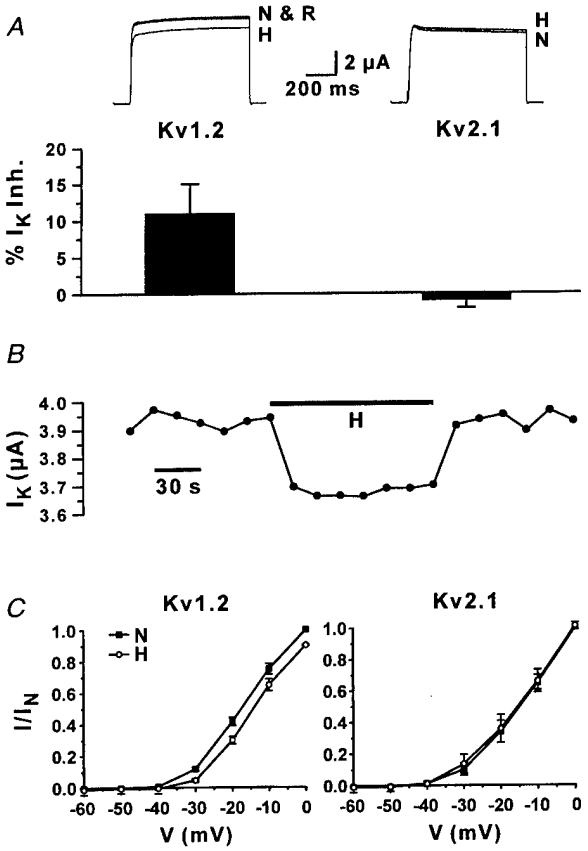

Figure 5. Oxygen sensitivity of Kv1.2 and Kv2.1 currents in *Xenopus* oocytes

A, hypoxic inhibition of K^+ currents (I_K) in oocytes injected with Kv1.2 or Kv2.1 cRNAs. K^+ currents were recorded in control conditions (N; 21 % O_2), after 2 min exposure to anoxia (H), and after returning to normoxia (R) with the two-electrode voltage-clamp technique. Currents were elicited with depolarizing voltage steps from a holding potential of −80 mV to 0 mV. The averaged data ($n = 8$) are shown in the bottom panel. *B*, time course of the effect of anoxia on the K^+ current amplitude in Kv1.2-injected oocytes. Depolarizing voltage steps were applied every 15 s (same protocol as in *A*). Bar (H) corresponds to the time of perfusion with the anoxic medium. *C*, current–voltage relationship of Kv1.2 (left) and Kv2.1 (right) cRNA-injected oocytes. K^+ currents were induced by depolarizing voltage steps from −60 to 0 mV (10 mV increments; holding potential 80 mV). Currents were measured in normoxia (21 % O_2) and 2 min after exposure to anoxia (H). I_N corresponds to the maximum I_K measured in normoxia. Values are reported as mean ± S.E.M. ($n = 5$ for Kv1.2, $n = 3$ for Kv2.1)

cRNA-injected oocytes is shown in Fig. 5*C*. The current–voltage (*I–V*) relationships were measured in normoxia (N), after 2 min exposure to anoxia (H) and 2 min after returning to the normoxic medium (R). For the sake of clarity, the *I–V* relationship after returning to normoxia is not shown in the figure. Anoxia induced inhibition of K^+ current at each potential only in oocytes injected with Kv1.2 ($n = 5$). No response, or a slight irreversible increase in K^+ current was observed in Kv2.1-injected oocytes ($n = 3$).

The single-channel properties of Kv1.2 channels and their response to hypoxia (10% O_2) were studied in the cell-attached configuration (Fig. 6). The single-channel *I–V* relationship for the Kv1.2 channels is shown in Fig. 6*A*. These channels have slope conductance of 18 pS (2·8 mM external K^+ concentration), which is comparable to the conductance of the K_{O_2} channel measured in PC12 cells (Conforti & Millhorn, 1997). Application of depolarizing voltage steps to +50 mV induced a slow-inactivating K^+ current that was inhibited by hypoxia ($n = 4$). The effect of hypoxia on the activity of a slow-inactivating single channel of 2 pA unitary current is shown in Fig. 6*B*. In this experiment, exposure to hypoxia (10% O_2) induced a 33% inhibition of the ensemble-averaged current amplitude and a 16% reduction in NP_o (unitary current amplitude was unchanged). When multiple Kv1.2 channels were present in a patch it was possible to record an outward K^+ current that resembled a macroscopic K^+ current ($n = 3$). The time course of the effect of hypoxia (10% O_2) on the Kv1.2 current amplitude in cell-attached patches is shown in Fig. 6*C*. Perfusion with hypoxic medium resulted in the immediate inhibition of the K^+ current. The inhibition reached steady-state values after *ca* 2 min of exposure to hypoxia. Currents recorded in normoxia (N) and in hypoxia (H) after steady-state inhibition was reached are reported as an inset. The mean K^+ current inhibition by hypoxia in cell-attached experiments was 65 ± 10% ($n = 7$).

DISCUSSION

The mechanisms by which O_2-sensitive cells detect a change in O_2 tension (P_{O_2}) and transduce this signal into the appropriate functional response remain unknown. However, it has become evident that O_2-sensitive K^+ (K_{O_2}) channels are key elements in the detection of changes in O_2 availability by excitable O_2-sensitive cells (Lopez-Barneo, 1996). Currently, neither the molecular composition of these important channels nor the mechanism(s) by which they respond to changes in P_{O_2} are known. The observation that the hypoxic inhibition of K_{O_2} channel activity occurs in excised patches from carotid body type I cells, PC12 cells and central neurons suggests it might occur via membrane-associated events (Ganfornina & Lopez-Barneo, 1992; Conforti & Millhorn, 1997; Haddad & Jiang, 1997). Various membrane-bound molecules have been proposed as the O_2 sensor, including NADPH-oxidase, metal-binding proteins and the auxiliary β-subunit of K_V channels (Acker, 1994; Haddad & Jiang, 1997; Gulbis *et al.* 1999). It has also been proposed that O_2 could interact directly with the K_{O_2} channel itself by modifying the redox state of amino acid residues in the pore-forming α-subunits and inducing a change in the channel molecular conformation (Ruppersberg

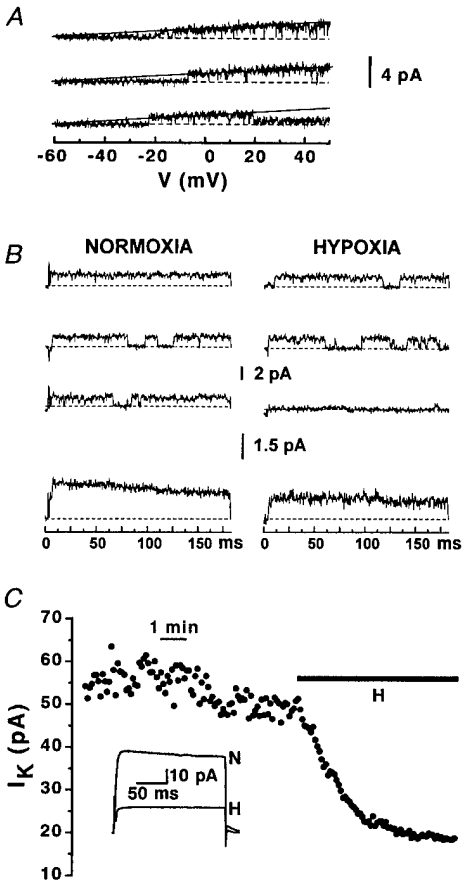

Figure 6. Response to hypoxia of Kv1.2 channels in *Xenopus* oocytes

A, conductance of the Kv1.2 channels. The *I–V* curves were obtained with ramp pulse depolarization from a holding potential of −60 mV to +50 mV, 800 ms duration. Experiments were performed in high K^+ bath solution and 2·8 mM K^+ pipette solution. Dashed lines represent the zero current. The recordings were fitted with a straight line, which had a slope value of 18 pS. *B*, the top panels show representative traces recorded during step depolarizing pulses (from a holding potential of −60 mV to +50 mV) in normoxia and 2 min after exposure to hypoxia (10% O_2). Leak and capacitative currents were subtracted from the record. The upward current deflections from the zero line (dashed) correspond to the opening of the channel. The corresponding ensemble-averaged currents (from 100 consecutive traces) are shown in the bottom panels. *C*, time course of the hypoxic inhibition of K^+ current recorded in a cell-attached patch containing multiple Kv1.2 channels. Bar indicates the time of perfusion with the hypoxic medium (10% O_2). K^+ currents were induced by depolarizing voltage steps from a holding potential of −60 mV to +50 mV. The averaged currents from 100 consecutive traces recorded in normoxia (N) and after steady-state I_K inhibition by hypoxia (H) are shown as an inset.

et al. 1991; Lopez-Barneo, 1996). Thus, elucidation of the molecular nature of K_{O_2} channels in different O_2-sensitive cells is an important step towards understanding their role in O_2 sensing. The current research provides evidence that the K_{O_2} channel in the O_2-sensitive PC12 clonal cell line is a K_V channel composed of Kv1.2 α-subunit(s). To our knowledge, this is the first direct evidence of the O_2 sensitivity of native Kv1.2 α-subunits of K^+ channels.

We previously reported that the K_{O_2} current in PC12 cells has slow-inactivating kinetics, is insensitive to Ca^{2+} and holding voltage, and is blocked by 5 mM externally applied TEA (Zhu *et al.* 1996). High doses of extracellular TEA are required for blockade of Kv1.2, 1.3 and 1.5 channels (Mathie *et al.* 1998). The current data confirm that the K_{O_2} channel in PC12 cells belongs to the Kv1 subfamily of K^+ channels. The K_{O_2} current in PC12 cells is inhibited by charybdotoxin, a potent blocker of Kv1.2 and Kv1.3 and large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels. Although K_{Ca} channels are present in PC12 cells, we have shown previously that, under our experimental conditions, their contribution to the total outward current is negligible (Zhu *et al.* 1996). We have also shown that the K_{Ca} channels in PC12 cells are not inhibited by hypoxia (Conforti & Millhorn, 1997).

Additional evidence supports the conclusion that the K_{O_2} channel in PC12 cells is formed by Kv1.2 α-subunit(s). We showed previously that the gene encoding the Kv1.2 α-subunit is selectively stimulated during prolonged exposure to hypoxia, and that the increased expression of the Kv1.2 α-subunit gene correlated with an enhanced response to hypoxia (Conforti & Millhorn, 1997). On the other hand, the Kv2.1 α-subunit is also expressed in PC12 cells but its expression does not increase during prolonged hypoxia (Conforti & Millhorn, 1997). Here we showed that Kv1.2 and Kv2.1 α-proteins are expressed in PC12 cells. Western blot analysis with the antibody against Kv1.2 revealed a single band of ~80 kDa. A band of similar size has been identified as a Kv1.2 α-subunit in Kv1.2 stably transfected cells and in pulmonary artery smooth muscle cells (Barry *et al.* 1995; Archer *et al.* 1998). The specificity of the anti-Kv1.2 antibody was established by us in immunohistochemical and Western blot experiments. We have also established the feasibility of using the anti-Kv1.2 antibody to selectively block the K^+ current carried by Kv1.2 channels. To our knowledge, this is the first evidence that this antibody can be used as a selective blocker of its own channels. Therefore, we next tested the hypothesis that the K_{O_2} channel in PC12 cells is composed of Kv1.2 α-subunit(s) by using antibodies against Kv1.2 as blockers of this channel and by comparing these results with similar experiments performed in the presence of anti-Kv2.1 antibody. We used the anti-Kv1.2 antibody (the same antibody that was used for Western blotting) that binds to the O_2-sensitive α-subunit to block K_{O_2} channel activity in PC12 cells. A similar approach has been used to establish the role of Kv2.1 in setting the resting potential of pulmonary artery smooth muscle cells (Archer *et al.* 1998). In addition, the same approach was successfully used to modify ion channel activity in neuronal and skeletal muscle cells (Vassilev *et al.* 1988; Naciff *et al.* 1996). An irrelevant antibody, which was shown previously to have no effect on K^+ and Ca^{2+} currents, was used as a negative control (Naciff *et al.* 1996). Dialysis of Kv1.2 and Kv2.1 antibodies through the patch pipette resulted in a decrease in K^+ current, which occurred gradually and reached a maximum in 8–10 min. A similar time course was reported for the effect on K^+ current of anti-annexin VI antibody delivered through the patch pipette (Naciff *et al.* 1996). Dialysis of PC12 cells with specific antibodies against the Kv1.2 α-subunit prevented the hypoxia-induced inhibition of voltage-activated K^+ current. Cells that were dialysed with anti-Kv2.1 antibody maintained their response to hypoxia. This important finding suggests that a functional Kv1.2 α-subunit is necessary for the response of the K_{O_2} channel to hypoxia and that the Kv2.1 channels are not O_2 sensitive.

The O_2 sensitivity of Kv1.2 was also confirmed in *Xenopus* oocytes. The O_2 sensitivity of Kv1.2 was compared to that of Kv2.1, which has been proposed as a possible O_2-sensitive K^+ channel in pulmonary artery smooth muscle cells (Patel *et al.* 1997; Archer *et al.* 1998). The Kv2.1 channel is also expressed in PC12 cells, although the current and previous data indicate that it does not mediate the O_2-sensitive current in these cells (Sharma *et al.* 1993; Conforti & Millhorn, 1997). In *Xenopus* oocytes anoxia inhibited the K^+ current carried by Kv1.2, and had no effect or even slightly increased the K^+ current carried by Kv2.1. The hypoxic inhibition of Kv1.2 current was reversed upon returning to normoxia. Although a relatively small inhibition is induced by anoxia in intact oocytes injected with Kv1.2, the time course of the response highly correlates with the arrival of the anoxic medium to the perfusion chamber and the return of normoxic conditions. Moreover, the anoxic inhibition of the K^+ current occurs over a whole range of potentials. The anoxia needed to inhibit the K^+ current in intact oocytes was obtained with the use of the O_2 chelator sodium dithionite, which is known to induce the formation of oxygen radicals (Archer *et al.* 1995). However, the inhibition of K^+ current is most probably not due to the formation of oxygen radicals, since Kv1.2 and Kv2.1 channels expressed in *Xenopus* oocytes have been shown to be insensitive to reactive oxygen species (Duprat *et al.* 1995). Furthermore, the activity of Kv1.2 channels in cell-attached experiments is inhibited by exposure to hypoxia obtained by saturating the perfusion medium with 10% O_2, without any sodium dithionite present. Single-channel experiments in oocytes injected with Kv1.2 cRNA showed that exposure to hypoxia (10% O_2; ~80 mmHg P_{O_2}) induced an inhibition of Kv1.2 ensemble-averaged current, which was associated with a reduction in NP_o and no change in unitary current amplitude. Detailed single-channel

analysis was not possible because many patches contained a small conductance endogenous channel. The presence of endogenous delayed-rectifier K^+ channels in *Xenopus* oocytes has been reported (Lu *et al.* 1990). The single-channel experiments were performed using the same K^+ gradient used previously to study the K_{O_2} channel in PC12 cells (Conforti & Millhorn, 1997). Thus, the Kv1.2 channel expressed in the oocyte had the same single-channel properties (conductance and inactivation kinetics) and displayed the same type of response to hypoxia as the K_{O_2} channel in PC12 cells. The lower sensitivity of recombinant Kv1.2 current in *Xenopus* oocytes observed in the two-electrode voltage-clamp experiments might be due to the presence of the vitelline membrane, which is likely to constitute a barrier to O_2 diffusion. It was previously reported that the follicular tissues surrounding the *Xenopus* oocytes (including the vitelline membrane) reduce the access of various compounds to the oocyte plasma membrane (Stuhmer & Parekh, 1995; Madeja *et al.* 1997). Moreover, Madeja *et al.* have shown that the vitelline membrane alone can be responsible for a substantial portion of this barrier effect. The current experiments using cell-attached patches of oocytes free of the vitelline membrane showed that hypoxia is able to induce a higher I_K inhibition than in intact oocytes. Although this might support the fact that the vitelline membrane itself might interfere with the O_2 sensitivity of the plasma membrane, the longer time course to reach steady-state inhibition induced by hypoxia in vitelline-free oocytes compared to intact oocytes and the irreversibility of the hypoxic effect suggests that other events might be activated during hypoxia in the absence of vitelline membrane, and that they eventually further inhibit the K^+ current. If this were the case, an alternative explanation of the lower O_2 sensitivity observed in oocytes compared to PC12 cells might reside in the different levels of expression of endogenous auxiliary subunits important in O_2 sensing. We have recent evidence that PC12 cells express Kvβ2 and Kvβ3 subunits (data not shown). It was recently suggested that Kvβ2 (which is known to associate to Kv1.2 channels) might have an important role in O_2 sensing (Gulbis *et al.* 1999). To our knowledge, the expression of these subunits in *Xenopus* oocytes has not been reported.

The experiments in *Xenopus* oocytes confirmed that the Kv1.2 channel is O_2 sensitive. In contrast, Kv2.1 channels expressed in oocytes were not inhibited by hypoxia. Our results in *Xenopus* oocytes contrast with those obtained using different expression systems. In monkey kidney COS cells Kv2.1 and Kv2.1–Kv9.3 currents were inhibited by hypoxia, while the Kv1.2 current was not O_2 sensitive (Patel *et al.* 1997). Recently, it has been shown that both Kv1.2 and Kv2.1 channels expressed in mouse L cells are inhibited by hypoxia (Hulme *et al.* 1999). The discrepancy between these expression systems might arise from the fact that they express different 'O_2 sensors' or different signalling pathways. Although the nature of the O_2 sensor remains obscure, it appears that the *Xenopus* oocytes behave in a manner similar to the O_2-sensitive PC12 cells. In both these cell types (PC12 cells and oocytes) only Kv1.2 channels are O_2 sensitive while Kv2.1 channels are not.

Relatively little is known about the subunits forming the O_2-sensitive K^+ channels in other chemosensitive cells. Most of the information available is derived from pulmonary artery smooth muscle cells. These cells express voltage-dependent CTX-insensitive outwardly rectifying K^+ channels inhibited by hypoxia (Patel *et al.* 1997; Archer *et al.* 1998). The Kv2.1 and Kv2.1–Kv9.3 subunits, present in pulmonary artery, have been proposed to form the K_{O_2} channel in this tissue (Patel *et al.* 1997; Archer *et al.* 1998). These subunits expressed in COS cells give rise to 8 and 14·5 pS K^+ channels (physiological K^+), respectively, which are inhibited by hypoxia (Patel *et al.* 1997). The conductances of the Kv2.1 and Kv2.1–Kv9.3 channels contrast with the conductance of the K_{O_2} channel in PC12 cells (20 pS). Archer *et al.* (1998) have proposed that pulmonary artery smooth muscle cells express two O_2-sensitive K^+ channels: Kv2.1, which might be important for initiating the hypoxia-induced depolarization, and Kv1.5, which might be important in the hypoxia-induced increase in intracellular Ca^{2+}. Although the presence of these subunits in pulmonary artery and their role in cell excitability have been well established, there is no direct evidence (e.g. loss of O_2 sensitivity upon selective blockade of these channels) that implicates them as O_2-sensitive K^+ channels. The different pharmacological profile of the K_{O_2} channel recorded in pulmonary artery smooth muscle cells (insensitive to CTX) compared to that of the K_{O_2} channels in PC12 cells does not exclude the possibility that the K_{O_2} channel in pulmonary artery might also include one or more Kv1.2 α-subunits (Patel *et al.* 1997; Archer *et al.* 1998). For example, the Kv1.5–K_{O_2} channel might be a heteromultimer formed by Kv1.5 and Kv1.2 α-subunits. In fact, it has been shown that a single CTX-insensitive Kv1.5 α-subunit can render the Kv1.2–Kv1.5 heteromeric channel insensitive to CTX (Russell *et al.* 1994). Recently, it has been shown that expression of Kv1.2 and Kv1.5 α-subunits in pulmonary artery smooth muscle cells is downregulated by chronic hypoxia (Wang *et al.* 1997). The downregulation of expression of these subunits correlates with the decreased O_2 sensitivity of pulmonary artery in rats exposed to chronic hypoxia. The hypothesis that the Kv1.5–K_{O_2} channel in pulmonary artery is indeed a heteromultimeric channel formed by Kv1.2 and Kv1.5 α-subunits, and that the Kv1.2 α-subunit is responsible for its O_2 sensitivity is supported by recent findings showing that co-expression of Kv1.2 and Kv1.5 α-subunits in mouse L cells give rise to O_2-sensitive Kv1.2–Kv1.5 heteromeric channels, while homomeric Kv1.5 channels are not O_2 sensitive (Hulme *et al.* 1999).

In the current study, we have presented direct evidence which indicates that the Kv1.2 α-subunit is an important

component of native O_2-sensitive K^+ channels expressed in chemosensitive cells. The same α-subunit might also be an important component of native K_{O_2} channels in other chemosensitive cells. Although the Kv1.2 α-subunit is expressed in many different tissues, it might play a special role in chemosensitive cells via a coupling with the O_2 sensor. Alternatively, the Kv1.2 α-subunit might be coupled to an O_2-sensitive signalling pathway activated only in chemosensitive cells.

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Corresponding author

L. Conforti: Department of Internal Medicine, Division of Nephrology and Hypertension, 231 Bethesda Avenue, University of Cincinnati, Cincinnati, OH 45267-0585, USA.

Email: laura.conforti@uc.edu

Hypoxia Differentially Regulates the Mitogen- and Stress-Activated Protein Kinases

Role of Ca^{2+}/CaM in the activation of MAPK and p38γ

P. William Conrad, David E. Millhorn, and Dana Beitner-Johnson
Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, PO Box 67-0576, Cincinnati, OH 45267-0576

Key words: MAPK, ERK, JNK, p38, SAPK

Abstract:

Hypoxic/ischemic trauma is a primary factor in the pathology of various vascular, pulmonary, and cerebral disease states. Yet, the signaling mechanisms by which cells respond and adapt to changes in oxygen levels are not clearly established. The effects of hypoxia on the stress-and mitogen-activated protein kinase (SAPK and MAPK) signaling pathways were studied in PC12 cells. Exposure to moderate hypoxia (5% O_2) was found to progressively stimulate phosphorylation and activation of p38γ in particular, and also p38α, two isoforms of the p38 family of stress-activated protein kinases. In contrast, hypoxia had no effect on enzyme activity of p38β, p38β_2, p38δ, or on JNK, another stress-activated protein kinase. Prolonged hypoxia also induced phosphorylation and activation of p42/p44 MAPK, although this activation was modest when compared to NGF and UV-induced activation. We further showed that activation of p38γ and MAPK during hypoxia requires calcium, as treatment with Ca^{2+}-free media or the calmodulin antagonist, W13, blocked the activation of p38γ and MAPK, respectively. These studies demonstrate that an extremely typical physiological stress (hypoxia) causes selective activation of specific elements of the SAPKs and MAPKs, and identifies Ca^{+2}/CaM as a critical upstream activator.

1. INTRODUCTION

Mammalian cell function is critically dependent on a continuous supply of oxygen. Even brief periods of oxygen deprivation (hypoxia/ischemia) can result in profound cellular and tissue damage. Thus, it is vital that organisms

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meet changes in O_2 tension with appropriate cellular adaptations. However, the specific intracellular pathways by which this occurs are not well-delineated. The stress- and mitogen-activated protein kinase (SAPK and MAPK) pathways play a critical role in responding to cellular stress and promoting cell growth and survival (Widmann et al., 1999; Su and Karin, 1996). We therefore investigated the effect of hypoxia on the SAPK and MAPK signaling pathways.

SAPKs and MAPKs are the downstream components of three-member protein kinase modules (Garrington and Johnson, 1999) (Figure 1). Five homologous subfamilies of these kinases have been identified, and the three major families include p38/SAPK2/RK, JNK/SAPK, and p42/p44 MAPKs/ERKs (Widmann et al., 1999; Su and Karin, 1996; Garrington and Johnson, 1999; Rouse et al., 1994; Raingeaud et al., 1995; Kyriakis and Avruch, 1996). In general, the stress-activated protein kinases (p38 and JNK) are activated primarily by noxious environmental stimuli, such as ultraviolet light, osmotic stress, inflammatory cytokines, and inhibition of protein synthesis (Hibi et al., 1993; Derijard et al., 1994; Kyriakis et al., Han et al., 1994). In contrast, p42/p44 MAP kinases are primarily stimulated by mitogenic and differentiative factors in a Ras-dependent manner (Raingeaud et al., 1995; Woodgett et al., 1996; Whitmarsh and Davis, 1994), although these enzymes can also be activated by certain environmental stressors (Widmann et al., 1999; Su and Karin, 1996; Garrington and Johnson, 1999). Thus, we hypothesized that hypoxia, a prevalent physiological stressor in many disease states, may regulate the activity of the SAPK and MAPK signaling pathways.

The PC12 cell line is a catecholaminergic, excitable cell type that has been widely used as an *in vitro* model for neural cells (Green, 1995). It has also been shown that PC12 cells are an O_2-sensitive cell type that provides a useful system to study the effects of hypoxia on catecholaminergic gene expression (Czyzk-Krzeska et al., 1994; Norris and Millhorn, 1995; Levy et al., 1995; Raymond and Millhorn, 1997; Beitner-Johnson and Millhorn, 1998). Very small reductions in atmospheric O_2 dramatically induce tyrosine hydroxylase gene expression and mRNA stability in this cell type (Czyzk-Krzeska et al., 1994). Hypoxia also induces activation of the CREB and c-Fos transcription factors in this cell type (Norris and Millhorn, 1995; Beitner-Johnson and Millhorn, 1998; Prabhakar et al., 1995). Finally, PC12 cells also express hypoxia-regulated ion channels, as shown by the finding that PC12 cells depolarize during hypoxia via an oxygen-regulated K^+ current (Zhu et al., 1996; Kumar et al., 1998) and secrete dopamine and norepinephrine (Kumar et al., 1998; Taylor and Peers, 1999). Thus, we have used this cell type to study the regulation of intracellular signaling systems by hypoxia.

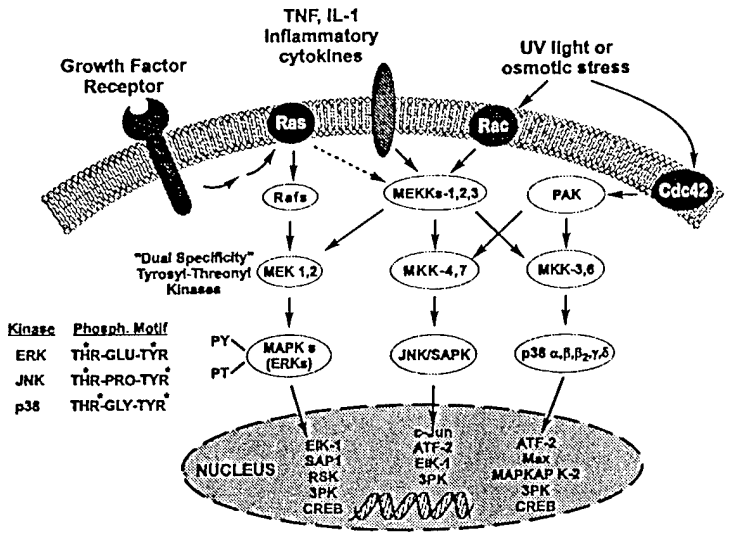

Figure 1. Overview of the MAPK and SAPK signalling pathways.

2. THE p38 FAMILY OF PROTEIN KINASES ARE DIFFERENTIALLY REGULATED BY HYPOXIA

The p38 family of protein kinases consists of several isoforms, including p38α, p38β, p38β2, p38γ/SAPK3/ERK6, and p38δ/SAPK4 (Rouse et al., 1994; Han et al., 1994; Li et al., 1996; Jiang et al., 1996; Jiang et al., 1997; Lechner et al., 1996; Mertens et al., 1996; Cuenda et al., 1997; Stein et al., 1997; Wang et al., 1997). To investigate the effects of hypoxia on the p38 family of protein kinases, PC12 cells were exposed to 5% O_2 for various times, between 20 min and 6 hr, followed by immunoblotting with an antibody specific for thr^{180}/tyr^{182}-phosphorylated p38α. It can be seen in Figure 2A that exposure to hypoxia progressively induced phospho-p38 immunoreactivity in two closely migrating bands. Phospho-p38 blots were then stripped and re-blotted with an antibody that equally recognizes phospho-and dephospho-p38α (*i.e.*, total p38α). Figure 2B shows that the lower phospho-p38 immunoreactive protein shown in Figure 2A corresponded to p38α, as determined by alignment of films using luminescent markers affixed directly to the blot. As shown in Figure 2B, hypoxia did not alter the total amount of p38α protein. Of the time points examined, maximal hypoxia-induced phosphorylation of p38α occurred at 6 hr, where there was an average 4.5-fold increase in p38α phospho-immunoreactivity (Figure 2C). The upper phospho-p38 immunoreactive

band was identified as p38γ, an isoform of p38α. Phospho-immunoreactivity of p38γ was more strongly increased by hypoxia, with an average of 12.7-fold increase over control levels by a 6 hr exposure to hypoxia (Figure 2C).

To further characterize the effects of hypoxia on p38 enzyme activity, PC12 cells were transfected with FLAG epitope-tagged versions of p38α, p38β, p38β_2, p38γ, or p38δ. Cells were then exposed to either normoxia (21% O_2) or hypoxia (5% O_2, 6h). The various kinases were then immunoprecipitated with an anti-FLAG antibody, and immune complex kinase assays were performed. As shown in Figure 3A, hypoxia stimulated both p38α and p38γ enzyme activity. In contrast to these results, hypoxia did not significantly alter p38β, p38β2 or p38δ enzyme activity. Hypoxia-induced changes in enzyme activity were not the result of differences in transfection efficiency as cell lysates blotted with anti-FLAG show equal amounts of the transfected protein (Figure 3B). It can be seen that the effect of hypoxia on the p38γ isoform is by far the most robust (average 5.9-fold activation, Figure 3C).

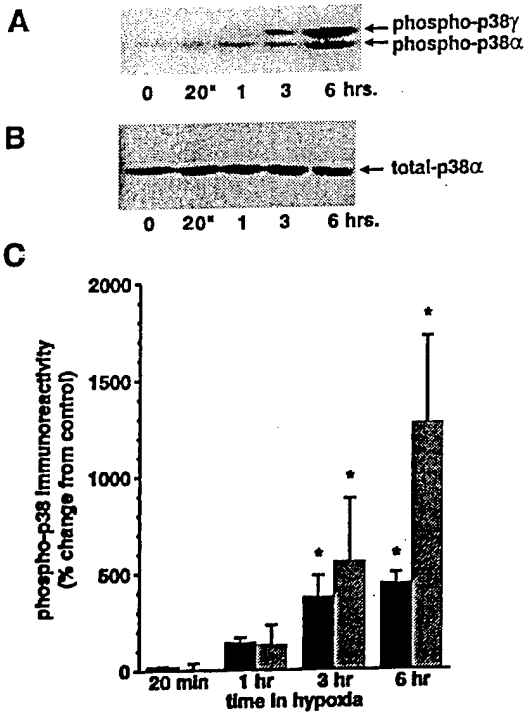

Figure 2. **Hypoxia induces phosphorylation of p38 and p38γ.** PC12 cells were exposed to normoxia or hypoxia (5% O_2) for various times, as indicated. Whole cell lysates were then immunoblotted for phospho-p38.

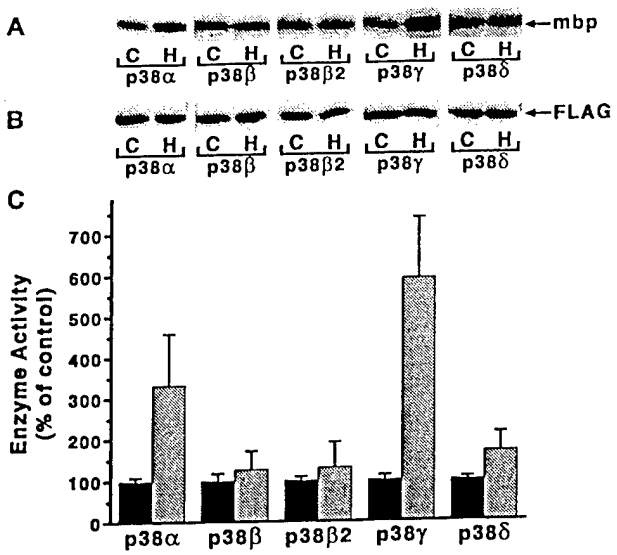

Figure 3. **Hypoxia specifically regulates members of the p38 family of protein kinases.** PC12 cells were transfected with FLAG-tagged constructs of the various p38 isoforms. Cells were then exposed to normoxia or hypoxia and subjected to immune complex kinase assays using myelin basic protein (mbp) as a substrate.

Our results demonstrate, for the first time, that physiological levels of hypoxia selectively activate p38γ and p38α. Phosphorylation of p38α has been shown to occur following ischemia in heart and kidney (Yin et al., 1997). Taken together with our findings, it is possible that the hypoxic component of ischemia, rather than the other types of substrate depletion (glucose, ATP, etc.), results in the activation of p38α and p38γ.

3. HYPOXIA HAS NO EFFECT ON JNK ACTIVITY

The other major stress-activated signaling pathway acts through the c-Jun N-terminal kinase (JNK) family of protein kinases (Widmann et al., 1999; Su and Karin, 1996; Garrington and Johnson, 1999). Like p38, the JNK family is activated by a number of stressors, but is distinctive in its ability to phosphorylate the transcription factor c-Jun (Kyriakis and Avruch, 1996; Hibi et al., 1993; Derijard et al., 1994). To evaluate the effect of hypoxia on JNK, PC12 cells were exposed to hypoxia for various times, from 20 min to 6 hr, and JNK enzyme activity was measured in an immune complex kinase assay. Unlike its effects on p38, hypoxia did not significantly alter JNK enzyme activity, whereas exposure of cells to UV light markedly increased JNK activity (Figure 4).

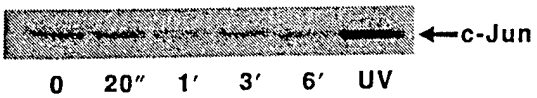

Figure 4. **JNK activity is not affected by hypoxia.** PC12 cells were exposed to normoxia or hypoxia for varying amounts of time, as indicated. JNK was immunoprecipitated and tested for kinase activity using c-Jun as a substrate.

It has been reported previously that ischemia/reperfusion in the kidney and hypoxia/reoxygenation in cardiac myocytes induces activation of JNK (Yin et al., 1997; Pombo et al., 1994). These groups found JNK activity to be activated by the reoxygenation event, but not during the initial hypoxia or ischemia. It has also recently been reported that severe hypoxia ($pO_2 \leq 0.01\%$) transiently activated JNK in human squamous carcinoma cells (Laderoute et al., 1999). In contrast, we found that neither hypoxia nor hypoxia plus reoxygenation (data not shown) stimulated JNK enzyme activity in PC12 cells. Clearly, various stressors can have different effects, depending on the specific cell type and its environment.

4. HYPOXIA INDUCES PHOSPHORYLATION AND ACTIVATION OF p42/p44 MAPK

To determine the effect of hypoxia on p42/p44 MAPK, PC12 cells were again exposed to either normoxia (21% O_2), or hypoxia (5% O_2) for various times, between 20 min and 6 hr. Samples of whole cell lysates were immunoblotted with either an antibody specific for tyrosine phosphorylated (activated) p42/p44 MAPK or an antibody that equally recognizes phospho- and dephospho-p42/p44 MAPK (total MAPK). Hypoxia had no significant effect on the levels of either phospho-p42/p44 MAPK at the earliest time points studied. However, exposure to hypoxia for six hours caused an increase in the tyrosine phosphorylation of p42/p44 MAPK (Figure 5A). The total amount of p42/p44 MAPK was not affected by hypoxia, as shown in Figure 5B. MAPK enzyme activity was measured directly by immune complex kinase assay. Figure 5C shows that p42 MAPK enzyme activity, like MAPK phosphorylation state, increased following six hours of hypoxia. To compare the effects of hypoxia with the prototypical activators of MAPK, we also evaluated p42/p44 MAP kinase phosphorylation in response to nerve growth factor (NGF) and UV light. In contrast to the rather modest effect of hypoxia, these stimuli caused a robust phosphorylation of p42/p44 MAP kinase (Fig. 5D).

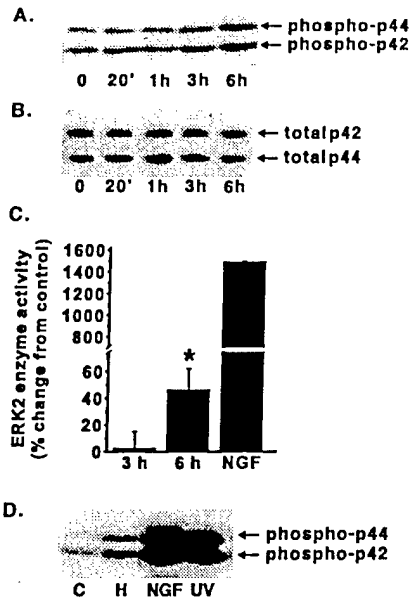

Figure 5. **MAPK is modestly activated by hypoxia.** PC12 cells were exposed to hypoxia for varying amounts of time, as indicated. Whole cells lysates were then immunoblotted for phospho-MAPK (panel A) or total-MAPK (panel B). Lysates were also subjected to immune complex kinase assays (panel C). In panel D, the hypoxia-induced phosphorylation of MAPK was compared to that of nerve growth factor (ngf) or UV light.

5. ACTIVATION OF p38γ AND MAPK BY HYPOXIA IS Ca^{+2}/CaM-DEPENDENT.

Previous experiments have shown that hypoxic exposure of PC12 cells results in membrane depolarization and calcium influx (Zhu et al., 1996; Raymond and Millhorn, 1997; Kumar et al., 1998). This increase in intracellular calcium is known to be a critical mediator of gene expression and transcription factor activation. Thus, we hypothesized that Ca^{2+}-influx upon hypoxic depolarization was involved in the signaling cascade leading to p38γ activation. To test this hypothesis, cells were incubated in Ca^{+2}-supplemented media or Ca^{2+}-free media (supplemented with 1 mM EGTA). Cells were then exposed to normoxia or hypoxia and subjected to immune-complex kinase assay. Figure 6A shows that the hypoxia-induced activation of p38γ is attenuated by incubation in Ca^{2+}-free media, suggesting a critical role for intracellular calcium in the activation of p38γ. Lysates immuno-blotted with anti-FLAG antibodies showed that expression of FLAG-p38γ was the same (data not shown).

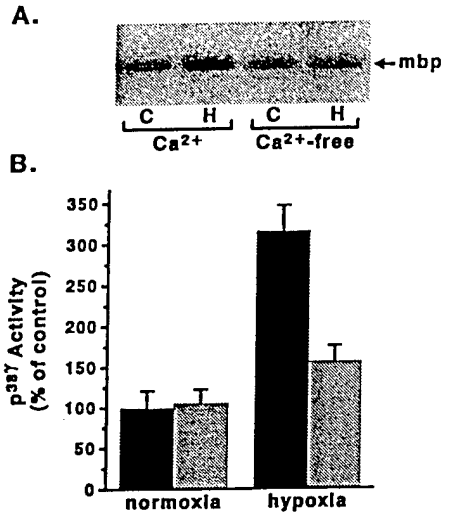

Figure 6. **Calcium is critical for the hypoxia-induced activation of p38γ.** PC12 cells were transfected with FLAG-tagged p38γ or the empty expression vector. Cells were incubated in Ca^{2+}-supplemented or Ca^{2+}-free media and then exposed to normoxia or hypoxia and then assayed for kinase activity.

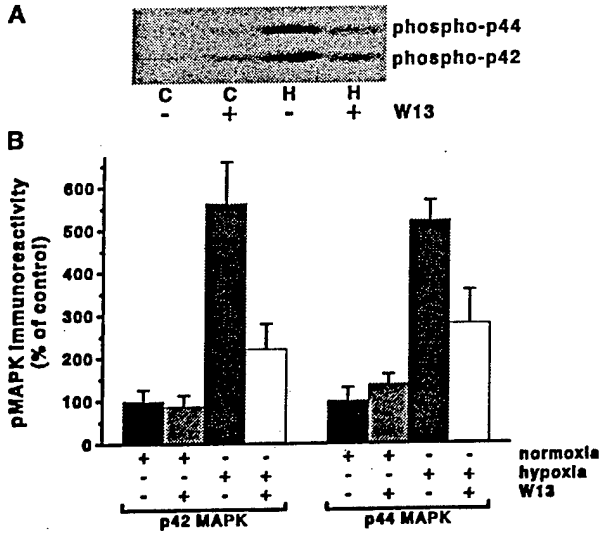

Figure 7. **Hypoxia-induced phosphorylation of MAPK is calmodulin-dependent.** PC12 cells were exposed to normoxia or hypoxia for 6 hours in the presence or absence of the calmodulin antagonist, W13. Whole cell lysates were then immunoblotted for phospho-MAPK. Results are shown quantitatively in panel B.

Egea et al. have shown that KCl-induced depolarization of PC12 cells results in MAPK activation via a calmodulin-dependent mechanism (Egea et al., 1998; Egea et al., 1999). Thus, we hypothesized that calmodulin could be involved in the hypoxia-induced activation of MAPK. Figure 7 shows that pre-treatment of PC12 cells with the calmodulin antagonist, W13 (20 μg/μl), caused a pronounced reduction in the hypoxia-induced phosphorylation of both p42 and p44 MAPK. These results are shown quantitatively in Figure 7B, and show that p42/p44 MAPK phosphorylation during hypoxia is calmodulin-dependent.

6. CONCLUSIONS

Taken together, these studies demonstrate that hypoxia, an extremely typical physiological stress, causes specific regulation of the stress- and mitogen-activated protein kinase signaling pathways. We also show that one isoform of p38, p38γ, is particularly strongly activated by hypoxia. In addition, the traditional growth factor-stimulated kinase, MAPK, is also phosphorylated and activated by hypoxia. Future studies are aimed at delineating the specific mechanisms by which a reduction in O_2 levels causes regulation of these pathways, as well as determining the mechanism by which Ca^{2+}/CaM target the MAPK and SAPK pathways.

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Review Article

Multiple Molecular Penumbras After Focal Cerebral Ischemia

Frank R. Sharp, Aigang Lu, Yang Tang, and *David E. Millhorn

*Department of Neurology, and *Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, Ohio, U.S.A.*

Summary: Though the ischemic penumbra has been classically described on the basis of blood flow and physiologic parameters, a variety of ischemic penumbras can be described in molecular terms. Apoptosis-related genes induced after focal ischemia may contribute to cell death in the core and the selective cell death adjacent to an infarct. The HSP70 heat shock protein is induced in glia at the edges of an infarct and in neurons often at some distance from the infarct. HSP70 proteins are induced in cells in response to denatured proteins that occur as a result of temporary energy failure. Hypoxia-inducible factor (HIF) is also induced after focal ischemia in regions that can extend beyond the HSP70 induction. The region of HIF induction is proposed to represent the areas of decreased cerebral blood flow and decreased oxygen delivery. Immediate early genes are induced in cortex, hippocampus, thalamus, and other brain regions. These distant changes in gene expression occur because of ischemia-induced spreading depression or depolarization and could contribute to plastic changes in brain after stroke. **Key Words:** Stroke—Apoptosis—Genes—Stress genes—Hypoxia.

On the eve of knowing the sequence of the mouse and human genomes, the prospects for this information helping to diagnose and treat stroke and other polygenic neurological disorders has enormous potential. This is not meant to be a review of gene regulation following ischemia. Rather, it is an attempt to show how specific changes of gene expression may be used to infer mechanisms of injury or recovery after stroke that might lead to novel therapy.

Gene induction in brain, particularly stroke, cannot be studied in isolation. That is, the spatial, temporal, and cellular basis for the changes of expression must be known before speculations regarding therapeutic potential can be addressed. For example, genes induced after temporary ischemia in brain might reflect the prominent role of free radicals and oxidative stress (Chan, 1994), whereas the same genes might play a less important role in permanent arterial occlusion (Chan et al., 1993). Genes induced in inflammatory cells in the core of an infarct have different implications for mechanisms of injury and stroke therapy than do genes induced in neurons outside an infarct during the same time periods (del Zoppo, 1997; Dirnagl et al., 1999). Genes induced days after ischemia may be related to plasticity and recovery rather than to damage.

This paper could have been titled multiple molecular, spatial, temporal, and cellular penumbras after focal ischemia. Every gene in every cell can vary spatially and temporally with varying degrees of ischemia, making any representation a tremendous oversimplification (Fig. 1). Figure 1 attempts to condense large amounts of data. It is important to devise strategies to look at the most important genes because the changes of the majority of genes after stroke probably do not mediate either injury or protection. At this point it is difficult to know what the most important genes are. We refer to genes that are either the most familiar, or that have been the most studied, with the realization that much still needs to be learned and that many important genes for understanding the pathogenesis of stroke have yet to be identified.

It was often difficult to determine where most genes were induced after a stroke. The imprecise descriptions in most studies as to which cells in which brain regions express a gene hampers the interpretation of the factors that might induce these genes. We have made some assumptions about where each set of genes is induced after ischemia in order to compare this presumptive data with more precise information on gene induction in different

Received February 9, 2000; accepted March 23, 2000.
Address correspondence and reprint requests to Dr. Frank R. Sharp, Department of Neurology, University of Cincinnati Medical Center, 231 Bethesda Avenue, Cincinnati, OH 45267-0536, U.S.A.

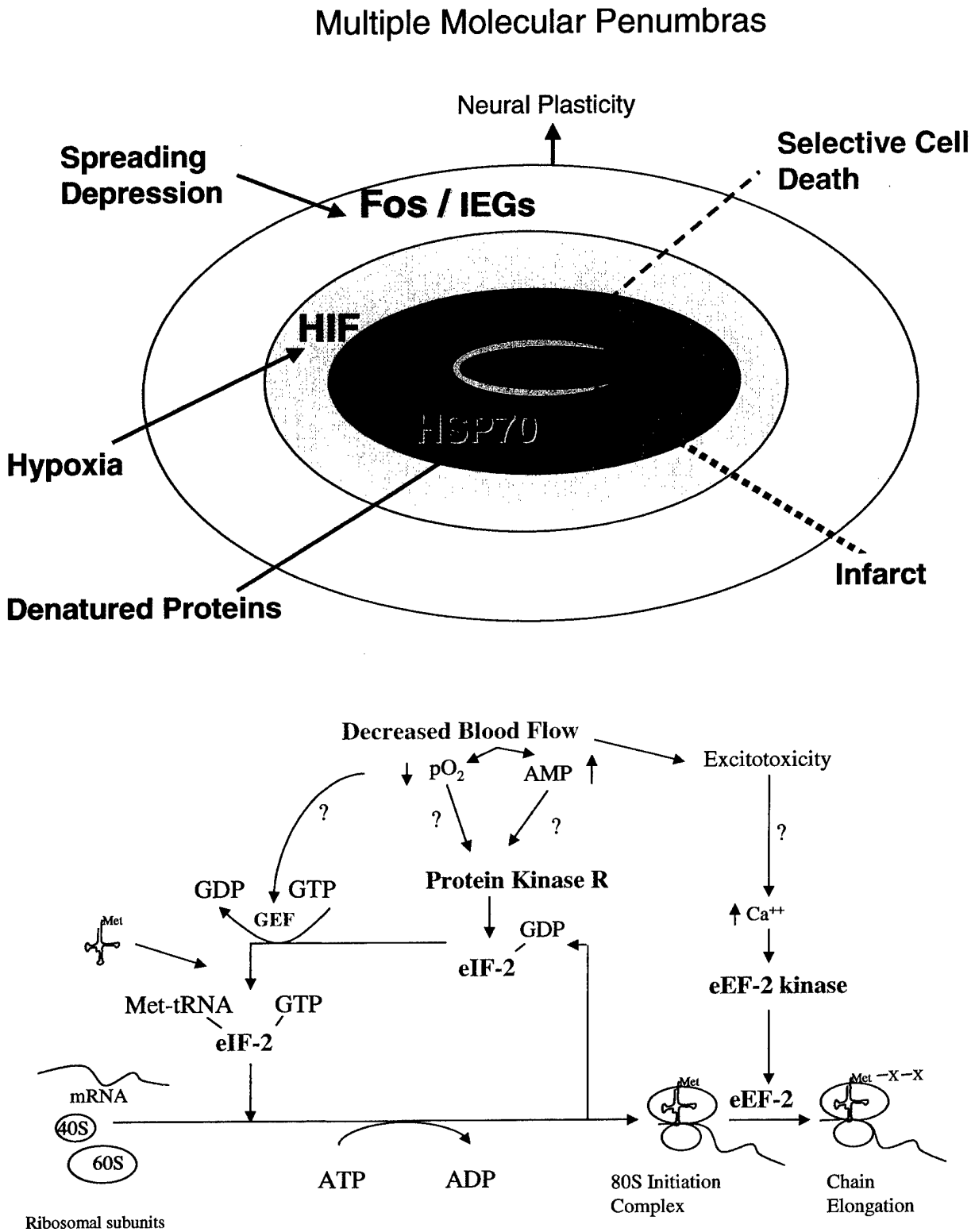

FIG. 2. Protein synthesis on the ribosomes can be completely suppressed during focal ischemia through inhibition of several elongation factors, including eIF-2, GEF, and eEF. EIF-2 phosphorylation by PKR is affected by increases of adenosine monophosphate and decreased oxygen that occur during ischemia. Increased concentrations of glutamate released during ischemia can directly down-regulate eEF-2 kinase that regulates eEF-2 function during ischemia.

FIG. 1. Schematic of multiple molecular penumbras after a stroke. A zone of selective neuronal cell death borders the infarct. The zone of protein denaturation extends outside of this and is demarcated by HSP70 protein expression in injured neurons. Hypoxia inducible factor is induced in areas where blood flow is persistently decreased and oxygen delivery is impaired. This may be co-incident with HSP70 or extend over more widespread regions depending on collaterals. Ischemia-induced spreading depression induces c-fos and many other immediate early genes at some distances from the infarct, including the ipsilateral rat occipital and frontal lobes, contralateral cortex and many subcortical structures.

brain regions. Genes that have well described mechanisms of induction through oxygen, free radicals, denatured proteins, pH, and so on can provide molecular and biochemical insights into the injury. Though there are assumptions about how the genes may be induced in the intact brain, the inferences may be useful.

Though mRNA is frequently studied, in terms of effectors that mediate injury, it is essential that protein expression, or protein function (enzymatic activity and so on), be examined. Hence, if a gene is believed to mediate injury after stroke, its protein must be expressed. Because most proteins are not expressed in the core of a stroke, it is important to examine protein expression in the core, at the margins, and at some distance from the strokes.

Relatively few genes are discussed because of the enormity of the subject. Global ischemia was purposely excluded in order to focus on focal ischemia penumbras. However, there are broad reviews available on gene regulation after cerebral ischemia (Chan, 1994; Dirnagl et al., 1999; Koistinaho and Hokfelt, 1997; Kogure and Kato, 1993; Massa et al., 1996; del Zoppo, 1997; Chen and Simon, 1997; Feuerstein et al., 1997; Nowak and Jacewicz, 1994; Nowak, 1999). The speed and widespread availability of information today will cause this review to be out of date even before publication; however, we hope the general ideas will prove useful.

INFARCT CORE - PROTEIN SYNTHESIS

A decrease or block of protein synthesis is one of the first biochemical changes to occur after focal cerebral ischemia (Fig. 2). This occurs when blood flow decreases approximately 50% (Mies et al., 1991). A decrease in ATP is not the signal for a block in protein synthesis because ATP does not decrease until flow falls to 20% of the normal level (Mies et al., 1991; Jacewicz et al., 1986). Ribosomal protein synthesis appears to be the sensitive step that responds to this reduced blood flow, occurring because of in inactivation of initiation factor 2 (eIF2), guanine nucleotide exchange factor (eIF-2-GTP complex factor), and eukaryotic elongation factor (eEF-2) (Marin et al., 1997; Massa et al., 1996). Glutamate-dependent phosphorylation of eEF-2 (Marin et al., 1997) provides a direct link between ischemia-induced increases of extracellular glutamate and ischemia-induced inhibition of protein synthesis (Fig. 2). Phosphorylation of eIF-2 by Protein Kinase R also provides a control point in protein synthesis that is sensitive to oxygen and/or adenosine monophosphate (Srivastava et al., 1998).

There is little or no synthesis of new proteins in neurons or astrocytes in the "core" of a cerebral infarct (Kleihues and Hossmann, 1971; Cooper et al., 1977; Mies et al., 1990, 1991; Hossmann, 1993, 1994). Most cerebral ischemia scientists use a change of histologic staining—hematoxylin and eosin, Nissl, or mitochondrial staining—to define the edges and volumes of infarction. Staining proteins by immunocytochemistry and Western blots within the core of an infarction is a function of the half-life of a protein. Proteins with short half-lives will disappear rapidly, without synthesis or rapid degradation. Because of their slow degradation proteins with long half-lives will continue to be detected long after histologic evidence of tissue infarction.

Protein synthesis continues in cells that survive an infarct. In some infarcts some or all of the blood vessels survive. When this occurs, protein synthesis can continue within blood vessels. For example, heat shock protein 70 (HSP70) continues to be expressed in blood vessels in an infarct (Gonzalez et al., 1989, 1991; Kinouchi et al., 1993*a,b*), as well as iNOS, eNOS (Iadecola et al., 1996) and many other genes. Importantly, there may be expression of cell adhesion molecules, cytokines, and chemokines by vascular cells within infarcts and at the margins of infarcts. Finally, inflammatory cells inside of infarcts, including neutrophils and macrophages, mount a specific genomic response to the dying and dead neurons and glia.

CORE - ADHESION MOLECULES, CYTOKINES, CHEMOKINES

Intercellular adhesion molecule-1 (ICAM-1) is expressed by vessels in the core of the infarction and at the edges of an infarction (Yang et al., 1999c). ICAM-1 mRNA and endothelial leukocyte adhesion molecule-1 (ELAM-1) and selectin are induced by 3 hours and 6 hours, respectively, after ischemia and peak at 6 to 12 hours (Zhang et al., 1995*b*; del Zoppo, 1997; Feuerstein et al., 1997; Wang et al., 1994*a*; Wang and Feuerstein, 1995; Amberger et al., 1997). ICAM-1 protein is expressed mainly within the core of the infarct on endothelial cells (Kim, 1996) and plays a role in neutrophil invasion of ischemic tissue. Cytokine-induced neutrophil chemo-attractant protein (CINC) is also induced mainly within an infarct and at its margins (Liu et al., 1993; Yamasaki et al., 1995). CD11 positive neutrophils appear

within a day at the infarct site and are numerous by 3 days (Kato et al., 1996). Many studies that show that a reduction in inflammatory cells or inhibition of adhesion molecules lessens injury in experimental models of stroke suggest that adhesion molecules and inflammatory cells play a role in mediating focal ischemic brain injury (Chopp et al., 1996; del Zoppo, 1997; Feuerstein et al., 1997; Kitagawa et al., 1998; Soriano et al., 1999). This suggestion is balanced by a recently completed study of an anti-ICAM antibody in humans that failed to show benefit. This might relate in part because maximal treatment of infarction could be dependent upon reperfusion of the core so that antibodies reach all areas of ischemia, particularly because anti-ICAM antibodies work best after temporary ischemia (Zhang et al., 1995*a*, 1999).

Integrin alpha beta 3 is expressed primarily in the core of an infarct and is likely related to vascular responses (Abumiya et al., 1999). Monocyte-chemoattractant protein-1 and macrophage inflammatory protein-1 alpha (MIP-1) are induced primarily in the core and adjacent areas of ischemia (Kim et al., 1995). MIP-1 is induced first in the core, where the greatest damage occurs, and then in the regions adjacent to the infarction, (Takami et al., 1997) where damage is less severe and possibly where macrophages and microglia engulf single cells or small groups of cells that might die more slowly. CD18 positive macrophages, which immunostain for heme oxygenase-1 (Bergeron et al., 1997), begin to appear at 2 to 3 days in the core of an infarct and are quite numerous by 7 days (Kato et al., 1996).

MATRIX METALLOPROTEINASES

The matrix metalloproteinases (MMPs) include MMP-7 (matrilysin), MMP-3,-10,-11,-13 (stromelysins), MMP-14 (membrane MMP), and MMP-2 and MMP-9 (gelatinase A and B, respectively) (Rosenberg et al., 1996; Mun-Bryce and Rosenberg, 1998). MMP-2 and MMP-4 have been the subject of recent studies because they attack type IV collagen, laminin, and fibronectin, the major components of the basal lamina around cerebral blood vessels. MMP-2 is expressed constitutively in brain and may play a role in ischemia (Clark et al., 1997; Gasche et al., 1999; Rosenberg et al., 1996). MMP-9, the 92kD type IV collagenase, is not expressed in normal brain. After ischemia, ProMMP-9 is induced in the core within 2 hours with enzymatic activity and mRNA induction being detected by 4 hours (Fujimura et al., 1999a; Gasche et al., 1999). Induction of MMP-9 mRNA could be mediated by a NF-kB site in the MMP-9 promoter (Mun-Bryce & Rosenberg, 1998) (Fig. 3). Activation of MMP-9 correlates with blood–brain barrier break down (Gasche et al., 1999) and in at least one study correlated with areas of hemorrhagic conversion after focal ischemia (Heo et al., 1999). MMPs may be important for producing increases of blood brain barrier permeability and brain edema after stroke (Gasche et al., 1999). MMPs may also promote tissue invasion of neutrophils and macrophages, and contribute to hemorrhages that result after reperfusion of ischemic tissue (Mun-Bryce & Rosenberg, 1998; Heo et al., 1999).

Therapeutic significance of the core

Many of the above genes are expressed mainly in the core of an infarct. When there is no reperfusion of the core, there would seem little hope of rescuing the core with current technology and little reason to think that manipulating the above genes might affect the outcome. However, if the core were reperfused either spontaneously or with tPA, the induction of these inflammatory molecules (Fig. 3) might prove to be important therapeutic targets (Zhang et al., 1999).

ZONE OF SELECTIVE NEURONAL DEATH ADJACENT TO INFARCTS

The edges of infarcts appear to be fairly well demarcated on Nissl and hematoxylin and eosin-stained tissue sections at the gross level. However, on hematoxylin and eosin-stained sections there are eosinophilic cells, most of which are neurons, that are outside an infarct. This appears to represent a rim of selective neuronal cell death (Nedergaard, 1987; Nedergaard et al., 1987) (see Fig. 1). These histologic findings have been supported by TUNEL staining. TUNEL-stained neurons with fragmented DNA are found immediately outside the areas of infarction, at most a centimeter from the infarct, and vary in number depending upon the severity of ischemia and the brain region examined (Li et al., 1995c; Li et al., 1995b; States et al., 1996). Consequently, there is a zone of selective neuronal death that occurs adjacent to, and just outside of histologically defined infarct margins.

APOPTOSIS, DNA DAMAGING INDUCIBLE AND DNA REPAIR GENES

DNA damage-inducible and DNA repair genes tend to be expressed either within the core or within the regions adjacent to the infarction. It is possible that these genes contribute to selective neuronal cell death, or contribute to tissue infarction itself, or both. Bax, the pro-apoptotic gene, is induced in the core of infarcts (Gillardon et al., 1996) and in cells just outside the infarct that have evidence of DNA fragmentation by TUNEL staining (Matsushita et al., 1998; Isenmann et al., 1998). Bcl-2 tends to decrease in cells that appear to be lethally injured. Bcl-2 and Bcl-xl, the anti-apoptotic genes, tend to be induced in cells that are immediately adjacent to an infarct (Asahi et al., 1997) and probably survive ischemia (Chen et al., 1995) (Isenmann et al., 1998). Bcl-2 can be induced in the entire middle cerebral artery (MCA) territory with

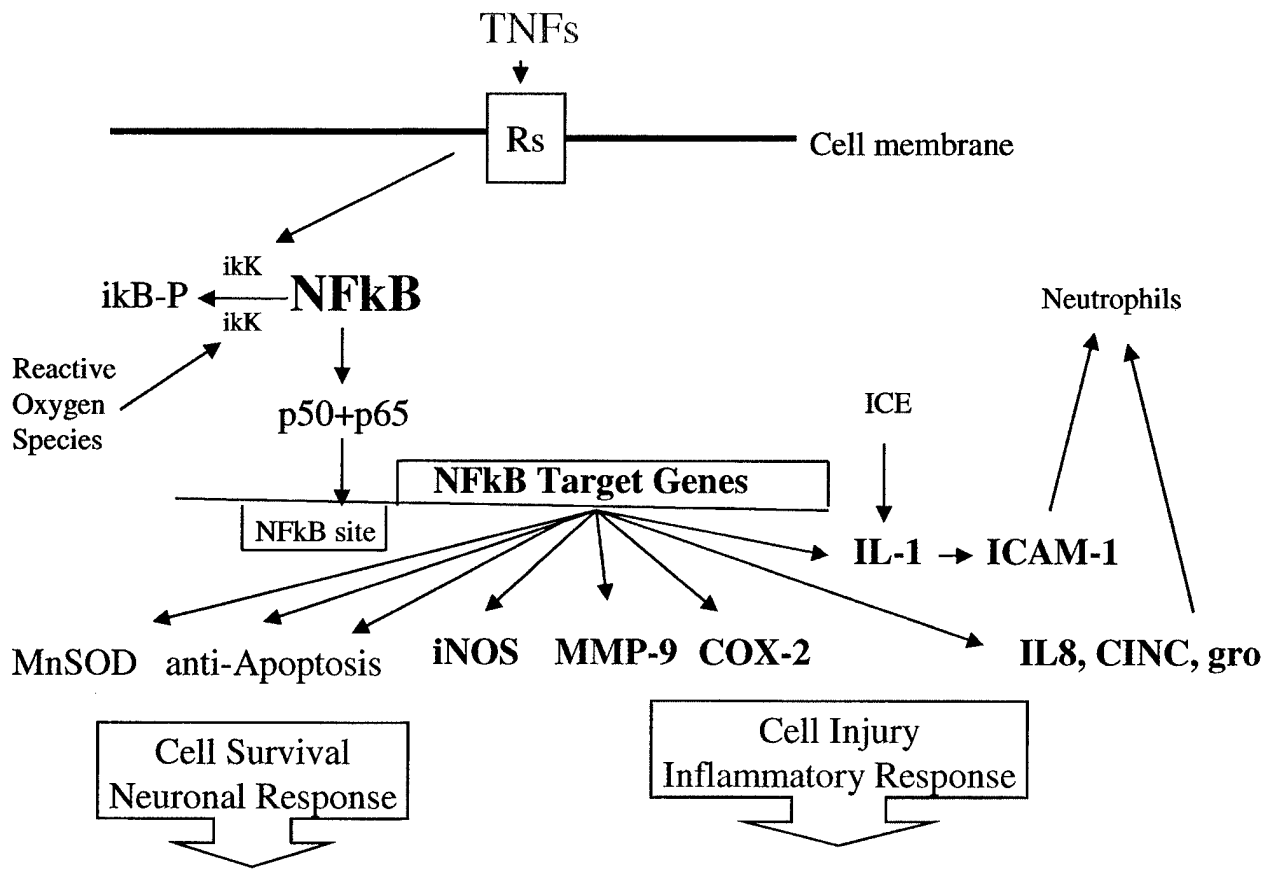

FIG. 3. Postulated mechanisms by which TNF and NFkB might mediate either cell survival or cell injury. This would depend upon which cells these genes are induced in and which target genes are induced. Note that NFkB is activated by phosphorylation of ikB and its release resulting in NFkB activation and binding of the p50-p65 complex to NFkB sites in target genes.

less severe degrees of ischemia (Chen et al., 1995). The cleaved portion of caspase 3, the protease associated with programmed cell death, is found in the MCA core and in the region adjacent to the core (Asahi et al., 1997; Namura et al., 1998).

Genes associated with DNA damage are expressed in and around the core after focal ischemia. Gadd45, a protein induced in response to DNA damage, was expressed at the edge of infarcts in cells that had little evidence of DNA fragmentation (Hou et al., 1997). This contrasted with another study that suggested that p53, Bax, MDM2, and Gadd45 were induced in cells that were dead or expected to die (Li et al., 1997). DNA repair proteins decrease in cells expected to die in the core (Fujimura et al., 1999*b,c*) and are induced in cells immediately adjacent to the core that appear to survive the ischemia (Li et al., 1997). Cell cycle genes and proteins can be induced in cells that survive ischemia and possibly in proliferating cells like microglia and astrocytes (Wiessner et al., 1996).

There is conflicting data about some of these genes. One study shows that p21 mRNA and protein and cyclin G1 increase; whereas p53 and Bax messenger RNA and protein levels, and protein levels of p27, cyclin-dependent kinase 5, p35, and cyclin E decrease in the infarct core and border areas after middle cerebral artery occlusion (MCAO) (van Lookeren Campagne and Gill, 1998). Some of the disparity between these studies could be attributed to differences in how much of the core or adjacent surviving brain was sampled, because protein levels in the core for most genes would decrease. Also of interest is that at least one study shows induction of GADD45, growth arrest and DNA damage-inducible in neurons throughout an ischemic hemisphere, in areas inside and outside any region of ischemia (Jin et al., 1996). This pattern of gene induction is similar to that of immediate early genes (IEGs) described below; hence, it is likely induced in response to spreading depression rather than damaged DNA (though once induced it could respond to DNA damage after focal ischemia).

CD95 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) have also been studied after reversible MCAO in adult rats (Martin-Villalba et al., 1999). Both CD95 ligand and TRAIL were expressed in the rim around the infarct. Recombinant CD95 ligand and TRAIL proteins induced apoptosis in primary neurons and neuron-like cells *in vitro*. FK506 prevented postischemic expression of these death-inducing ligands

both *in vivo* and *in vitro* and abolished phosphorylation, but not expression, of the c-jun transcription factor involved in the transcriptional control of CD95 ligand. In addition, in lpr mice expressing dysfunctional CD95 reversible MCAO resulted in infarct volumes significantly smaller than those found in wild-type animals (Martin-Villalba et al., 1999).

Therefore, the evidence suggests that this narrow zone of selective neuronal injury, where various apoptosis-related and DNA damage and repair genes are expressed, could be important for stroke outcome. This is supported by recent studies showing that inhibiting caspases or introducing bcl-2 into brain improve outcome from stroke (Cheng et al., 1998; Lawrence et al., 1996*a*; Endres et al., 1998; Martinou et al., 1994). It is possible that the region of selective cell death might convert to an area of infarction with continuing or worsening ischemia (Du et al., 1996). Adjacent, previously unaffected regions could then develop selective cell death and so on. It remains to be seen whether manipulating apoptotic-related proteins will improve stroke outcomes.

NOS AND NO

Immediately after focal infarction, NO (nitric oxide) is derived mainly from neuronal nNOS (NOS-1) and endothelial eNOS (NOS-3) in the core and margin of the infarct (Iadecola, 1997; Ashwal et al., 1998). Inhibiting nNOS at this point appears to improve stroke outcome (Iadecola et al., 1994, 1997; Dirnagl et al., 1999), with NO injury occurring through actions on the *N*-methyl-*D*-aspartate (NMDA) receptor and acting as a free radical (Coeroli et al., 1998; Dirnagl et al., 1999). eNOS appears to protect at this early stage by releasing NO to dilate vessels and accounting for nonspecific nitric oxide synthase (NOS) inhibitors either worsening (inhibiting eNOS) or improving (inhibiting nNOS) stroke when given before or just after ischemia (Iadecola et al., 1994, 1997; Dirnagl et al., 1999).

Inducible NOS (iNOS, NOS-2) is induced many hours after ischemia (Nagafuji et al., 1994; Iadecola, 1997). It is induced either in neutrophils or macrophages, in the core of the infarct, or in microglia, blood vessels, or astrocytes at the margins of the infarct (Iadecola et al., 1996; Coeroli et al., 1998; Forster et al., 1999; Loihl et al., 1999). Inhibition of iNOS (NOS-2) appears to improve stroke (Iadecola et al., 1995, 1996, 1997; Dirnagl et al., 1999).

TNF AND NFkB

Some cytokines are expressed only within the infarct, whereas others are expressed at a considerable distance, possibly even in the opposite hemisphere. In addition, there are many differences in the temporal induction of the various genes (Hill et al., 1999). Tumor necrosis factor-α (TNF-α) is induced early, whereas interleukin-1 (IL-1) and transforming growth factor (TGF) are delayed (Hill et al., 1999). TGF expression can persist for weeks after a stroke (Wang et al., 1995*b*), as can interferon-inducible protein-10 (Wang et al., 1998*b*).

TNF-α

TNF-α is induced in the core and in the region adjacent to the infarction in neurons, astrocytes, and endothelial cells (Feuerstein et al., 1994; Gong et al., 1998; Liu et al., 1994; Feuerstein et al., 1997) within 1 to 6 hours the ischemia (Yang et al., 1999*a*). TNF-α expression can be observed in neurons and macrophages (Liu et al., 1994). Although TNF-α is mainly induced in the ischemic hemisphere, it can also be induced in the ipsilateral hippocampus (Gong et al., 1998) and the contralateral nonischemic hemisphere (Zhai et al., 1997).

TNF-α has often been thought to mediate injury and apoptotic cell death (Dawson et al., 1996; Mizuno and Yoshida, 1996; Yang et al., 1998), although more recent studies suggest that it can also be protective. Some investigators have shown that administration of TNF worsens infarcts, and binding TNFs with ligands or antibodies decreases stroke size (Barone et al., 1997; Dawson et al., 1996; Nawashiro et al., 1997). However, other studies have suggested that mice with knockouts of their TNF receptors have larger infarcts and greater injury because of excitotoxins (Bruce et al., 1996; Sullivan et al., 1999). These differences in the actions of TNF could depend upon the following: (1) which TNF is induced; (2) which receptor it acts on; and (3) which cells the TNF is induced in (Fig. 3). TNF induction in neutrophils and endothelial cells could mediate injury, whereas TNF induction in neurons could be protective (Bruce et al., 1996; Mattson, 1997) (Fig. 3).

NFkB

Stimulation of TNF-α receptors leads to activation of NFkB, with phosphorylation and release of ikB from the NFkB complex (May and Ghosh, 1999; Li and Karin, 1999). The p50 and p65 dimer of NFkB then acts on NFkB target genes (Ghosh et al., 1998) (see Figure 3). The precise role of NFkB in ischemia remains unclear because some studies show that NFkB might mediate injury, whereas others suggest that it could protect brain.

For example, one study shows that NFkB/p65 decreases after focal ischemia (Botchkina et al., 1999*a*). However, other studies show that, although absolute amounts of p50 and p65 may not be predictive, NFkB DNA binding activity is increased after focal and global ischemia (Schneider et al., 1999; Carroll et al., 1998; Gabriel et al., 1999; Howard et al., 1998; Salminen et al., 1995). In one study stroke volumes were decreased in p50 knockout mice (Schneider et al., 1999) suggesting a harmful role for NFkB. However, blocking NFkB activity can exacerbate excitotoxic injury (Botchkina et al.,

1999*b*), and stroke and excitotoxic injury in p50 knockout mice has been reported to be worse (Yu et al., 1999).

In addition to being regulated by TNF and reactive oxygen species (Schreck et al., 1991; Meyer et al., 1993), NFkB is also regulated by ikB kinases (ikKs). ikKs phosphorylate ikB, which activates the p50-p65 complex of NFkB. TNF activates NFkB by activating ikKs (Schottelius et al., 1999). Aspirin and NSAIDs down-regulate NFkB and TNF (Shi et al., 1999) in part by inhibiting ikKs (Pierce et al., 1996; Stevenson et al., 1999). IL-10 decreases NFkB activity in part by inhibiting ikKs, and also by blocking NFkB binding to target promoter elements (Schottelius et al., 1999).

Although NFkB is a major sensor and effector of oxidative stress in cells, it is not entirely clear how this occurs (Li and Karin, 1999). Thioredoxin (TRX) is a small disulfide protein induced in response to oxidative stress. TRX interacts with and activates NFkB (Weichsel et al., 1996). Although TRX expression decreases in an infarct, it is markedly induced adjacent to an infarct (Takagi et al., 1998). TRX overexpression in transgenic mice protects them against focal ischemia (Takagi et al., 1999).

NFkB could mediate cell protection and cell damage because of its many downstream target genes. Genes with NFkB binding sites in their promoters that would protect cells include MnSOD (Darville et al., 2000; Xu et al., 1999); calbindin (Bruce-Keller, 1999); bcl family genes, including bcl-2 and bcl-xl (Wang et al., 1998a; Chen et al., 1999*a*; Zong et al., 1999; Tsukahara et al., 1999; Tamatani et al., 1999); and TNFR-associated factors (TRAFs) and inhibitor of apoptosis proteins (IAPs) (Wang et al., 1998*a*).

A number of other genes can be regulated at least in part by NFkB can mediate cellular injury. These include IL-1, ICAM-1, CINC, IL-8, and gro, that promote neutrophil adhesion (Roebuck, 1999); COX-2, that metabolizes arachidonic acid (Lee and Burckart et al., 1998; Kotake et al., 1998; Plummer et al., 1999); MMP-9, that cleaves type 4 collagen at the blood–brain barrier (Mun-Bryce & Rosenberg, 1998; Lee and Burckart, 1998); heme oxygenase-1, that metabolizes heme to release iron; and iNOS, that releases NO and contributes to oxidative stress (Kotake et al., 1998). Lastly, the pro-apoptotic gene bcl-xs has a NFkB element in its promoter that is activated during brain ischemia (Dixon et al., 1997).

NFkB and TNF might promote cell injury or cell protection depending upon the cells and the circumstances of their induction (Li & Karin, 1999). For example, COX-2 is induced in smooth muscle cells but not through NFkB (Chen et al., 1999*b*). As a working hypothesis, it is conceivable that TNF and NFkB expression in neutrophils and endothelial cells might induce neural injury related genes after stroke (Schneider et al., 1999). However, TNF and NFkB expression in neurons may induce neuroprotective genes and prevent injury caused by stroke and excitotoxins (Bruce-Keller, 1999) (Bruce et al., 1996) (Mattson, 1997) (Yu et al., 1999*a*). This dual action of TNF and NFkB is shown in Fig. 3.

COX-2

Phospholipids are metabolized to arachidonic acid (AA) by phospholipase A2. AA is metabolized to prostaglandins by COX-2 and metabolized to leukotrienes by 5-lipoxygenase. COX-2 is induced by focal ischemia (Kinouchi et al., 1999*b*; Planas et al., 1995). COX-2 inhibitors decrease stroke volumes in some, but not all, studies (Nagayama et al., 1999; Hara et al., 1998). Cell protection produced by COX-2 inhibitors appears to be linked to iNOS mediated injury (Nagayama et al., 1999). It should be noted that spreading depression induces COX-2 throughout a hemisphere (Miettinen et al., 1997; Koistinaho et al., 1999) so that variable degrees of focal ischemia may induce COX-2 throughout one-half of the brain. This may explain why COX-2 is induced at great distances from the region of ischemia in rodents (Koistinaho et al., 1999) and in human stroke patients (Sairanen et al., 1998). This also suggests that spreading depression, not AA itself, is the likely stimulus for COX-2 induction after focal ischemia.

INTERLEUKINS

IL-1

IL-1 is markedly induced after focal ischemia (Szaflarski et al., 1995; Betz et al., 1996; Rothwell and Relton, 1993; Yabuuchi et al., 1994; Rothwell et al., 1997). IL-1 is induced in the ischemic ipsilateral cortex and in the contralateral, nonischemic cortex (Zhai et al., 1997). It peaks at 6 hours after ischemia and persists for several days (Wang et al., 1994*b*). The bilateral IL-1 induction appears to occur in cerebral endothelial cells and microglia (Giulian et al., 1986; Zhang et al., 1998*b*). This suggests that although ischemia may only occur in one hemisphere, adverse cytokine responses may appear in the opposite hemisphere (Zhai et al., 1997). This could occur through ischemia induced spreading depression as described below to explain bilateral induction of fos and other IEGs after stroke. IL-1 appears to worsen ischemic injury (Betz et al., 1996; Stroemer and Rothwell, 1998), and to produce selective neuronal cell death and edema (Holmin and Mathiesen, 2000). Blocking IL-1 decreases ischemic injury (Loddick et al., 1997; Rothwell et al., 1997; Betz et al., 1996; Yang et al., 1999*b*). This could occur in part because IL-1 induces ICAM-1 and other proinflammatory molecules (Rothwell et al., 1997; Yang et al., 1999*c*) (Fig. 3).

IL-6 is also induced diffusely in brain after ischemia (Loddick et al., 1998). It is induced in neurons and microglia and is found in the ischemic hemisphere, the

ipsilateral hippocampus, and contralateral cortex (Suzuki et al., 1999). Mice deficient in IL-6 have markedly decreased astrocytic and microglial responses to injury (Klein et al., 1997) and administration of IL-6 protected against stroke (Loddick et al., 1998).

IL-10 is also induced by ischemia, although it is induced only in the ischemic hemisphere (Zhai et al., 1997). This monocyte chemo-attractant is induced mainly in regions of injury at early times after stroke and continues expression for days after stroke (Wang et al., 1998*b*).

TGF AND PLASMINOGEN ACTIVATOR INHIBITOR

TGF-b1 mRNA is induced in the ischemic MCA territory, including cortex and striatum, and in the ischemic cingulate cortex (Lehrmann et al., 1998). Microglia and macrophages are the major source of TGF-β1 after ischemia (Lehrmann et al., 1998). Expression of mRNA was detected by 6 to 12 hours after ischemia (Wang et al., 1995b), was highly expressed at 1 week, and continued expression in a rim around the infarct at 3 weeks after infarction (Lehrmann et al., 1998). TGFβ appears to protect against focal ischemia (Ruocco et al., 1999).

The plasminogen activator inhibitor-1 (PAI-1) is a TGF target gene. Focal ischemia induces PAI-1 without any effect on protease nexin-a, neuroserpin, or tissue plasminogen activator. PAI-1 is expressed in astrocytes (Docagne et al., 1999). PAI-1 was modulated by TGF-β1 treatment through a TGF-β-inducible element contained in the PAI-1 promoter (CAGA box) (Docagne et al., 1999). TGF-beta and activin induced the overexpression of PAI-1 in astrocytes; whereas bone morphogenetic proteins, glial cell line-derived neurotrophic factor, and neurturin did not. Protective effects of TGFβ may be mediated in part through binding of TGFb to PAI-1 and its downstream effects. Others have confirmed that focal ischemia induces PAI-1 mRNA without effects on tPA or u-PA mRNAs (Ahn et al., 1999), although u-PA enzymatic activity increases and tPA enzymatic activity decreases after stroke (Rosenberg et al., 1996a). TGF could induce PAI-1mRNA through the combined actions of two sets of transcriptional activators, Smad3 and Smad4 in cooperation with AP-1 (Fig. 6) (Zhang et al., 1998*a*).

GROWTH FACTORS

bFGF

bFGF is induced mainly in astrocytes in the MCA territory and ipsilateral hippocampus after ischemia (Lin et al., 1997). Others have noted global up-regulation of bFGF in the ischemic hemisphere (Lippoldt et al., 1993), including nonischemic regions of cingulate cortex, temporal cortex, and some nonischemic subcortical structures (Speliotes et al., 1996). This suggests that bFGF is probably induced by ischemia induced spreading depression (see below). Although bFGF protected against stroke in rodent models, it failed to protect in a recent human trial. However, it is still possible that bFGF and other growth factors might promote more rapid stroke rehabilitation and perhaps improve long-term recovery (Fisher and Finklestein, 1999; Ay et al., 1999).

BDNF

BDNF is also induced throughout an ischemic hemisphere (Hsu et al., 1993; Kokaia et al., 1998; Guegan et al., 1998; Kokaia et al., 1993). It is likely that BDNF is induced through spreading depression induction of Fos (An et al., 1993*a*) that then induces BDNF through an AP-1 site in its promoter (Cui et al., 1999). bFGF may be induced through a similar mechanism of spreading depression induction of fos that then acts on AP-1 sites in the promoter of the bFGF gene. Inflammatory cytokines like IL-1 also induce bFGF through an AP-1 site in its promoter (Faris et al., 1998). Once induced, BDNF, bFGF, and other growth factors have a large number of target genes that they also regulate (Aho et al., 1997; Semkova and Krieglstein, 1999; Shieh and Ghosh, 1999; Black, 1999).

Glial derived nerve growth factor is also induced after stroke (Abe et al., 1997), as is EGFr (Planas et al., 1998). Insulin growth factors and binding proteins (Gluckman et al., 1992; Lee et al., 1996), platelet derived growth factor (Iihara et al., 1994), ciliary neurotrophic factor (Lin et al., 1998) and growth inhibitory factor (metallothionein III) (Yuguchi et al., 1997) are also induced after focal ischemia. Insulin growth factor like receptor-II (IGF-II) is induced in pyramidal neurons primarily in the core (Stephenson et al., 1995) Interestingly, growth inhibitory factor is induced throughout the hemisphere after a focal stroke, again suggesting possible induction of this gene through spreading depression. Fibroblast growth factor and ciliary neurotrophic factor have been reported to attenuate the thalamic atrophy that occurs after MCAOs in animals (Yamada et al., 1991; Kumon et al., 1996). When tested, the administration of most of the neurotrophic factors protects against focal ischemia (Semkova and Krieglstein, 1999; Wang et al., 1997).

HEAT SHOCK PROTEINS

HSP70 - zone of protein denaturation and renaturation

The induction of heat shock proteins (HSPs) after focal and global ischemia continues to be of interest because they are unique among most of the genes studied because they are specifically induced in cells responding to injury (Nowak and Jacewicz, 1994; Massa et al., 1996; Nowak, 1999; Welch and Brown, 1996), and these genes

protect against a wide variety of injuries (Massa et al., 1996; Rajdev et al., 1997, 2000; Chen and Simon, 1997).

HSP70 is the major inducible heat shock protein, being expressed at low levels in all cells (Welch and Gambetti, 1998; Craig et al., 1993). Any injury that contributes to protein denaturation appears to produce transcriptional activation of hsp70, including ischemia, heat shock, heavy metals, hypoglycemia, low pH, and disease states (Lindquist, 1992; Brown, 1995; Morimoto et al., 1997; Welch and Gambetti, 1998). The presence of the denatured proteins appears to be the major stimulus for hsp70 induction (Ananthan et al., 1986). The transcriptional activation of hsp70 occurs through heat shock factors (HSFs). HSFs are bound to HSP90 in normal cells in an inactivated state (Zou et al., 1998; Gass et al., 1994; Schumacher et al., 1996). With the appearance of denatured proteins, HSP90 binds to the denatured proteins releasing HSFs (Zhou et al., 1996; Zou et al., 1998). HSFs are activated—that is, phosphorylated and form a trimer—and bind to heat shock elements on hsp70 and other heat shock genes to stimulate the heat shock response (Zou et al., 1998).

Thus, the zone of HSP70 induction after focal ischemia can be viewed as the zone of protein denaturation associated with the injury (see Figures 1 and 4). Since HSP70, in concert with HSP90 and other heat shock protein chaperones, acts to renature the denatured proteins, hsp70 induction represents the zone of protein denaturation and attempted protein renaturation. After permanent ischemia or severe temporary focal ischemia hsp70 mRNA may not be expressed in the core of an infarct if ATP is limiting (Nowak, 1999; Welsh et al., 1992; Kobayashi and Welsh, 1995). However, even with vessel occlusions that lead to MCA infarction, hsp70 mRNA can be expressed inside and outside the region of infarction, with most of the hsp70 mRNA within the infarct being expressed in vessels (Kinouchi et al., 1993*a,b*, 1994*b*).

HSP70 protein is expressed mainly in blood vessels and sometimes in scattered microglia and astrocytes in areas inside an infarction (Kinouchi et al., 1993*a*; Soriano et al., 1994; Planas et al., 1997). HSP70 protein is expressed in glia at the margins of infarcts, and HSP70 protein is expressed in glia and neurons outside areas of infarction (Kinouchi et al., 1993*a,b*; Li et al., 1992, 1993; Soriano et al., 1994). As noted above, the neuronal expression of HSP70 protein can be interpreted as a molecularly defined penumbra of protein denaturation (Sharp et al., 1999) (Figs. 1 and 4). The zone of protein denaturation (HSP70) extends beyond the zone of selective neuronal cell death. This is based upon the finding that after a stroke, TUNEL-stained cells occur immediately adjacent to the infarct, whereas HSP70-stained neurons are found at much greater distances from the infarct in the same brains (States et al., 1996; Planas et al., 1997; Li et al., 1993, 1995*a,c*).

HSP70 expression appears to protect cells against various types of injury (Xu and Giffard, 1997; Chen et al., 1996; Mailhos et al., 1994; Wagstaff et al., 1998; Yenari et al., 1998). However, it may not protect against apoptosis or relatively severe injury (Wagstaff et al., 1998). Most important, HSP70 overexpression can protect heart (Marber et al., 1995; Trost et al., 1998) and hippocampus (Plumier et al., 1997*d*) against ischemia. We have found that transgenic mice that overexpress HSP70 protein in brain are protected against strokes produced by permanent MCAO (Rajdev et al., 1998, 2000).

HSP27. There are many other heat shock proteins than HSP70. For example, HSP27 is another inducible HSP expressed at low levels in most brain cells. Notably, HSP27 is expressed at high levels in motor neurons in brainstem and spinal cord (Plumier et al., 1997*d*). HSP27 is also induced inside and outside the areas of infarction after focal ischemia in rodents (Plumier et al., 1997*a,b,c*), although its role is clearly different than HSP70. HSP27 is expressed almost entirely in astrocytes (Plumier et al., 1997*a*) and can be induced by a noninjurious stimulus like spreading depression (Plumier et al., 1997*b*). Hence, the expression of HSP27 does not appear to reflect the region of protein denaturation (Plumier et al., 1997*b*), but reflects a region of cell stress in astrocytes through an undescribed mechanism. HSP27 could associate with actin and astrocyte specific structural proteins to protect glia during stress, and perhaps even contribute to activated astrocyte phenotype (Huot et al., 1996; Loktionova and Kabakov, 1998; Sakamoto et al., 1998).

HSC70. HSC70 is the constitutive heat shock protein found in all cells. It probably chaperones all proteins as they are being formed on the ribosome to prevent abnormal folding during protein synthesis (Beckmann et al., 1990). HSC70 is modestly induced after global and focal ischemia (Abe et al., 1993; Kawagoe et al., 1993) and many other types of injury. Modest ischemia, including that which produces ischemia induced tolerance, can induce HSC70 (Abe et al., 1993; Aoki et al., 1993*a,b*; Chen et al., 1996; Chen and Simon, 1997; Kato et al., 1994).

HSP32 (HO-1). Heme oxygenase-1 (HO-1) is a complex, inducible heat shock protein (also called HSP32) that metabolizes heme to biliverdin, carbon monoxide, and iron (Maines, 1996,1997). The HO system is analogous to NOS because HO-2 is a constitutive form of HO similar to NOS-1 that is found in neurons and is not generally inducible (Ewing and Maines, 1997). HO-1 and NOS-2 are both genes induced mainly in glia that release diffusible gases. There is a HO-3 gene, though it has not been characterized (McCoubrey et al., 1997). HO-1 induction by heat shock (Maines, 1988; Ewing and

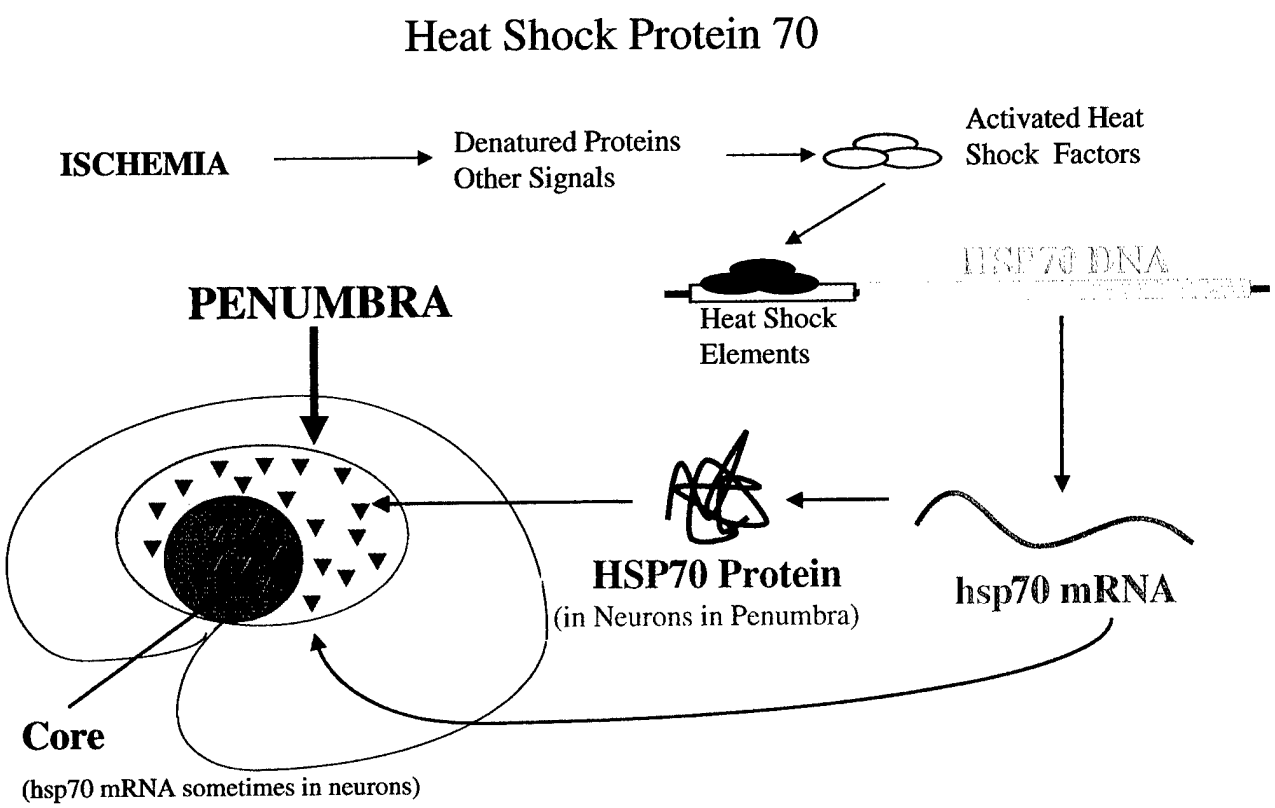

FIG. 4. The denatured protein penumbra. Induction of HSP70 after stroke delineates a region that includes the core of the infarct and regions adjacent to the infarct where the presence of denatured proteins within the cells signals the induction of hsp70. hsp70 mRNA can be detected in the core of infarct with moderate focal ischemia, but may not be synthesized after permanent vessel occlusions when ATP is rapidly depleted. hsp70 mRNA and protein are expressed in some glia and in many neurons at some distance from the infarct in a penumbra defined entirely upon the expression of a molecule.

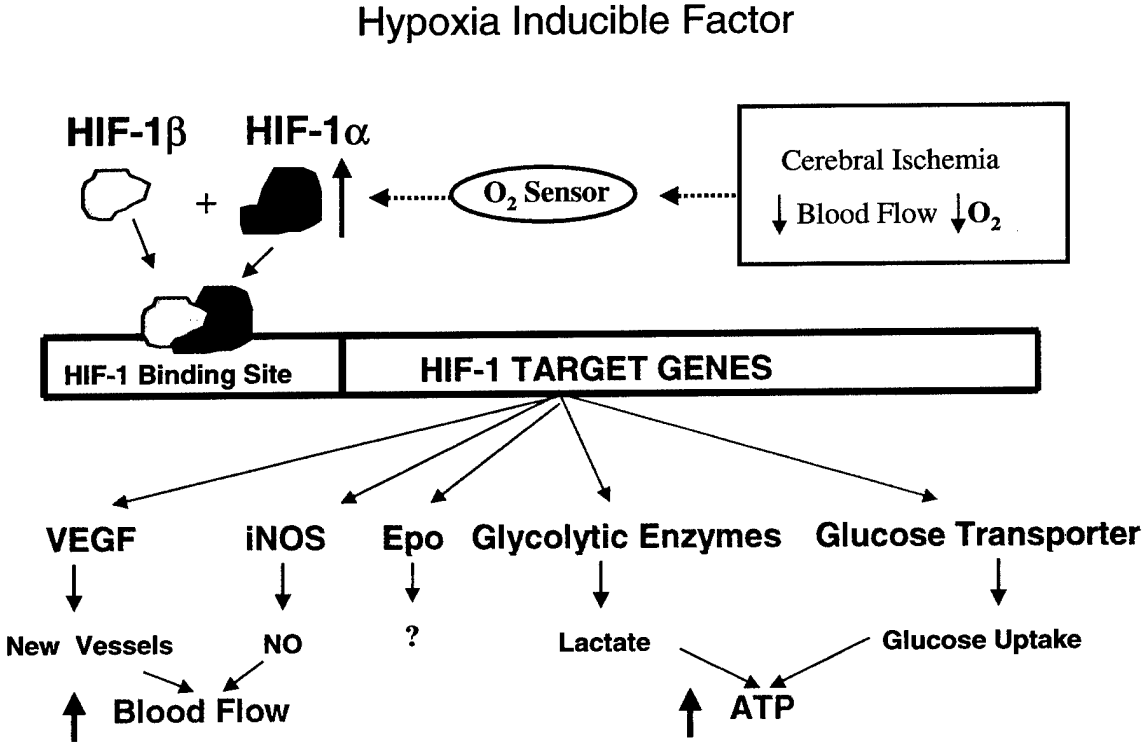

FIG. 5. Proposed mechanism by which stroke induces the hypoxia inducible-factor (HIF) in brain. Decreased blood flow decreases oxygen delivery that is presumed to be detected by a sensor of molecular oxygen. This induces HIF-1α, but not HIF-1β mRNA. The HIF-1α and HIF-1β proteins form a dimer that then bind to response elements in target genes. This interaction then induces the target genes, including the glucose transporter and glycolytic enzymes.

Maines, 1991) and ischemia (Maines et al., 1993) suggests that cells must metabolize heme containing proteins during times of stress and must deal with the subsequent release of iron (Dwyer et al., 1992).

HO-1 plays an important role in metabolizing heme released from hemoglobin after subarachnoid hemorrhage and intracerebral hemorrhage in brain (Matz et al., 1996; Matz et al., 1997; Turner et al., 1998). Heme-iron acts on the HO-1 promoter and is a potent HO-1 inducer (Alam et al., 1994). The major source of heme in ischemic brain however, probably comes from the heme found in mitochondrial electron transport heme proteins released after cell injury.

HO-1 is also induced after cerebral ischemia (Paschen et al., 1994; Geddes et al., 1996). After focal ischemia HO-1 is induced in vessels in the core of an infarct, and in microglia, scattered neurons, and astrocytes at the margin of an infarct (Nimura et al., 1996). At 1 day after a stroke, HO-1 protein is induced in microglia well outside regions of ischemia, including cingulate cortex—a pattern postulated because of spreading depression (Nimura et al., 1996). In fact, spreading depression can induce HO-1 (Koistinaho et al., 1999), c-fos, and COX-2 as described below (Koistinaho et al., 1999). This HO-1 induction could occur through spreading depression-fos-activated AP-1 sites in its promoter (Alam and Den, 1992). Although hypoxia can induce HO-1 through a hypoxia-inducible factor site (Lee et al., 1997) we found little evidence for hypoxia induction of HO-1 in neonatal brain (Bergeron et al., 1997).

HO-1 continues to be expressed in microglia and macrophages at very long time periods after stroke (Koistinaho et al., 1996; Bergeron et al., 1997). This could occur through NFkB sites in the HO-1 promoter (Lavrovsky et al., 1994), suggesting that HO-1 may play a role in inflammation as well (Ewing and Maines, 1993; Willis et al., 1996).

HYPOXIA INDUCIBLE FACTOR - ZONE OF HYPOXIA

HIF-1 is a recently recognized transcription factor that is induced by changes in molecular oxygen levels in tissue (Semenza, 1999; Ratcliffe et al., 1998; Wang et al., 1995*a*). Mutations in the HIF-1α gene result in the inability to induce erythropoietin and increase red blood cells after hypoxia (Semenza, 2000). HIF-1α mRNA is induced by hypoxia and not by inhibitors of mitochondrial respiration. This suggests that HIF-1 is activated by molecular oxygen sensor, possibly a heme-protein (Bunn and Poyton, 1996; Huang et al., 1999).

Once induced, HIF-1α protein binds to HIF-1β, which is constitutively expressed in most cells (Wood et al., 1996). The HIF-1 dimerization that stabilizes both proteins leads to binding to hypoxia response sequences in various target genes. Hypoxia-inducible genes that have HIF-1 sites in their genes and may be regulated at least in part by hypoxia include erythropoietin (Huang et al., 1997), tyrosine hydroxylase (Millhorn et al., 1997), inducible NOS (iNOS, NOS2) (Melillo et al., 1997; Keinanen et al., 1999), vascular endothelial growth factor (VEGF) (Forsythe et al., 1996), glucose transporter-1 (GLUT-1), HO-1 (HSP32) (Lee et al., 1997), transferrin (Rolfs et al., 1997; Lok and Ponka, 1999) and all of the glycolytic enzymes including phosphofructokinase and lactate dehydrogenase (Firth et al., 1994, 1995; Semenza et al., 1996) (Fig. 5).

During reoxygenation, the HIF-1 protein complex has a very short half-life, a matter of minutes (Semenza, 2000). The rapid proteolysis of HIF occurs through the proteasome (Salceda and Caro, 1997; Srinivas et al., 1999; Kallio et al., 1999) that is regulated by the Van Hippel Landau tumor suppressor protein (Maxwell et al., 1999).

HIF is induced in brain after focal ischemia (Bergeron et al., 1999). Although HIF-1α and HIF-1β mRNA are both present in normal brain, only HIF-1α mRNA is induced in the cingulate cortex adjacent to an infarct after suture-induced MCAOs (Bergeron et al., 1999). HIF-1α and HIF-1β protein increase, possibly because of hypoxia-induced stabilization of the dimer. HIF-1 target genes also were induced in the cingulate cortex after MCA inclusion, including GLUT-1, phosphofructokinase, lactate dehydrogenase and others (Bergeron et al., 1999). Because blood flow decreased in this region of HIF-1 expression outside the infarction, we proposed that this region of HIF-1 gene expression represented a region of chronically decreased blood flow that was also chronically hypoxic and that resulted in HIF-1α gene induction. Hence, the region of HIF-1α gene expression after a stroke could be interpreted to be the region of chronic hypoxia around the region of infarction (Figs. 1 and 5).

The region of HIF-1α expression could be co-incident with HSP70 expression. However, the region of HIF-1 expression may be larger than that for HSP70 (Fig. 1), because after suture-induced MCAOs, HIF-1α was expressed in the cingulate cortex (Bergeron et al., 1999) where HSP70 is rarely induced (Kinouchi et al., 1994*a*). The HIF induction in cingulate that occurs with the suture occlusion MCA model is likely caused by anterior cerebral artery ischemia without infarction because of a patent anterior communicating artery (Longa et al., 1989).

It is possible that many experimental models of ischemia may not induce HIF. Global ischemia models produce transient ischemia with only temporary hypoxia that may not induce HIF. Transient focal ischemia models in which blood flow and oxygen delivery are rapidly restored may not induce HIF.

Other hypoxia inducible factors have also been recognized, including HIF-2/HRF and EPAS-1 (Tian et al., 1997; Ema et al., 1997; Flamme et al., 1997). Because

EPAS1 is expressed in capillary endothelial cells (Tian et al., 1997), it might play a primary role in inducing vascular target genes that might be somewhat different or only partially overlap the target genes for HIF-1 (Conrad et al., 1999; Kobayashi et al., 1999). For example, EPAS1, HIF-related genes, or both might regulate VEGF expression in vessels (Ema et al., 1997; Flamme et al., 1997; Badr et al., 1999); whereas HIF-1 itself might regulate glycolytic enzymes to a greater degree in neurons (Bergeron et al., 1999; Badr et al., 1999). EPAS-1 is crucial for normal survival and oxygen sensing during development (Tian et al., 1998). Metal transcription factor-1 is another transcription factor that appears to mediate metal response element responses to hypoxia in metallothionein genes (Murphy et al., 1999).

Genes with HIF-EPAS sites often have other sites in the gene that mediate gene activation through other mechanisms. These include a glucose-regulated element in the glucose transporter GLUT-1 gene (Ebert et al., 1995) and NFkB sites in iNOS and HO-1. The HIF site in p53 does not appear to mediate hypoxic induction of p53 (Wenger et al., 1998). Ischemic induction of HO-1 in brain does not appear to occur through hypoxia, hence, the HIF site in the HO-1 gene (Bergeron et al., 1997). These results are important for showing that the presence of a particular promoter or enhancer element in a gene does not mean that that element is used, or that a particular stimulus uses that promoter element in a particular cell (Chen et al., 1999*b*).

Although the genes induced by HIF-1 and other hypoxia responsive transcription factors generally tend to increase blood flow, glucose delivery, and maintenance of energy after chronic hypoxia (Fig. 5), the role of HIF-1 in acute focal cerebral ischemia is unclear. For example, increased NO from iNOS (Palmer et al., 1998), dopamine from tyrosine hydroxylase (Millhorn et al., 1997) and lactate from lactate dehydrogenase (Semenza et al., 1996) may worsen ischemia. However, increased glucose transporter expression (Lawrence et al., 1996*b*; Vannucci et al., 1998) or erythropoietin (Bernaudin et al., 1999; Semenza, 1994) expression might protect brain. The role of HIF-1 remains to be determined. HIF-1 may have harmful roles in some cell types and beneficial roles in other cell types, with the ultimate harm or protection depending on the model, timing, and mode of the HIF induction in brain (Halterman et al., 1999; Zaman et al., 1999). HIF-1 could play a role in mediating hypoxia-induced tolerance to cerebral ischemia (Gidday et al., 1994,1999; Bergeron et al., 1999).

VEGF

VEGF is a potential HIF-1 target gene (Levy et al., 1995, 1997) that is induced by focal ischemia. VEGF is induced in the core and ischemic border zone after focal ischemia Kovacs et al., 1996; Cobbs et al., 1998), although some have reported bilateral cortical induction of VEGF after focal ischemia (Lennmyr et al., 1998). In primate ischemic models, noncapillary vessels in the ischemic core and the periphery of an infarct express VEGF mRNA (Abumiya et al., 1999). VEGF mRNA is located in both microglia-macrophages and in endothelial cells in regions adjacent to rodent infarcts (Plate et al., 1999). The VEGF receptors Flt-1 and Flk-1 were induced after ischemia as well, Flt-1 on neurons, glia, and endothelial cells; and Flk-1 mainly on glial and endothelial cells (Lennmyr et al., 1998). Induction of the VEGF receptors and other VEGF target genes could be mediated by Ets-1, a vascular-related transcription factor (Valter et al., 1999). The regulation of VEGF may involve multiple sites on the gene and may involve several transcription factors (Dibbens et al., 1999). HIF-like or EPAS-1 induction of VEGF could mediate the formation of new vessels after stroke (LaManna et al., 1998; Ment et al., 1997; Shweiki et al., 1992). However, because the formation of new vessels is considerably delayed, it seems unlikely that this would influence the outcome of an acute infarct. Expression of VEGF could influence the permeability of existing vessels and contribute to ischemia-induced edema, however (Ment et al., 1997; LaManna et al., 1998; van Bruggen et al., 1999).

IMMEDIATE EARLY GENE INDUCTION

Hemispheric spreading depression

After focal ischemia, a number of IEGs, including the c-fos gene, are induced throughout the entire hemisphere of the rat brain, and in the frontal, parietal, occipital, and limbic cortex including cingulate cortex (An et al., 1993; Hsu et al., 1993; Welsh et al., 1992; Kinouchi et al., 1994*a*; Lindsberg et al., 1996). Because most rodent MCAO models only produce infarction in the MCA distribution, it has been suggested that spreading depression accounts for induction of c-fos and other genes in the nonischemic portions of the hemisphere (Gass et al., 1992; Kinouchi et al., 1994*d*; Mancuso et al., 1999). This is supported by evidence showing that preventing ischemia induced spreading depression with NMDA antagonists, like MK-801, prevents c-fos induction in frontal and occipital poles after MCAOs (Gass et al., 1992; Kinouchi et al., 1994*d*; Collaco-Moraes et al., 1994). In addition, spreading depression produced by applying potassium chloride to the cortex, or by producing small cortical lesions induces these genes throughout the entire hemisphere (Herdegen et al., 1993; Sharp et al., 1989, 1990; Herrera and Robertson, 1989, 1990; Kobayashi et al., 1995; Koistinaho et al., 1999).

A large number of genes in addition to c-fos are induced throughout an ischemic hemisphere; hence, these genes are likely to be induced by spreading depression or repeated ischemic depolarizations (Koistinaho and Hok-

felt, 1997). These genes include junB (Comelli et al., 1993; Hsu et al., 1993; Kamii et al., 1994*a*; Kinouchi et al., 1994*a*), Zac1 and PACAP (Gillardon et al., 1998), NGFIA,B,C (Lin et al., 1996; Honkaniemi et al., 1997), egr (Honkaniemi et al., 1997), Rheb (Kinouchi et al., 1999*a*), Arc (Kunizuka et al., 1999) and probably other IEGs. Hsp27, COX2, and PKC are induced by spreading depression (Plumier et al., 1997*c*; Miettinen et al., 1997; Koponen et al., 1999).

Some of the genes induced by ischemia induced spreading depression are likely to be fos-jun target genes (Fig. 6). BDNF, bFGF and GFAP are induced throughout a hemisphere after spreading depression (Kraig et al., 1991; Kokaia et al., 1993) and may play a role in protecting brain against stroke (Matsushima et al., 1998). Since BDNF, bFGF and GFAP have AP-1 sites in their promoters, members of the fos and jun families could induce these genes. However, the induction of any of these genes could be complex. Although GFAP could be induced through AP-1 sites, there are also NFkB-like sites in the GFAP gene that renders it responsive to both TGF-1 and IL-1 (Krohn et al., 1999). Other genes with AP-1 sites in their promoters that could be induced by fos-jun family members could include dynorphin, enkephalin, NPY, iNOS, HO-1, APP, tyrosine hydroxylase, GAP43, NGF, and many others (Nowak, 1999; Morgan and Curran, 1995). Hypoxia induction of tyrosine hydroxylase occurs specifically through junB/c-fos dimers binding to the AP-1 site in the tyrosine hydroxylase promoter (Norris and Millhorn, 1995; Millhorn et al., 1997).

c-jun

c-jun mRNA also appears to be induced throughout an ischemic hemisphere (Gass et al., 1992; An et al., 1993; Kinouchi et al., 1994*a*; Munell et al., 1994). The role of c-jun and its other family members is complex because different family members likely have different target genes in different cells. In addition, c-jun can form a homodimer as well as complex with c-fos family members. Therefore, it is not too surprising that c-jun expression has been associated with cell survival as well as cell death. C-jun is expressed in axotomized motor neurons that will survive axotomy, and c-jun is expressed in ischemia-induced tolerance models in which cells survive ischemia (Herdegen and Leah, 1998; Sommer et al., 1995; Kato et al., 1995*a*). However, phosphorylated c-jun (c-jun-P) appears to be expressed in cells that undergo apoptosis and may be expressed in ischemic cells that are dying or dead (Gillardon et al., 1999; Matsuoka et al., 1999; Domanska-Janik et al., 1999; Walton et al., 1999). Phosphorylated c-jun is coexpressed with possible jun/AP-1* target genes, APP, and CPP32 (caspase 3) in ischemic neurons (Walton et al., 1999).

Hippocampus

A number of genes are induced in hippocampus after MCAOs in rodents, particularly using the suture model. This includes c-fos and c-jun family members, the zinc finger immediate early genes, and a variety of other genes including COX-2 (Kinouchi et al., 1994*a,b,c*, 1999*a,b*; Honkaniemi et al., 1997; Kamii et al., 1994*b*; Koistinaho et al., 1999). There are several possible explanations for such gene induction. First, there may be some hippocampal ischemia using the suture model. This is supported by TUNEL positive CA1 neurons in hippocampus using this model that is also associated with bilateral induction of HSP70 in CA1 pyramidal neurons (States et al., 1996). In addition, models that produce infarctions restricted to cortex do not generally induce the IEGs in hippocampus (Gass et al., 1992; Lindsberg et al., 1996).

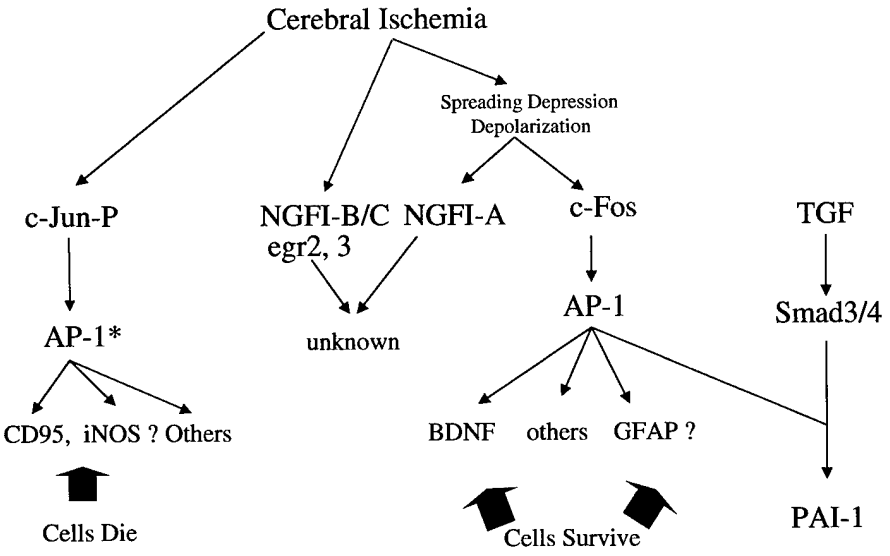

FIG. 6. Ischemia induces a large number of transcription factors in brain. Spreading depression appears to be the stimulus for the induction of c-fos, NGFI-A and a large number of other immediate early genes. Though c-jun may be widely induced after focal ischemia, phosphorylated c-jun (c-jun-P) is associated with cell death in many paradigms and probably has different AP-1* mediated target genes than c-fos mediated AP-1 target genes in areas of spreading depression where there is no cell death. Notably, AP-1 can interact with many other transcription factors including Ets, Smad3/4, and others to presumably regulate different sets of target genes.

It is also possible that IEGs are induced in hippocampus through excitatory mechanisms. Middle cerebral artery occlusions can produce repeated cortical spreading depressions that depolarize entorhinal cortex (Busch et al., 1995). Activation of entorhinal cortical inputs to hippocampus could account for the induction of many genes in hippocampus after MCAOs. This is supported by the suppression of hippocampal gene induction by MK-801 that prevents cortical spreading depression (Kinouchi et al., 1994*c*; Gass et al., 1992).

Gene induction in contralateral cortex and subcortical structures

Some studies also demonstrate that ipsilateral thalamus, ipsilateral substantia nigra, and contralateral cortex show induction of c-fos and other immediate early genes after an ipsilateral MCAO (Kinouchi et al., 1994*a,b,c*). NGFI-A is induced bilaterally after MCAOs (Lin et al., 1996). IL-1 and TNF-α are not only induced in the ischemic hemisphere, but they are also induced in the contralateral hemisphere at lower levels (Buttini et al., 1996; Zhai et al., 1997). HSP27 can be induced in both hemispheres after a unilateral MCA stroke (Kato et al., 1995*b*). Arc is induced bilaterally in hippocampus and amygdala after MCAOs (Kunizuka et al., 1999). GLUT-1 and GLUT-3 can be induced in both hemispheres after unilateral stroke (Lee and Bondy, 1993; Urabe et al., 1996). Changes of gene expression in cerebellum are of interest because of the well-described phenomenon of cerebellar diaschisis (Ginsberg, 1990). However, there is little information on gene regulation in cerebellum after stroke. cGMP changes in cerebellum after MCAOs (Kader et al., 1993) and biliverdin reductase is induced in cerebellum after permanent MCAOs (Panahian et al., 1999).

The gene induction in the contralateral hemisphere, cerebellum, and many other subcortical regions is not caused by ischemia. Gene induction in these remote regions may be caused in part by acute ischemia-induced depolarization in the period immediately after a focal stroke. At longer times, changes in gene expression likely represent plastic changes in neurons and glia that must occur in the contralateral cortex, pons, cerebellum, spinal cord, and other brain regions that are connected directly or indirectly to the cortex and basal ganglia that were infarcted by the MCAOs. These changes of gene expression offer fruitful possibilities for possibly enhancing brain plasticity and behavioral recovery after stroke. Changes in GABA receptor subunits many days after stroke may be related to plastic responses of GABAergic neurons that could mediate recovery mechanisms (Neumann-Haefelin et al., 1999). There are bilateral changes of NMDA receptors in cortex after focal ischemia (Que et al., 1999) that may play a role in plastic changes in cortex and cortical motor and sensory maps (Nudo and Friel, 1999; Johansson, 2000).

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Review

The molecular basis of O_2-sensing and hypoxia tolerance in pheochromocytoma cells

P. William Conrad, Laura Conforti, Shuichi Kobayashi, Dana Beitner-Johnson, Randy T. Rust, Yong Yuan, Hie-Won Kim, Richard H. Kim, Karen Seta, David E. Millhorn*

Department of Molecular and Cellular Physiology, University of Cincinnati, College of Medicine, P.O. Box 67-0576, Cincinnati, OH 45267-0576, USA

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Abstract

Hypoxia is a common environmental stimulus. However, very little is known about the mechanisms by which cells sense and respond to changes in oxygen. Our laboratory has utilized the PC12 cell line in order to study the biophysical and molecular response to hypoxia. The current review summarizes our results. We demonstrate that the O_2-sensitive K^+ channel, Kv1.2, is present in PC12 cells and plays a critical role in the hypoxia-induced depolarization of PC12 cells. Previous studies have shown that PC12 cells secrete a variety of autocrine/paracrine factors, including dopamine, norepinephrine, and adenosine during hypoxia. We investigated the mechanisms by which adenosine modulates cell function and the effect of chronic hypoxia on this modulation. Finally, we present results identifying the mitogen- and stress-activated protein kinases (MAPKs and SAPKs) as hypoxia-regulated protein kinases. Specifically, we show that p38 and an isoform, p38γ, are activated by hypoxia. In addition, our results demonstrate that the p42/p44 MAPK protein kinases are activated by hypoxia. We further show that p42/p44 MAPK is critical for the hypoxia-induced transactivation of endothelial PAS-domain protein 1 (EPAS1), a hypoxia-inducible transcription factor. Together, these results provide greater insight into the mechanisms by which cells sense and adapt to hypoxia.

Keywords: Gene expression; Signal transduction; Transcription; Adenosine; Pheochromocytoma; PC12 cells; Mitogen-activated protein kinase (MAPK); p38; EPAS1

* Corresponding author. Tel.: +1-513-558-5636; fax: +1-513-558-5738.
E-mail address: david.millhorn@uc.edu (D.E. Millhorn).

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1. Introduction

The research in our laboratory has focused on how cells sense and respond to hypoxia. Hypoxia is a condition having both environmental (e.g. altitude) and pathological (e.g. ischemia) origins. Cell survival requires that hypoxia elicit appropriate changes in gene expression and cell function. We have utilized PC12 cells, an O_2-sensitive cell line, in order to study the biophysical and molecular mechanisms by which cells respond to hypoxia. In the current review, we discuss three different aspects of the hypoxic response. First, we provide evidence for the role of O_2-regulated potassium channels (Ko_2), particularly Kv1.2, in O_2 sensing. We next discuss the effects of hypoxia on modulation of adenosine-induced responses. We demonstrate that adenosine receptor binding plays a role in modulating hypoxia responsiveness and, perhaps, in protecting cells against the harmful effects of hypoxia. Finally, we discuss the effects of hypoxia on regulation of various signal transduction pathways and gene expression. Taken together, these results encompass a wide spectrum of hypoxia-induced changes, from the immediate sensing mechanism to the effects of chronic hypoxia on membrane excitability and gene expression. Although these studies address three distinct responses, there are almost certainly interactions and cross-talk between them. For example, each of these responses is dependent upon or modulates intracellular Ca^{2+} levels. A detailed discussion of these relationships is also included.

2. PC12 cells contain the O_2-sensitive Kv1.2 K^+ channel

The ability of cells to continually sense their environment and make appropriate changes in gene expression and cell function is critical for their survival. Cells have therefore evolved the capacity to exquisitely sense changes in their extracellular milieu. An example of this sensing ability is found in specialized O_2-sensitive or chemoreceptor cells. These cells are localized in specific tissues within the body, including the carotid body, the pulmonary vasculature and pulmonary neuroepithelial bodies (for review see Lopez-Barneo, 1994). A decrease in pO_2 stimulates these cells, resulting in cardiovascular and pulmonary responses that optimize the delivery of O_2 to vital organs. Such rapid responses have evolved to prevent global or localized O_2 deficits that can produce irreversible cellular damage.

The PC12 cell line has been used as a model to study O_2-chemosensory mechanisms. There are a number of phenotypic similarities between type I carotid body cells and PC12 cells, including the presence of O_2-sensitive K^+ channels (Lopez-Barneo et al., 1988; Conforti and Millhorn, 1997). In addition, both PC12 cells and type I cells respond to hypoxia with an increase in tyrosine hydroxylase gene expression (Czyzyk-Krzeska et al., 1992, 1994). Finally, both cell types depolarize and secrete the neurotransmitter dopamine in response to hypoxia (Krammer, 1978; Kumar et al., 1998; Taylor and Peers, 1998). We have therefore utilized PC12 cells to study the biophysical and molecular mechanisms by which cells sense and respond to hypoxia.

The presence of O_2-sensitive K^+ channels in chemosensitive cells is critical to sensing hypoxia. O_2-sensitive K^+ (Ko_2) channels have been identified in several chemosensitive cells. Inhibition of Ko_2 channel activity is an important early event in the process of O_2 chemoreception, initiating the process of cell depolarization, Ca^{2+} influx, neurotransmitter release, muscle contraction, regulation of protein kinases, and alterations in gene expression. Therefore, Ko_2 channels have been proposed to be key elements in the detection of changes in O_2 availability by chemosensitive cells. Work from our lab has shown that PC12 cells express O_2-sensitive potassium channels. Additional experiments identified the Kv1.2 α subunit as an important component of the Ko_2 in PC12 cells.

In order to identify the Ko_2 channel in PC12 cells, patch clamp experiments were performed at the single-channel level. Four types of voltage-dependent outward K^+ channel were identified in PC12 cells: a slow-inactivating or delayed-rectifier K^+ channel (20 pS conductance, K_{dr}), a transient or fast-inactivating K^+ channel (20 pS), a small conductance (14 pS) and a Ca^{2+}-activated K^+ channel (105 pS). In order to identify the K^+ channel inhibited by hypoxia, patches containing different types of channels were exposed to 10% O_2. The activity of the slow-inactivating 20 pS K^+ channel was inhibited by reduced pO_2. These were the most frequently observed channels in our recordings, and their current kinetics suggest that they belong to the delayed-rectifier family of

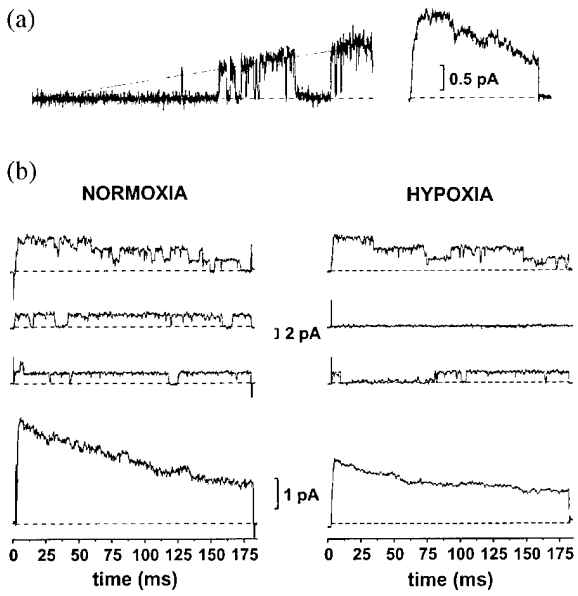

Fig. 1. A delayed-rectifier K^+ channel in PC12 cells is inhibited by hypoxia. (a) The left trace shows the i–V relationship obtained with ramp pulse depolarization (from a V_h of −60 mV to +50 mV, 800 m duration, 2.8 mM K^+ in the pipette) of a delayed-rectifier type of K^+ channel (K_{dr}). The recording was fitted with a straight line having a slope value of 19 pS. Outward K^+ current was evoked in the same patch with step pulse depolarization from a V_h of −60 mV to +50 mV, 180 m duration. The corresponding ensemble-averaged slow-inactivating outward current is shown in the right trace. Data shown are representative of a characteristic population of K^+ channels in PC12 cells. (b) Shown are representative traces recorded during step depolarizing pulses (from a V_h of −60 mV to +50 mV, 180 m duration) in normoxia and 2 min after exposure to hypoxia (10% O_2). Leak and capacitative currents were subtracted from the record. Upward current deflections from the zero line correspond to the opening of the channel. Dashed lines represent the zero current. The ensemble-averaged currents from 100 consecutive traces are shown in the bottom panel.

K^+ channels (K_{dr}). Fig. 1a shows the *i–V* relationship for the Ko_2 channel. Application of a step depolarization caused the channel to open early on in the pulse depolarization and remain open for most of the pulse duration. Fig. 1b shows representative recordings from the 20-pS K_{dr}, channel in normoxia and after 2 min exposure to hypoxia. We also found that the inhibitory effect of hypoxia on the 20 pS K_{dr} channel persisted in inside-out patches (Conforti and Millhorn, 1997), excluding cytoplasmic soluble factors as mediators of the hypoxic response.

The molecular composition of Ko_2 channels is still poorly understood. Recently, a K^+ channel composed of Kv2.1 and the silent Kv9.3 α subunit was proposed as a possible Ko_2 channel in pulmonary artery smooth muscle cells (Patel et al., 1997). Other subunits, including Kv1.2 and Kv1.5, have also been proposed to form Ko_2 channels in the pulmonary artery. Recently, it has been shown that the Kv1.2 α subunit confers O_2-sensitivity to the Kv1.5 K^+ channel and that both Kv1.2 and Kv2.1 K^+ channels expressed in mouse *L* cells were inhibited by hypoxia (Hulme et al., 1999). Our laboratory has identified the Kv1.2 α subunit as an important component of the native Ko_2 channel in PC12 cells. Because O_2-sensitive cells adapt to prolonged hypoxia, including a modified response to subsequent exposure to hypoxia (e.g. enhanced chemosensitivity of the carotid body; Stea et al., 1995), we hypothesized that the Ko_2 channel gene expression might be regulated during prolonged exposure to hypoxia. Fig. 2a shows the expression of the different Kv genes in PC12 cells exposed to normoxia or hypoxia (18 h, 10% O_2). The expression of the Shaker Kv1.2, but not the other K^+ channel genes, was increased by prolonged exposure to hypoxia (Fig. 2a). The increased expression of the Kv1.2 gene correlated with an enhanced response to hypoxia in those cells exposed to 10% O_2 for 18 h prior to electrophysiological measurements (Fig. 2b,c). These data provided the first evidence that the Kv1.2 gene encodes the α subunit(s) of the slow-inactivating Ko_2 channel in PC12 cells. Previous results support the involvement of the Kv1 subfamily of K^+ channels, as the Ko_2 current in PC12 cells was blocked by 5 mM TEA (Zhu et al., 1996). Furthermore, the Ko_2 in PC12 cells is inhibited by charybdotoxin, a potent blocker of Kv1.2 and Kv1.3 and large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels (Conforti et al., 2000). Although K_{Ca} channels are present in PC12 cells, we have shown previously that, under our experimental conditions, their contribution to the total outward current is negligible (Zhu et al., 1996). We have also shown that the K_{Ca} channels in PC12 cells are not inhibited by hypoxia (Conforti and Millhorn, 1997). Taken together, these data support a role for Kv1.2 in forming the Ko_2 in PC12 cells.

In addition to expression of the Kv1.2 gene, PC12 cells also express the Kv2.1 α subunit, which has been proposed as a possible K^+ channel in pulmonary artery smooth muscle cells (Patel et al., 1997; Archer et al., 1998). Thus, we were interested in comparing the ability of Kv1.2 and

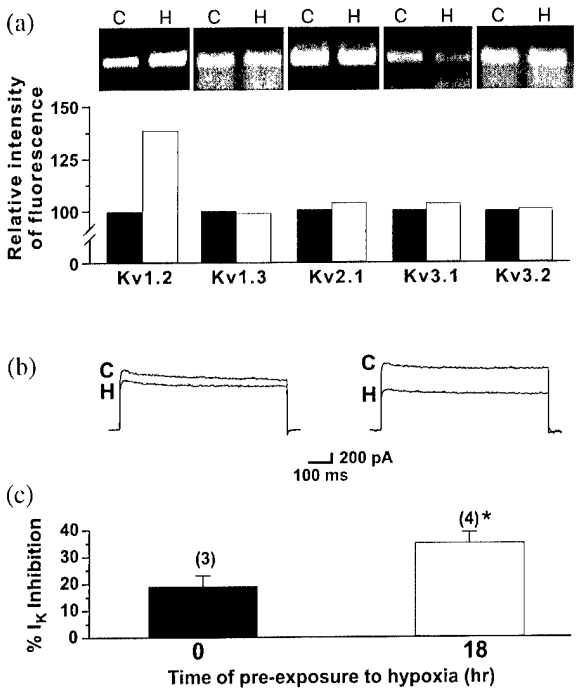

Fig. 2. The Kv1.2 gene is induced by hypoxia and correlates with increased O_2-sensitivity of PC12 cells to hypoxia after prolonged exposure to hypoxia. (a) The effect of prolonged hypoxia on Kv gene expression in PC12 cells was determined by RT-PCR of total RNA. Equal amounts of RNA from PC12 cells maintained in a normoxic (C) or hypoxic (10% O_2) incubator for 18 h (H) were simultaneously tested for the Kv genes of interest. PCR products were collected before reaching saturation (30 cycles for Kv1.2, 25 cycles for all other genes). Agarose gels for the Kv gene PCR products are shown in the top panel. Each pair corresponds to the gene label on the abscissa of the graph below. The pairs are separated by a white lane for clarity, but were all analyzed in the same gel. The relative intensity of the ethidium bromide fluorescence of each band is reported in arbitrary units with respect to the control (set as 100) and shown in the bottom panel. The data are the mean of seven experiments for Kv1.2 and four experiments for the other genes. (b) O_2 sensitivity was tested in cells maintained in normoxic conditions (left panel) or after 18 h exposure to 10% O_2 (right panel). K^+ currents (I_K) were measured in whole-cell voltage clamp before and after 1 min exposure to hypoxia (H, 10% O_2). Cells were depolarized to +50 mV (V_h, −70 mV) for 800 ms C). The bottom panel shows the amount of hypoxic inhibition of I_K in cells maintained in a hypoxic environment (18 h pre-exposure to hypoxia; 35%) compared with cells grown in a normoxic incubator (0 h pre-exposure to hypoxia; 19%). $^*P < 0.05$ using Student's unpaired t-test. The number of cells is given in parentheses.

Kv2.1 to form the Ko_2 in PC12 cells. Expression of Kv1.2 and Kv2.1 α proteins in PC12 cells was determined by immunoblot analysis (Fig. 3a). Analysis with an affinity-purified antibody against Kv1.2 revealed a single band of approximately 80 kDa. Antibodies against Kv2.1 α subunit detected a single band of approximately 110 kDa. Specificity of the Kv2.1 antibody has been shown previously (Archer et al., 1998), while the specificity of the Kv1.2 antibody was established by immunoblot and immunohistochemical analysis (Fig. 3a,b). The ability of the anti-Kv1.2 antibody to selectively block Kv1.2 K^+ channels was assessed in *Xenopus* oocytes. Recombinant Kv1.2 K^+ current amplitude was significantly decreased in oocytes injected with anti-Kv1.2 antibody (Fig. 3c). The same concentration of anti-Kv1.2 antibody did not reduce K^+ current amplitude measured in oocytes expressing Kv2.1 K^+ channels (Fig. 3c).

We next tested the hypothesis that the Ko_2 channel in PC12 cells is composed of Kv1.2 α subunit(s) by comparing the efficiency of anti-Kv1.2 and anti-Kv2.1 antibodies in blocking the Ko_2 current. A similar approach has been used to establish the role of Kv2.1 in setting the resting potential of pulmonary artery smooth muscle cells (Archer et al., 1998). Others have also used this approach to modify ion channel activity in neuronal and skeletal muscle cells (Vassilev et al., 1988; Naciff et al., 1996). Whole-cell voltage-clamp experiments were performed with anti-Kv1.2 or anti-Kv2.1 antibodies delivered to the cell by dialysis through the patch pipette. Fig. 4 shows representative experiments performed in the presence of an anti-Kv1.2 antibody in the pipette. The left panel shows K^+ currents recorded in normoxia upon breaking into whole-cell configuration (N_o). Within 8–10 min after breaking into the whole-cell configuration, dialysis of Kv1.2 antibody through the patch pipette resulted in a $32 \pm 6\%$ decrease in K^+ current amplitude. Subsequent exposure to hypoxia (H, 10% O_2) did not inhibit the K^+ current. The averaged inhibition of the K^+ current by hypoxia in cells dialyzed with antibody against Kv1.2 was $4 \pm 3\%$ ($n = 6$, Fig. 4c).

Identical experiments were performed with an anti-Kv2.1 antibody in the patch pipette. Within 8–10 min after breaking into the whole-cell configuration, dialysis of anti-Kv2.1 antibody through the patch pipette resulted in a $39 \pm 3\%$ ($n = 3$) decrease in K^+ current amplitude. Subsequent exposure to hypoxia (10% O_2) inhibited the K^+ current of $24 \pm 2\%$ ($n = 3$). This amount of inhibition is significantly different from that observed in cells dialyzed with the anti-Kv1.2 antibody ($P \leq 0.01$). Control experiments using

an irrelevant antibody (rabbit anti-sheep IgG) in the pipette are shown in Fig. 4c. Ten minutes after breaking into the whole-cell configuration, no decrease in K^+ current amplitude was observed, but application of hypoxia caused a

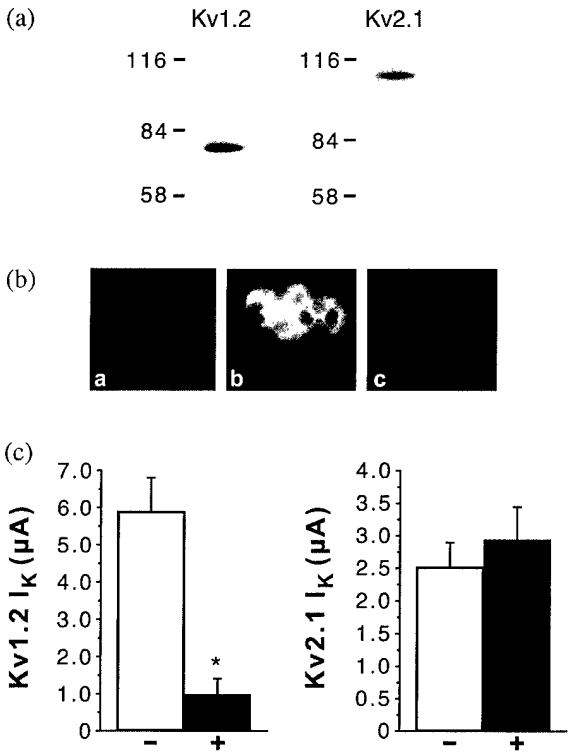

Fig. 3. The Kv1.2 and Kv2.1 α subunits of K^+ channels are expressed in PC12 cells. (a) Representative Kv1.2 and Kv2.1 immunoblots in PC12 cell extracts (40 μg protein). (b) Immunostaining analysis of Kv1.2 in PC12 cells. (Panel a): PC12 cells were subjected to all steps in the staining protocol, except that the primary antibody was omitted (background staining). (Panel b): Kv1.2 immunostaining. (Panel c): Immunostaining of PC12 cell with antiKv1.2 antibody pre-incubated with the antigen against which the antibody is directed. The intensity of the fluorescent signal is comparable to the background fluorescence observed in panel a. (c) The effect of anti-Kv1.2 antibodies on recombinant Kv1.2 and Kv2.1 K^+ channel function was evaluated. The left panel shows that the anti-Kv1.2 antibody quantitatively blocks K^+ current in oocytes expressing Kv1.2 K^+ channels. Kv1.2 K^+ currents were recorded in control oocytes (−, $n = 6$) and oocytes injected with anti-Kv1.2 antibody (0.01 μg in 50 nl) 2 h before recording (+, $n = 4$). $^*P < 0.01$ using Student's unpaired t-test. The right panel shows the lack of effect of anti-Kv1.2 antibody on K^+ currents in oocytes expressing Kv2.1 K^+ channels. Kv2.1 K^+ currents were recorded in control oocytes (−, $n = 5$) and oocytes injected with anti-Kv1.2 antibody (0.01 μg in 50 nl) 2 h before recording (+, $n = 6$). Kv2.1 K^+ currents were elicited from with voltage steps from −80 mV HP to −10.0 mV in two-electrode voltage-clamp experiments.

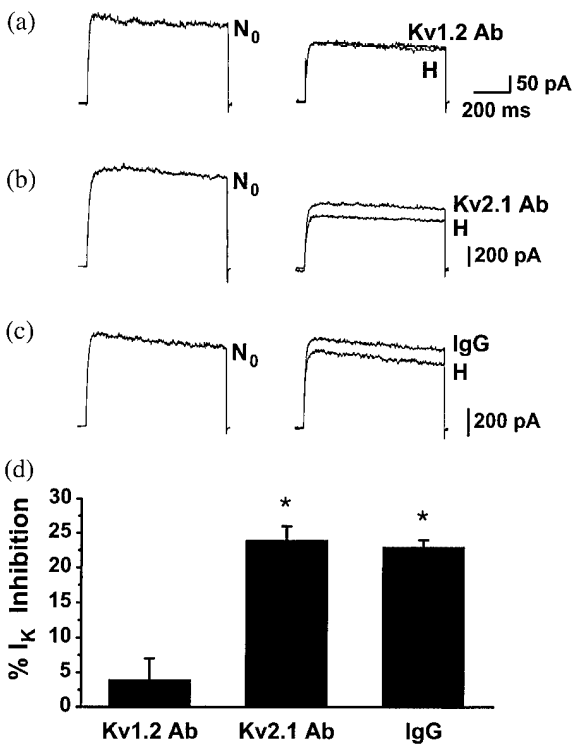

Fig. 4. Antibodies against Kv1.2, but not Kv2.1, block the effect of hypoxia on the K^+ current. K^+ currents were elicited with voltage steps from −70 mV HP to +50 mV (every 5 s) in experiments performed in presence of anti-Kv1.2 antibody (a), anti-Kv2.1 antibody (b) or irrelevant antibody (c) in the pipette. The representative K^+ current traces were recorded in normoxia (21% O_2) upon breaking into whole-cell configuration (left panel, N_0, in normoxia 8–10 min into whole-cell configuration (right panel, labeled with the name of the antibody used in each experiment), and after exposure to hypoxia (10% O_2, H). (d) Averaged amount of current inhibition by hypoxia in the presence of each antibody. $^*P \leq 0.001$

reversible inhibition of the K^+ current. This level of inhibition of K^+ current is not statistically different from the inhibition induced by hypoxia in the presence of the anti-Kv2.1 antibody. Taken together, these data indicate that the Kv1.2 α subunit plays a critical role in the response of PC12 cells to hypoxia. Future experiments are aimed at defining the mechanism by which Kv1.2 responds to hypoxia. In addition, we are also interested in investigating the interactions between Kv1.2 and its downstream effectors.

3. Hypoxia modulates the adenosine system

Adenosine is an endogenous metabolite of ATP that has been proposed to have a protective func-

tion in neurons (Scanziani et al., 1992; Lupica et al., 1992). Adenosine is released in the central nervous system in response to ischemia and hypoxia, where it acts to decrease pre- and post-synaptic excitability, thereby protecting neurons against the metabolic stress associated with O_2 deprivation (Scanziani et al., 1992; Lupica et al., 1992). The cellular effects of adenosine are mediated via specific receptors cell surface receptors, classified as A_1, A_{2A}, A_{2B}, and A_3 (Bruns, 1990; Dalziel and Westfall, 1994; Palmer and Stiles, 1994; Fredholm, 1995; Olah and Stiles, 1995). The A_1 and A_3 receptors are generally coupled to the G_i protein and mediate inhibition of adenylate cyclase activity, while the A_2 receptor family is coupled to the G_S protein, which stimulates adenylate cyclase activity.

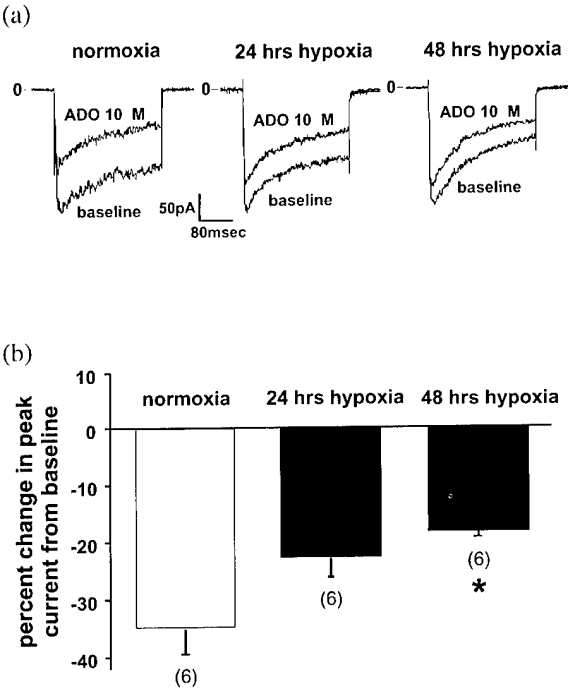

Fig. 5. Hypoxia attenuates the adenosine-induced inhibition of voltage-dependent Ca^{2+} current. (a) Representative traces show the effect of adenosine (ADO) on I_{Ca}. I_{Ca} was measured every 30 s by 160-ms test pulses from a V_h of -80 to $+20$ mV, using 20 mM Ba^{2+} as a charge carrier. Peak current amplitude was measured for evaluation. In normoxic controls, adenosine (10 μM) caused a decrease in the amplitude of I_{Ca} (left panel). This effect was reduced when the cells were pretreated with hypoxia (10% O_2) for 24 h (middle panel) or 48 h (right panel). (b) The effect of adenosine on I_{Ca} is shown quantitatively. The response to adenosine was evaluated as the percentage inhibition from baseline inward current. The number in parentheses indicates the number of cells examined. Data are expressed as mean ± S.E.M., $^*P < 0.05$.

We studied the effects of adenosine on the responses of these cells to both acute and chronic hypoxia. PC12 cells express the A_{2A} and A_{2B}, but not the A_1, or A_3 adenosine receptor subtypes (Hide et al., 1992, Kobayashi et al., 1998a). One of the immediate cellular events in response to hypoxia in this cell type is an elevation in intracellular calcium levels ($[Ca^{2+}]_i$, Zhu et al., 1996). Acutely, the exogenous application of adenosine attenuated both the total cellular calcium current (I_{Ca}) and the hypoxia-induced increase in $[Ca^{2+}]_i$ in PC12 cells (Kobayashi et al., 1998a). These effects are mediated via the A_{2A} receptor and are dependent on protein kinase *A* (PKA, Kobayashi et al., 1998a).

The effect of adenosine on I_{Ca} following pre-exposure to hypoxia (10% O_2, 24 or 48 h) was also investigated. Fig. 5 shows that the inhibition of I_{Ca}, by adenosine was reduced when the cells had been pre-exposed to chronic hypoxia. These results suggested that chronic hypoxia might also attenuate the effect of adenosine on the hypoxia-induced increase in $[Ca^{2+}]_i$. To test this hypothesis, intracellular Ca^{2+} levels were analyzed in cells that were pre-exposed to hypoxia in the presence or absence of adenosine (Kobayashi et al., 1998b). The $[Ca^{2+}]_i$ in PC12 cells is sharply increased in response to anoxia (< 10 torr O_2). Interestingly, the increase in $[Ca^{2+}]_i$ in response to acute anoxia was greater in PC12 cells that had been pre-exposed to 48 h of moderate hypoxia (10% O_2). Furthermore, pre-exposure to hypoxia for 48 h blunted the inhibitory effect of adenosine on $[Ca^{2+}]_i$ in response to anoxia (Fig. 6). Thus, pre-exposure to prolonged hypoxia attenuates the acute inhibitory effects of I_{Ca} and $[Ca^{2+}]_i$ in PC12 cells (Kobayashi et al., 1998b).

We next designed experiments to further elucidate the mechanism by which chronic hypoxia impairs the actions of adenosine on I_{Ca} and $[Ca^{2+}]_i$. As mentioned above, PC12 cells do not express either the A_1 or A_3 adenosine receptor subtypes, but do express the A_{2A} and A_{2B} receptor subtypes (Kobayashi et al., 1998a). As summarized in Fig. 7, activation of either the A_{2A} or the A_{2B} receptor causes activation of the G_S guanine nucleotide binding protein, which stimulates adenylyl cyclase. Increased production of cyclic AMP then leads to activation of PKA. We found

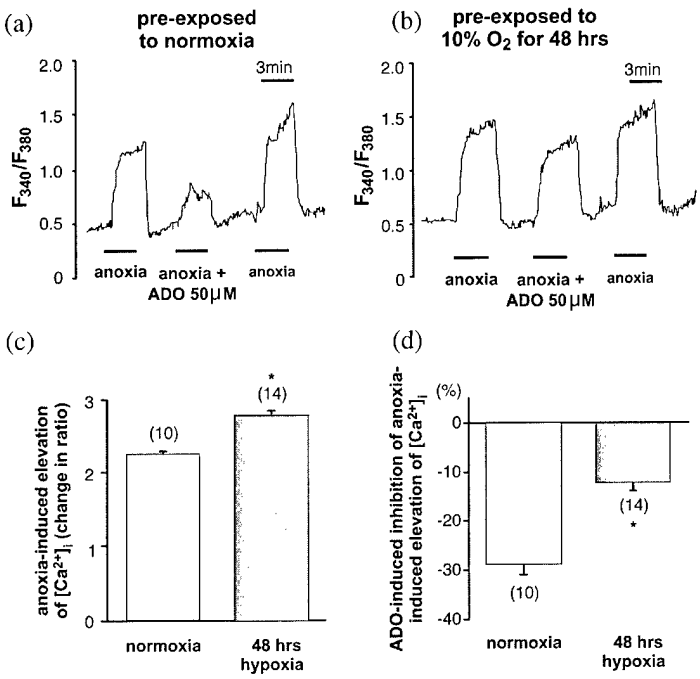

Fig. 6. Effect of adenosine on the anoxia-induced increase of intracellular free Ca^{2+}. (a) Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the fluorescent Ca^{2+} indicator, Fura-2. The ratio of F_{340}/F_{380} reflects $[Ca^{2+}]_i$. Shown is a representative recording of $[Ca^{2+}]_i$ in response to anoxia ± ADO (50 μM). (b) A representative recording of $[Ca^{2+}]_i$ is shown from a cell pre-exposed to 10% O_2 for 48 h. These results are summarized in (c,d). The numbers in parentheses indicate the number of cells examined. Data are expressed as mean ± S.E.M., $^*P < 0.05$.

that chronic hypoxia (24 to 48 h exposure to 5 or 10% O_2) significantly reduced PKA immunoreactivity and enzyme activity in PC12 cells (Beitner-Johnson et al., 1998; Kobayashi et al., 1998b). However, chronic hypoxia had no effect on either forskolin or adenosine-stimulated adenylyl cyclase activity in PC12 cells (Kobayashi et al., 1998b). Down-regulation of $G_{S\alpha}$ is also not the mechanism by which chronic hypoxia affects adenosine signaling, as prolonged hypoxia had no effect on $G_{S\alpha}$ immunoreactivity levels (Kobayashi et al., 1998b). Finally, chronic hypoxia reduced the efficacy of 8-bromo-cAMP for inhibition of I_{Ca} (Kobayashi et al., 1998b). Taken together, these results are consistent with the hypothesis that down-regulation of PKA is the mechanism by which chronic hypoxia attenuates the effect of adenosine on I_{Ca} and $[Ca^{2+}]_i$ (see Fig. 7). The down-regulation of the PKA signaling pathway has been hypothesized to be one of the defense mechanisms by which cells survive episodes of hypoxia (Hochachka et al., 1996). In our model system, the reduced actions of adenosine on I_{Ca} and $[Ca^{2+}]_i$ and the concomitant down-regulation of PKA in response to chronic hypoxia might function to activate specific cell survival programs.

Acute hypoxia induces the release of adenosine from PC12 cells into the extracellular milieu (Kobayashi et al., 2000). This effect is enhanced when cells are pre-exposed to chronic hypoxia (Kobayashi et al., 2000). Thus, it appears that adaptation to chronic hypoxia involves not only the modulation of I_{Ca} and $[Ca^{2+}]_i$, as discussed previously, but also the production and release of adenosine. We therefore investigated the mechanisms that underlie the regulation of adenosine synthesis and release by hypoxia. Fig. 8a schematically illustrates the various metabolic pathways leading to adenosine synthesis and degradation. We hypothesized that one or more of these enzymes was regulated by hypoxia such that adenosine metabolism was shifted toward net production.

One enzyme involved in the metabolic regulation of adenosine is 5′-nucleotidase (5′NT), which catalyzes the hydrolysis of AMP to adenosine and phosphate (Zimmermann, 1992). This reaction represents the final step in the hydrolysis of ATP to adenosine, a major pathway for the production

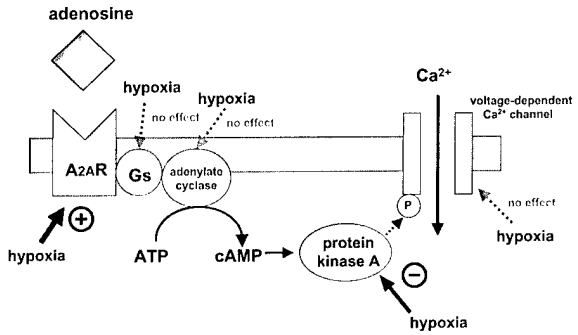

Fig. 7. Schematic summary of effects of hypoxia on adenosine signaling system. Binding of adenosine to the adenosine 2A receptor ($A_{2A}R$) leads to activation of the stimulatory G-protein, which in turn activates adenylate cyclase. This leads to increased synthesis of cAMP and activation of protein kinase *A*. As illustrated, protein kinase *A* can, under certain conditions, phosphorylate and thereby regulate voltage-dependent calcium channels. The solid arrow indicates an element that is upregulated by hypoxia, the outlined arrow indicates an element that is downregulated by hypoxia, and the dashed arrows designate cellular elements that are not affected by hypoxia.

of adenosine from adenine nucleotides (Zimmermann, 1992). We performed Northern blots to determine whether chronic hypoxia affected the steady-state levels of 5′-NT mRNA. Fig. 8b (top panel) shows that long-term exposure to hypoxia (24 and 48 h) strongly increased 5′-NT mRNA levels. Additional experiments demonstrated that 5′-NT enzyme activity was similarly increased (Fig. 8b, bottom panel). The increase in 5′-NT activity includes both the membrane–bound and cytoplasmic forms of 5′-NT (Kobayashi et al., 2000). Thus, enhanced 5′-NT gene expression and activity during hypoxia represents one mechanism whereby PC12 cells can increase the available pool of cellular adenosine.

Adenosine kinase (AK) phosphorylates adenosine to produce 5′-AMP, and serves as a primary regulator of cellular adenosine levels. We used Northern blot analysis to determine whether AK gene expression is modulated by hypoxia. Fig. 8c (top panel) shows that hypoxia progressively decreased AK mRNA levels in PC12 cells. This was correlated with a decrease in AK enzyme activity (Fig. 8c, bottom panel). Negative regulation of AK by chronic hypoxia is consistent with increased levels of adenosine, suggesting this is a major mechanism by which hypoxia enhances adenosine production and release.

Adenosine deaminase (ADA) is also involved in the metabolic regulation of adenosine. This enzyme is responsible for the deamination of adenosine to inosine. ADA mRNA levels were largely unchanged during exposure to hypoxia though there was a modest but statistically significant decrease in both ADA protein levels and enzyme activity after 48 h exposure to hypoxia (Fig. 8d, bottom panel; Kobayashi et al., 2000). A decrease in ADA activity would be consistent with an increase in the amount of adenosine available for release into the extracellular milieu.

As discussed above, hypoxia primarily acts through the A_{2A} receptor ($A_{2A}R$) in PC12 cells (Kobayashi et al., 1998a). Thus, we performed experiments to determine whether hypoxia also regulates the adenosine system the level of gene expression of cell surface receptors. Fig. 9a shows that exposure to either 10 or 5% O_2 progressively increases $A_{2A}R$ mRNA levels, with a greater effect at 5% O_2 than at 10% O_2. Fig. 9b shows that there was also a corresponding increase in $A_{2A}R$ immunoreactivity following in response to 5% O_2. Thus, upregulation of the $A_{2A}R$ gene is another mechanism by which PC12 cells adapt to hypoxia.

The physiological consequences of increased adenosine production and receptor expression on cell function have not been established. However, this would be predicted to cause increased sensitivity to adenosine. We have shown that stimulation of the $A_{2A}R$ promotes the viability of PC12 cells during hypoxia (Kobayashi and Millhorn, 1999). Thus, it is possible that increases in both adenosine production and $A_{2A}R$ gene expression enhance the sensitivity of PC12 cells to adenosine, and thereby improve cell viability. Clearly, adenosine has important roles in modulating the cellular response to hypoxia.

4. Regulation of the SAPKs and MAPKs by hypoxia is critical for regulation of hypoxia-responsive genes

O_2 sensing and the modulation of intracellular Ca^{2+} currents are critical aspects of the hypoxic response. However, many of the changes in cell function that occur during hypoxia require changes in gene expression. Thus, the hypoxic signal needs to be transmitted to the nucleus where changes in gene expression occur. The stress- and mitogen-activated protein kinase (SAPK and MAPK) pathways play a critical role in responding to cellular stress and promoting

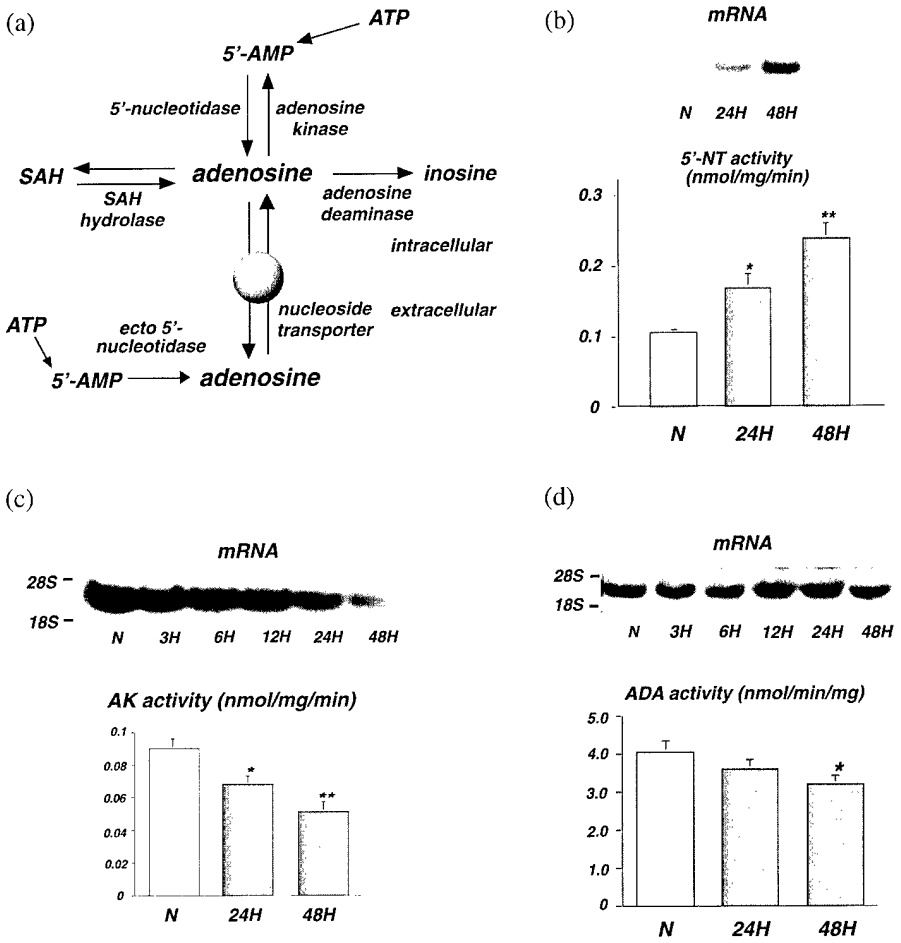

Fig. 8. Hypoxia modulates adenosine metabolism to increase production. PC12 cells were exposed to either normoxia (N, 21% O_2) or hypoxia (H, 5% O_2), for the amount of time indicated. (a) Schematic diagram showing the various metabolic pathways of ADO formation and degradation. (b) The effect of increasing amounts of hypoxia on 5′-NT mRNA is shown in the upper panel. The results are shown quintitatively in the lower panel. (c) The upper panel shows the effects of increasing amounts of hypoxia on AK mRNA. The lower panel shows results from AK enzyme activity assays. Cells were exposed to 5% O_2 for 24 and 48 h. AK activity was measured as the conversion of [^{14}C]ADO to [^{14}C]5′-AMP, in nmol/min mg of protein. Data are expressed as mean ± S.E.M. and represent $n = 6$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$. D) Shown in the upper panel is a representative Northern blot of ADA mRNA following increasing amounts of hypoxia. The lower panel shows results from ADA enzyme activity assays. The ADA activity was evaluated as conversion of [^{14}C]ADO to [^{14}C]INO. Data are mean ± S.E.M. $P < 0.05$. These results were reproduced from the *Journal of Neurochemistry* 74, 621–632, 2000, with permission.

changes in gene expression, cell growth and cell survival (Widmann et al., 1999; Su and Karin, 1996). Five homologous subfamilies of these kinases have been identified, and the three major families include: p38/SAPK2/RK; JNK/SAPK; and p42/p44 MAPKs/ERKs (Rouse et al., 1994; Raingeaud et al., 1995; Kyriakis and Avruch, 1996; Su and Karin, 1996; Garrington and Johnson, 1999; Widmann et al., 1999). In general, the SAPKs (p38 and JNK) are activated primarily by noxious environmental stimuli such as: ultraviolet light, osmotic stress, inflammatory cytokines, and inhibition of protein synthesis (Hibi et al., 1993; Derijard et al., 1994; Kyriakis et al., 1994; Han et al., 1994). However, increasing evidence suggests that, at least under certain conditions, these pathways can also be activated by mitogenic and neurotrophic factors (Logan et al., 1997; Xing et al., 1998). In contrast, p42/p44 MAP kinases are primarily stimulated by mitogenic and differentiative factors in a Ras-dependent manner (Raingeaud et al., 1995; Woodgett et al., 1996; Whitmarsh and Davis, 1994), although these enzymes can also be activated by certain environ-

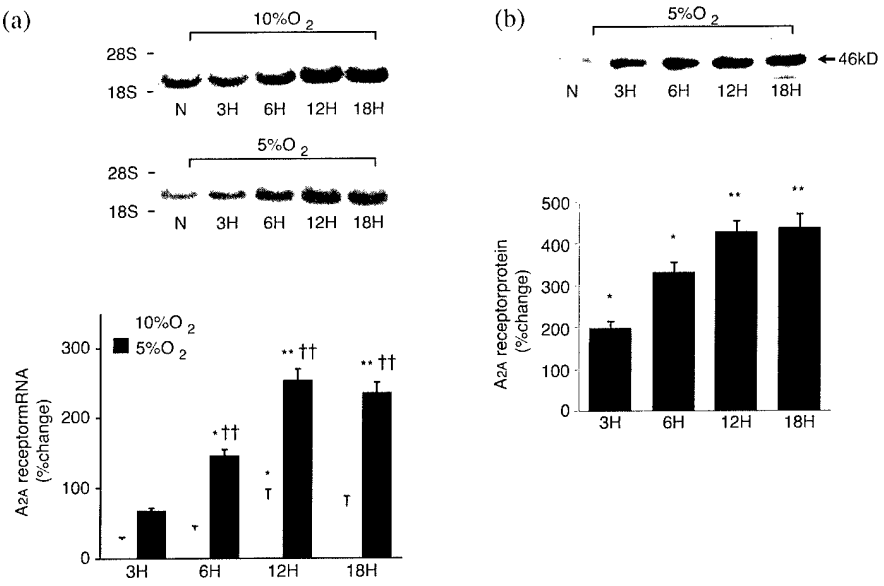

Fig. 9. Hypoxia increases expression of adenosine A2A receptor mRNA and protein in PC12 cells. PC12 cells were exposed to normoxia or hypoxia, (10 or 5% O_2) for increasing amounts of time, as indicated. (a) Cellular RNA was isolated and subjected to Northern blot analysis for adenosine A2A receptor expression. Representative blots following 10 or 5% O_2 are shown in the upper panels. These results are shown quantitatively in the lower panel, and represent averaged data from six separate experiments for each time point and O_2 level. Data are expressed as percent change from control and are expressed as mean ± S.E.M., $^*P < 0.05$, $^{**}P < 0.01$. *b*) PC12 cells exposed to increasing amounts of hypoxia were subjected to immunoblot analysis with an antibody specific for the adenosine A2A receptor. The upper panel shows a representative blot. The averaged results from four separate experiments are provided in the lower panel. Data are expressed as mean ± S.E.M., $^*P < 0.05$, $^{**}P < 0.01$, ††P < 0.01 from $10\%0_2$.

mental stressors (Su and Karin, 1996; Widmann et al., 1999; Garrington and Johnson, 1999). Thus, we hypothesized that hypoxia, a prevalent physiological stressor in many disease states, may regulate the activity of the SAPK and MAPK signaling pathways.

To characterize the effects of hypoxia on p38 enzyme activity, PC12 cells were transfected with FLAG epitope-tagged versions of p38α, p38β, p38β_2 p38γ, or p38δ. Cells were then exposed to either normoxia (21% O_2) or hypoxia (5% O_2, 6 h). The various kinases were then immunoprecipitated with an anti-Flag antibody, and immune complex kinase assays were performed. Fig. 10a shows that hypoxia stimulated both p38α and p38γ enzyme activity, as determined by the ability of each isoform to phosphorylate myelin basic protein. In contrast to these results, hypoxia did not significantly alter p38β, p38β_2 or p38δ enzyme activity (Fig. 10a,c). Hypoxia-induced changes in enzyme activity were not the result of differences in transfection efficiency as cell lysates blotted with anti-Flag show equal amounts of the transfected protein (Fig. 10b). It can be seen that the effect of hypoxia on the p38γ isoform is by far the most robust (average 5.9-fold activation, Fig. 10c).

We next evaluated the effect of hypoxia on JNK, another stress-activated protein kinase. PC12 cells were exposed to hypoxia for various times, from 20 min to 6 h, and JNK enzyme activity was measured in an immune complex kinase assay. Unlike its effects on p38, hypoxia did not significantly alter JNK enzyme activity, whereas exposure of cells to UV light markedly increased JNK activity (Conrad et al., 1999a).

To determine the effect of hypoxia on p42/p44 MAPK, PC12 cells were again exposed to either normoxia (21% O_2), or hypoxia (5% O_2) for various times, between 20 min and 6 h. Samples of whole cell lysates were immunoblotted with either an antibody specific for tyrosine phosphorylated (activated) p42/p44 MAPK or an antibody that equally recognizes phospho-and dephospho-p42/p44 MAPK (total MAPK). Hypoxia had no significant effect on the levels of either phospho-p42/p44 MAPK at the earliest time points studied (Fig. 11a). However, exposure to hypoxia for 6 h caused an increase in the tyrosine phosphorylation of p42/p44 MAPK (Fig. 11a). The total

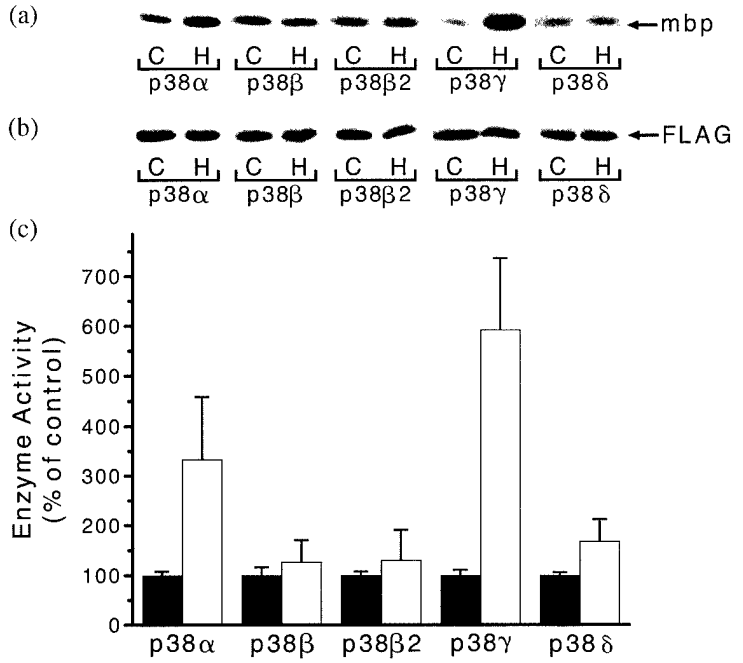

Fig. 10. Effect of hypoxia on enzyme activity of the various p38 isoforms. PC12 cells were transfected with either Flag-p38, Flag-p38β, Flag-p38β2, Flag-p38γ, Flag-p38δ, or the pCDNA3 vector. After 48 h, cells were exposed to either control conditions (C, 21% O_2) or hypoxia (H, 5% O_2, 6 h). (a) Enzyme activity of p38 isoforms, was determined in immunecomplex kinase assays by the amount of ^{32}p incorporation into myelin basic protein (mbp) as described in Experimental Procedures. (b) Whole cell lysates were immunoblotted for Flag as described in Experimental Procedures. (c) Protein kinase activity of the various p38 isoforms after exposure to normoxia (black bars) or hypoxia (shaded bars) are expressed as average percent of control ± S.E.M, and represent $n = 6–9$ dishes in each group, performed in at least two separate experiments.

amount of p42/p44 MAPK was not affected by hypoxia, as shown in Fig. 11b. An increase in the phosphorylation of MAPK is presumed to increase its enzyme activity. We next measured MAPK enzyme activity by immune complex kinase assay. Fig. 11c shows that p42 MAPK enzyme activity, like MAPK phosphorylation state, increased following 6 h of exposure to hypoxia.

The SAPKs and the MAPKs are known to mediate their effects via the activation of transcription factors. Thus, we were interested in identifying transcription factors regulated by these protein kinases during hypoxia. Endothelial PAS-domain protein 1 (EPAS1, also known as HIF2-α, HLF and HRF) is a recently identified hypoxia–inducible transcription factor (Tian et al., 1997; Ema et al., 1997; Flamme et al., 1997). EPAS1 is a basic helix–loop–helix transcription factor, which shares 48% sequence identity with hypoxia-inducible factor (HIF1-α), a transcription factor involved in the regulation of vascular endothelial growth factor (VEGF), glycolytic enzymes, and several other hypoxia-regulated genes (Tian et al., 1997). EPAS1 protein levels, like HIFl-α levels, are relatively low under basal conditions and accumulate upon exposure of cells to hypoxia (Wiesner et al. 1998). These factors then translocate to the nucleus and *trans*-activate target genes containing the sequence 5′GCCCTACGTGCTGTCTCA-3′, which is commonly referred to as the Hypoxia Response Element (HRE) (Semenza and Wang, 1992; Tian et al., 1997). Interestingly, EPAS1 is particularly abundant in the type I O_2-sensing cells of the carotid body (Tian et al., 1998). We have previously demonstrated the phenotypic similarities between the type I cells and PC12 cells. Thus, we hypothesized that EPAS1 would be regulated by hypoxia in PC12 cells.

As a first step towards characterizing the regulation of EPAS1 in PC12 cells, we evaluated EPAS1 protein levels following exposure to hypoxia. Fig. 12a shows that exposure to hypoxia (1% O_2) for 6 h resulted in a 12-fold increase in EPAS1 protein levels. It has previously been established that EPAS1 can *trans*-activate an HRE-

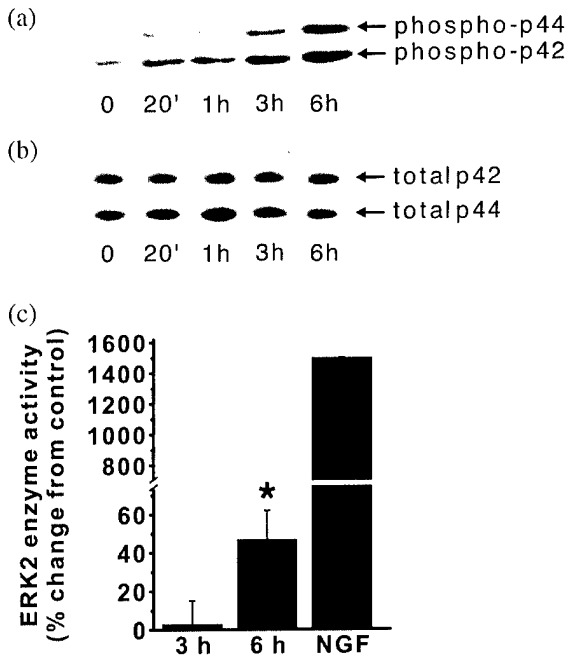

Fig. 11. Hypoxia modestly activates p42/p44 MAPK. PC12 cells were exposed to hypoxia (5% O_2) for various times between 0 and 6 h, as indicated. In (a) and (b), lysates were subjected to SDS–PAGE and immunoblotted with antibodies specific for either tyr[204]-phosphorylated p42/p44 MAPK or total (phospho- and dephospho-) MAPK, as described in Experimental Procedures. (a) Representative immunoblot showing phospho-p42/p44 MAP kinase immunoreactivity at the various time points studied. (b) Representative immunoblot showing total MAPK at the various time points studied. Similar results as those shown in (a) and (b) were observed in three separate experiments. (c) MAPK enzyme activity was determined in an immune-complex kinase assay by the amount of ^{32}P incorporation into myelin basic protein as quantified by phosphorimager. Data shown are representative of that obtained in two separate experiments and represent $n = 6$ dishes in each group.

luc reporter gene (Tian et al., 1997). We found that titrating the level of hypoxia from 21% O_2 to 1% O_2 resulted in a dose-dependent increase in HRE–luciferase activity (Fig. 12b).

The MAPK pathway is known to regulate a number of transcription factors, including c-fos, jun-B, CREB, and Elk-1 (Hipskind et al., 1994; Bernstein et al., 1994; Xing et al., 1996). We therefore hypothesized that the MAPK pathway might be important for EPAS1 activation during hypoxia. To test this hypothesis, PC12 cells were cotransfected with the HRE-luc reporter gene and a plasmid encoding the human EPAS1 cDNA or the empty expression vector, pcDNA3. Cells were then pre-treated with either PD98059 (50 μM) or vehicle, and exposed to normoxia or hypoxia (1% O_2) for 6 h. As reported by others (Tian et al., 1997) we found that expression of EPAS1 increased HRE-luc activity under both normoxic and hypoxic conditions (Fig. 13a). We also found that inhibition of MEK1, by PD98059,

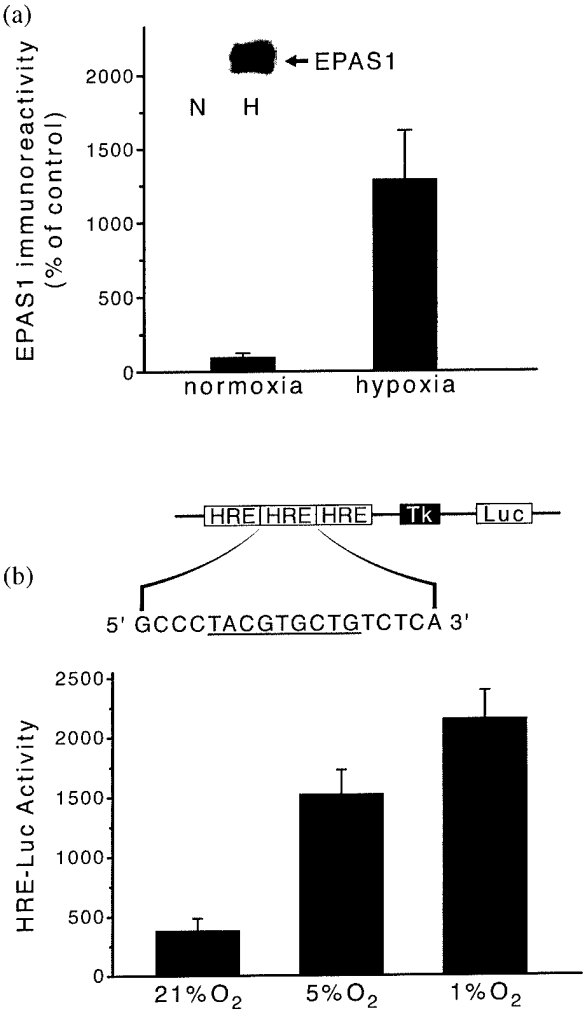

Fig. 12. EPAS1 protein accumulates and is activated by hypoxia. PC12 cells were exposed to normoxia (21% O_2) or hypoxia (1% O_2, 6 h) followed by SDS-PAGE and immunoblotting with an α-EPAS1 antibody. (a) Immunoblot showing the effect of hypoxia on EPAS1 immunoreactivity. Results are representative of $n = 6$ performed in two separate experiments. (b) PC12 cells were seeded in 24-well dishes and transfected with the HRE-luc reporter gene (250 ng/dish). 48 h post-transfection, cells were exposed to normoxia, or increasing levels of hypoxia, as indicated, and then assayed for luciferase activity as described in Experimental Procedures. Data are representative of results performed in three experiments.

completely blocked the effect of hypoxia on both basal and EPAS1-stimulated HRE-luc activity (Fig. 13a). However, the hypoxia-induced phosphorylation of EPAS1 is not blocked by PD98059 (Conrad et al., 1999b). Thus, although MAPK does not appear to mediate phosphorylation of EPAS1, our results strongly suggest that the MEKI–MAPK signaling pathway is critical for activation of EPAS1 and HRE-dependent gene expression.

To test this, we measured the effect of expressing a constitutively active MEK1 (pFC-MEK1) on basal and hypoxia-induced HRE-luc activity. MEK1 is a dual specificity protein kinase that directly phosphorylates and activates MAPK (Garrington and Johnson, 1999). Fig. 13b shows that expression of pFC-MEK1 enhanced based HRE-luc activity during both normoxia and hypoxia. However, when coexpressed with EPAS1, pFC-MEK1 caused a much larger increase in the *trans*-activation of the HRE-luc (data not shown). The relative increase in HRE-luc activity in the presence of pFC-MEK1 and EPAS1 was 13-fold higher than cells transfected with EPAS1 and exposed to normoxia (data not shown). In contrast, transfection with EPAS1 alone, followed by hypoxia, resulted in only a twofold increase in HRE-luc activity (data not shown). Taken together, these data strongly suggest that the MAPK pathway is involved in the hypoxia-induced *trans*-activation of EPAS1.

We were interested in identifying upstream protein kinases that led to MAPK and EPAS1 activation. MAPK is activated by growth factors in a Ras-dependent manner. In order to test whether Ras was involved in the EPAS1 *trans*-activation of the HRE-luc, PC12 cells were co-transfected with the EPAS1 expression plasmid, the HRE-luc plasmid, and increasing amounts of a dominant–negative Ras expression plasmid, RasN-17. Our results showed that increasing amounts of RasN-17 had no effect on the EPAS1 *trans*-activation of HRE-luc (data not shown; Conrad et al., 1999b). However, co-expression of the same amounts of RasN-17 blocked activation of a c-fos-luc reporter gene by nerve growth factor (NGF) in PC12 cells (Conrad et al., 1999b). Thus, EPAS1 activation by hypoxia occurs via a Ras-independent mechanism.

Several reports have shown that MAPK can be phosphorylated via a calmodulin-dependent

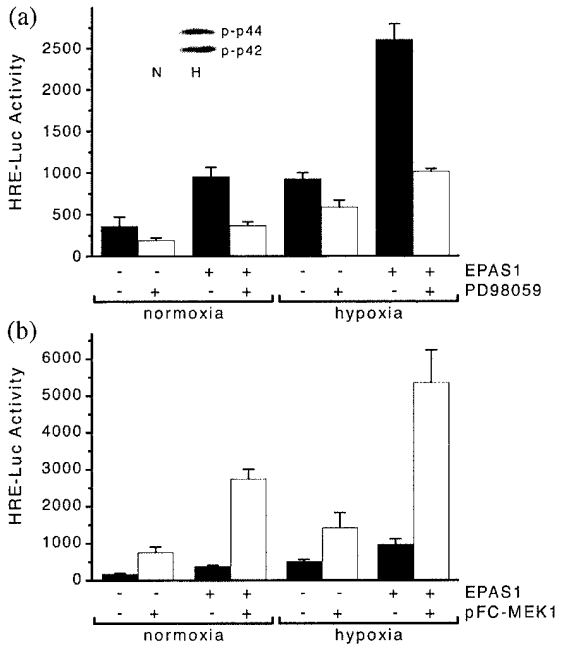

Fig. 13. p42/p44 MAPK is critical for EPAS 1 *trans*-activation. PC12 cells were exposed to either normoxia (21% O_2) or hypoxia (1% O_2). (a) Inset panel is a representative immunoblot (from $n = 6$) showing phospho-p42/p44 MAPK immunoreactivity following normoxia (N, 21% O_2) or hypoxia (H, 1% O_2, 6 h). PC12 cells were plated in 24-well dishes and transfected with the HRE-Luc reporter gene (250 ng/well) and either the EPAS1 cDNA (25 ng/well) or the empty expression vector, pcDNA3, as indicated. 48 h post-transfection, cells were exposed to normoxia or hypoxia (1% O_2, 6 h) in the presence or absence of PD98059 (50 μM), as indicated. Lysates were assayed for luciferase activity as described in Experimental Procedures. Data are representative of results obtained in four different experiments. (b) PC12 cells were transfected with the HRE-Luc reporter gene (250 ng/well), a constitutively active MEK1 construct (pFC-MEK1, 25 ng/well), the EPAS1 cDNA (25 ng/well), or the empty expression vector, pcDNA3, as indicated. Representative experiment showing the effect of constitutively-active MEK1 on EPAS1 *trans*-activation of the HRE reporter gene. Data are from one of three experiments.

mechanism. We therefore tested whether the calmodulin antagonist, W13, could block MAPK phosphorylation. Fig. 14a shows that treatment with W13 (20 μg/μl) caused a pronounced reduction in hypoxia-induced MAPK phosphorylation. Consistent with these findings, Egea et al. have shown that depolarization of PC12 cells results in MAPK activation via a calmodulin-dependent mechanism (Egea et al., 1998, 1999). Because previous studies demonstrated that EPAS1 *trans*-activation was dependent on MAPK,

we hypothesized that calmodulin inhibitors would also block EPAS1 activation. Fig. 14c shows that treatment with either W13, or calmidazolium chloride (CMZ, 1 μM), another calmodulin antagonist, inhibited both endogenous HRE activity, as well as the EPAS1 *trans*-activation of the HRE reporter gene (Fig. 14c). Thus, MAPK activation

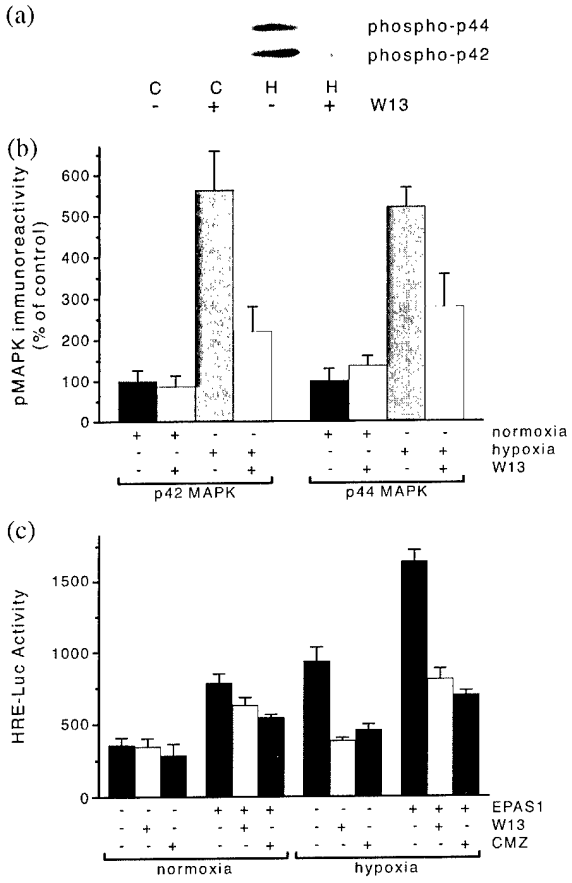

Fig. 14. MAPK phosphorylation and EPAS1 activity is calmodulin-dependent. PC12 cells were exposed to normoxia (21% O_2) or hypoxia (1% O_2, 6 h) in the presence or absence of the calmodulin antagonists W13 (20 μg/μl) or calmidazolium (1 μM). (a) Representative immunoblot showing the effect of W13 on phospho-MAPK immunoreactivity. (b) Immunoreactivity levels of phospho-MAPK following hypoxic exposure in the absence or presence of W13. Data are expressed as average percent change from control ± S.E.M., and represent $n = 6$ dishes analyzed in two separate experiments. (c) Representative experiment showing the effect of W13 and CW on EPAS1 *trans*-activation of the HRE-luc gene. PC12 cells were seeded in 24-well dishes and transfected with the HRE-luc reporter gene (250 ng/well), the EPAS1 cDNA (25 ng/well), or the empty expression vector, pcDNA3, as indicated. Cells were pretreated with W13 (20 μg/μl), CMZ (1 μM), or vehicle and then exposed to normoxia or hypoxia. Two other experiments gave similar results.

of EPAS1 occurs via a calmodulin-dependent pathway.

The mechanism by which MAPK regulates the EPAS1 transcription factor is not yet known. The precise physiologic role of EPAS1 is also still uncertain. However, knock-out studies have demonstrated that EPAS1 is involved in catecholamine homeostasis. Tian et al. (1998) demonstrated that EPAS1-deficient mice die during development of cardiac failure, secondary to decreased catecholamine production. They further showed that addition of a dopamine precursor, DOPA-S, to the drinking water of pregnant females, was able to rescue this phenotype. The expression of EPAS1 in the type I cells of the carotid body also suggests a role in catecholamine production. These cells respond to reduced arterial pO_2 by secreting catecholamines which intitiate the hyperventilatory response. Our lab has shown that the rate-limiting enzyme in catecholamine biosynthesis, tyrosine hydroxylase (TH), is induced by hypoxia in both carotid body cells and the type I cells. Thus, it is tempting to speculate that EPAS1 is involved in the transcriptional activation of TH. Preliminary data from our laboratory using an in vitro transcription system showed that EPAS1 can indeed activate transcription via the 5′ flanking region of the TH gene (Yuan et al., 2000).

It is important to note that both type I cells of the carotid body and PC12 cells are excitable catecholaminergic cells. Since EPAS1 has a specialized expression pattern (i.e. highly enriched in the type I cells of the carotid body), it is possible that this transcription factor has specific functions in excitable cells. The more widely distributed hypoxia-inducible factor-1 (HIF-1) may be the primary mechanism which gene regulates expression in non-excitable cells.

5. Summary and conclusions

Previous experiments have shown that exposing PC12 cells to hypoxia results in membrane depolarization and Ca^{2+} influx (Zhu et al., 1996; Raymond and Millhorn, 1997; Kumar et al., 1998). The results presented in this review demonstrate that the O_2-sensitive K^+ channel, Kv1.2, mediates this depolarization. The subsequent increase in intracellular Ca^{2+} is known to be a critical mediator of gene expression and transcription factor

activation. For example, the hypoxia-induced increase in TH gene expression is dependent on Ca^{2+} levels (Raymond and Millhorn, 1997). TH is the rate-limiting enzyme in catecholamine biosynthesis. Our lab has further shown that the immediate early genes c-fos and jun-B bind to the AP-1 site of the TH promoter (Norris and Millhorn, 1995). Removal of extracellular Ca^{2+} prevents the expression of c-fos and jun-B during hypoxia (unpublished data).

In addition to TH, we show that hypoxia also regulates the $A_{2A}R$ gene. We performed experiments aimed at delimiting the signaling pathways that mediate this increase. Our results indicate that removal of extracellular Ca^{2+}, chelation of intracellular Ca^{2+}, and pretreatment with PKC inhibitors, block the hypoxia-induced increase in $A_{2A}R$ (Kobayashi and Millhorn, 1999). These results provide further evidence that Ca^{2+} is a critical mediator of hypoxia-regulated gene expression. In addition to its dependence on Ca^{2+} for gene expression, activation of the $A_{2A}R$ then modulates $[Ca^{2+}]_i$. Thus. $A_{2A}R$ is able to regulate its own expression via its regulation of $[Ca^{2+}]_i$.

In this review, we have described some of the biophysical and biochemical changes that occur during hypoxia. Together, these events contribute to the hypoxic-response. These changes include membrane depolarization and the role of Kv1.2, as well as the modulation of membrane excitability by the adenosine A_{2A} receptor. We also demonstrate that the MAPK pathway is activated, causing *trans*-activation of the transcription factor, EPAS1. Our results show that the phosphorylation of MAPK and the subsequent *trans*-activation of EPAS1 are dependent on Ca^{2+}/CaM. Although these hypoxia-induced changes are separated spatially (membrane vs. cytoplasm) and temporally (acute vs. chronic hypoxia), they are both Ca^{2+}-dependent. Each of these events is responsible for either controlling Ca^{2+} levels within the cytoplasm or is critically dependent on the level of $[Ca^{2+}]_i$.

Although PC12 cells continue to be a model whereby Ca^{2+}-activated signal transduction pathways can be studied, we have also identified Ca^{2+}-independent pathways. Most notable among these is the cAMP response element binding protein (CREB), a transcription factor that is phosphorylated in response to hypoxia (Beitner-Johnson and Millhorn, 1998). Our experiments demonstrate that the hypoxia-induced phosphorylation of CREB persists in the absence of Ca^{2+} (Beitner-Johnson and Millhorn, 1998). The signaling mechanism that is responsible for CREB phosphorylation is unknown and may be a novel CREB kinase (Beitner-Johnson and Millhorn, 1998).

Finally, it is important to note that PC12 cells are an excitable cell line that depolarizes upon exposure to hypoxia. Thus, the critical role of Ca^{2+} is likely specific to these and possibly other excitable cell lines. Other O_2 sensitive cells lines (HEP3B, HEPG2) are not excitable and are therefore likely to use other mechanisms in order to activate downstream protein kinases and target genes. The challenge for future studies will be the elucidation of the mechanisms by which Ca^{2+} leads to activation of downstream targets. In addition, it will be interesting to identify other genes that are induced by hypoxia in a Ca^{2+}-dependent manner.

Acknowledgements

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Identification of hypoxia-responsive genes in a dopaminergic cell line by subtractive cDNA libraries and microarray analysis

D. Beitner-Johnson, K. Seta, Y. Yuan, H.-W. Kim, R.T. Rust, P.W. Conrad, S. Kobayashi, D.E. Millhorn*

Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH 45267-0576, USA

Abstract

Transplantation of dopamine-secreting cells harvested from fetal mesencephalon directly into the striatum has had limited success as a therapy for Parkinson's disease. A major problem is that the majority of the cells die during the first 3 weeks following transplantation. Hypoxia in the tissue surrounding the graft is a potential cause of the cell death. We have used subtractive cDNA libraries and microarray analysis to identify the gene expression profile that regulates tolerance to hypoxia. An improved understanding of the molecular basis of hypoxia-tolerance may allow investigators to engineer cells that can survive in the hypoxic environment of the brain parenchyma following transplantation. © 2001 Published by Elsevier Science Ltd.

Keywords: Oxygen/pheochromocytoma; Genomics; Tyrosine hydroxylase; Carotid body; Type I cells

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by the loss of dopaminergic neurons that project from the substantia nigra to the striatum. One of the most promising therapies involves the transplantation of dopamine-secreting cells harvested from fetal mesencephalon directly into the striatum [1–4]. Although this approach has been used successfully to alleviate PD-associated motor dysfunction both in patients and animal models, the majority of these cells die within a short-time following [5,6]. Recently a different approach was taken by Lopez-Barneo and associates who transplanted autografts, namely cell aggregates from the carotid body, into the putamen of monkeys which had been treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rats which had been treated with 6-hydroxydopamine [7,8]. Each of these compounds induces degeneration of dopaminergic neurons, and these treatments have been used to mimic Parkinson's disease in experimental animals. These investigators found that the transplanted carotid body cells led to re-innervation of putamen and caudate nucleus with dopaminergic fibers, resulting in long-term amelioration of parkinsonian-like motor symptoms in both monkey and rat. The carotid body is an oxygen-sensing organ that is located at the bifurcation of the common carotid artery, and contains a high concentration of cells (glomus cells) that synthesize and release dopamine in response to hypoxia. Not only are the carotid body cells able to survive in the hypoxic environment of the brain parenchyma, but hypoxia is a stimulus that actually enhances dopamine synthesis and release in these oxygen-sensing cells. Thus, tolerance to hypoxia is a beneficial property, which may enhance the survivability of transplanted cells.

Here we shall describe our effort to identify the molecular mechanisms that confer the oxygen-sensing and the hypoxia tolerant phenotype. Most of our work has been performed in pheochromocytoma (PC12) cells, which have an oxygen-sensing phenotype that is virtually indistinguishable from the carotid body cells [9–15]. A primary characteristic of oxygen-sensing cells is that gene expression patterns are altered during hypoxia. Here we summarize some of our work on signal transduction and gene regulation during hypoxia. We also describe our more recent work using a combination of subtracted cDNA libraries and microarray analysis to identify the gene expression profile that mediates a hypoxia-tolerant phenotype. From the work of Lopez-Barneo and associates, it is now clear that this is a critical consideration for the long-term viability of transplanted cells [7,8].

* Corresponding author. Tel.: +1-513-5586009; fax: +1-513-5585738.
E-mail address: david.millhorn@uc.edu (D.E. Millhorn).

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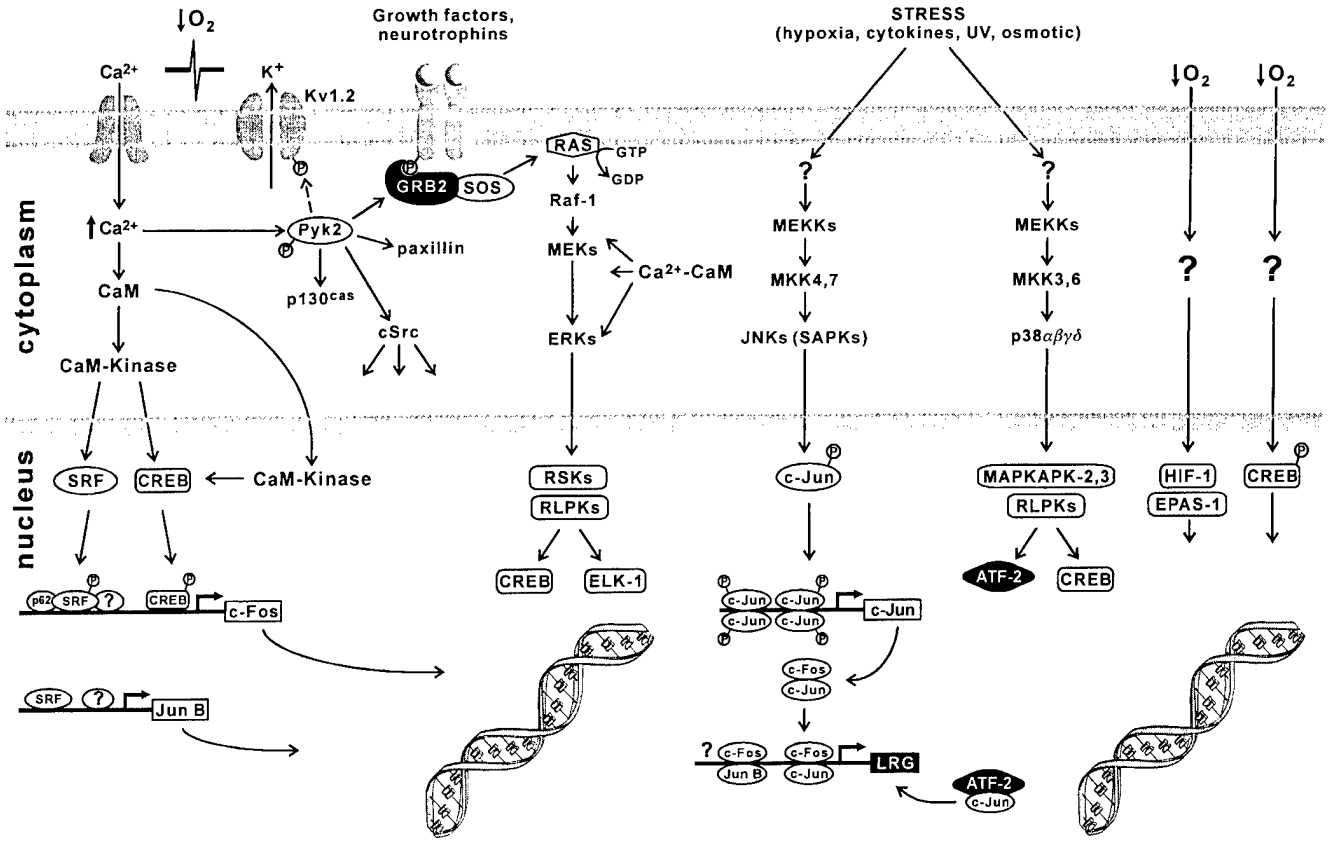

Fig. 1. Summary of certain intracellular signaling pathways and target genes that are regulated by hypoxia. In PC12 cells, exposure to hypoxia leads to a rapid inhibition of conductance of the Kv1.2 K^+ channel. This is accompanied by depolarization and calcium influx, which, in turn, regulates a variety of calcium-dependent signaling pathways and gene expression. Certain members of the mitogen-activated protein kinase (MAPK) and p38 stress-activated protein kinase (SAPK) signaling pathways are also specifically activated by hypoxia. Transcription factors that are regulated by hypoxia include CREB,. *c-fos*, *junB*, HIF-1a and EPAS1.

2. Regulation of gene expression by hypoxia: a brief overview

Hypoxia induces tyrosine hydroxylase (TH) gene expression, and thereby stimulates dopamine biosynthesis in PC12 cells and carotid body type I [9,10]. We showed that TH gene expression is regulated by hypoxia at both the level of transcription and mRNA stability [10]. Moreover, we showed that hypoxia-induced transactivation of the TH gene requires protein binding to a putative Hypoxia Response Element (HRE) and a nearby Activator Protein 1 (AP1) site [11]. Supershift analyses revealed that binding of the transcription factors *JunB* and *c-fos* to the AP1 element is markedly increased during hypoxia [11]. Furthermore, mutation of the AP1 site prevented hypoxia-induced transcription of a reporter gene [11]. We also found that both *junB* and *c-fos* gene expression is regulated by reduced O_2 in PC12 cells [16].

We have also made progress in understanding the signal transduction pathways that link reduced O_2 to gene regulation in PC12 cells (Fig. 1). The earliest measured signaling event in response to hypoxia in both carotid body type I cells and PC12 cells is inhibition of a specific potassium (K^+) channel that mediates membrane depolarization [8,17]. We now have strong evidence that this K^+ channel is Kv1.2 [12,19]. The primary physiological consequence of the membrane depolarization that occurs during hypoxia is an increase in intracellular free calcium (Ca^{2+}) levels [18], which can regulate gene expression via Ca^{2+}-dependent signal transduction pathways. For example, we found that hypoxia-induced gene expression for the immediate early genes, *c-fos* and *junB*, as well as expression of the TH and glucose transporter-1 (Glut-1) genes which require increased intracellular free Ca^{2+} [16,20]. There is evidence that the Ca^{2+}-dependent regulation of gene expression that occurs during hypoxia is mediated by either a Ca^{2+}-calmodulin protein kinase or a protein kinase C (PKC) pathway. Thus, membrane depolarization and increases in cytosolic Ca^{2+} are involved in regulation of gene expression during hypoxia in excitable cells such as PC12 cells and carotid body type I cells.

It is important to recognize that hypoxia is a metabolic stress that can impair normal cellular functions. This raises an important question, namely, how do cells adapt and

survive in low O_2 environments such as brain parenchyma? It is entirely possible that de novo gene expression plays a major role. For this reason, it is important to identify the signal transduction pathways and the specific target genes that might be involved in mediating this important adaptive mechanism. We focused our studies on the three parallel mitogen- and stress-activated protein kinase pathways, which include the mitogen-activated protein kinase (MAPK), c-jun N-terminal kinase (JNK), and the p38 kinase pathway. It is has been previously shown that the survival of transplanted mesencephalic dopaminergic neurons is enhanced by growth factors [6]. We found both the MAPK and p38 (p38α and p38γ) protein kinase pathways, but not the JNK pathway, to be activated by hypoxia in PC12 cells [21,22]. Moreover, we also identified a novel cyclic AMP Response Element Binding protein (CREB) kinase that is stimulated by hypoxia [13].

A primary function of signal transduction pathways is to activate protein factors in the nucleus that are involved in regulation of transcription. In the case of hypoxia, our goal is to determine if the hypoxia-regulated signal transduction pathways lead to transactivation of genes that mediate hypoxia-tolerance in PC12 cells. A number of hypoxia-related transcription factors have been identified and have been shown to play a role in mediating the cellular response to hypoxia. These transcription factors include HIF-1α, *c-fos*, *JunB* and CREB [11,13,23]. HIF-1α has been shown to be critical for hypoxia-induced regulation of a number of genes including glycolytic enzymes, vascular endothelial growth factor (VEGF), and erythropoietin [24–26]. Recently, endothelial PAS-domain protein 1 (EPAS1) was identified as a hypoxia-inducible transcription factor [27,28]. EPAS1 (also termed HIF-2α) is a basic helix-loop-helix protein that shares 48% sequence identity with HIF1α. EPAS1 protein levels, like HIF1α, are relatively low under normoxic conditions and accumulate upon exposure of cells to hypoxia [22]. These factors then translocate to the nucleus and trans-activate target genes containing the sequence 5′-GCCCTACGTGCTGTCTCA-3′, which is commonly referred to as the Hypoxia Response Element (HRE) [27,29]. The tissue distribution for HIF-1α is rather broad, whereas the distribution of EPAS1 is much more restricted. Interestingly, EPAS1 is most intensely expressed in the carotid body [28]. This transcription factor is localized in both the catecholaminergic type 1 O_2-sensing cells and in the endothelial cells of this highly vascularized organ [28]. EPAS1 is much more abundant than HIF1α in both the carotid body oxygen-sensing cells and PC12 cells [22,28]. It has been hypothesized that EPAS1 in the carotid body senses hypoxia and translates this signal into an altered pattern of gene expression, leading to increases in circulating catecholamine levels [28].

We have therefore investigated the potential role of EPAS1 as a transactivator of hypoxia responsive genes in PC12 cells. We found that EPAS1 protein levels and phosphorylation state are dramatically increased in PC12 cells that have been exposed to hypoxia [22]. Interestingly, the activation of EPAS1 during hypoxia appears to be mediated by the MAPK pathway via a calmodulin-sensitive pathway rather than through a classical Ras-dependent mechanism. It is also important to note that EPAS1 is not directly phosphorylated by MAPK, suggesting that the effects of MAPK are indirect, possibly through recruiting other proteins critical for activation of EPAS1. We have used an in vitro transcription system to investigate the role of EPAS1 in transactivation of TH expression [30]. We found that EPAS1 accumulation is required, but insufficient to induce transactivation of TH, which also requires EPAS1 phosphorylation. HIF1α does not appear to be activated by hypoxia in PC12 cells.

Although a number of hypoxia signal transduction pathways and transcription factors have been identified, the role of these pathways and factors in mediating a cell phenotype that is tolerant to hypoxia remains unclear. This, in large part, is due to a lack of understanding of the full repertoire of genes required to confer this special phenotype. Relatively few hypoxia-regulated genes have been identified and studied. Thus, it is clear that a more comprehensive understanding of the molecular basis of hypoxia tolerance will require identification of the gene profile that regulates this adaptive response. Here, we shall describe some of our recent work using subtractive cDNA libraries and microarray analysis to identify the genes that regulate the cellular response to hypoxia.

3. Identification of hypoxia-responsive genes in a dopaminergic cell line using custom subtractive cDNA libraries and microarray analysis

It is likely that many genes will be regulated in response to a complex physiological stimulus like hypoxia. However, of the 10,000 to 30,000 genes estimated to be expressed in any given cell type [31,32], the majority are likely *not* to be regulated by the hypoxia. Thus, to improve the percentage of cDNA sequences on our arrays that are relevant to our studies, we have constructed custom subtractive cDNA libraries and arrayed these cDNA sequences on glass slides. Subtractive hybridization is a powerful technique that enables isolation of cDNAs that are differentially expressed in two populations of cells or tissues. Theoretically, most all of the genes regulated by hypoxia in this cell type should be included in these libraries, and most all of the genes that are not regulated by hypoxia should be excluded.

Briefly, in this approach, which is also termed 'subtractive suppression hybridization' (SSH), RNA from control ('driver') and hypoxia-exposed ('tester') samples is converted to cDNA by reverse transcription. The driver cDNA (in excess) and tester cDNAs are then hybridized together. The resulting hybrids, which are the cDNA

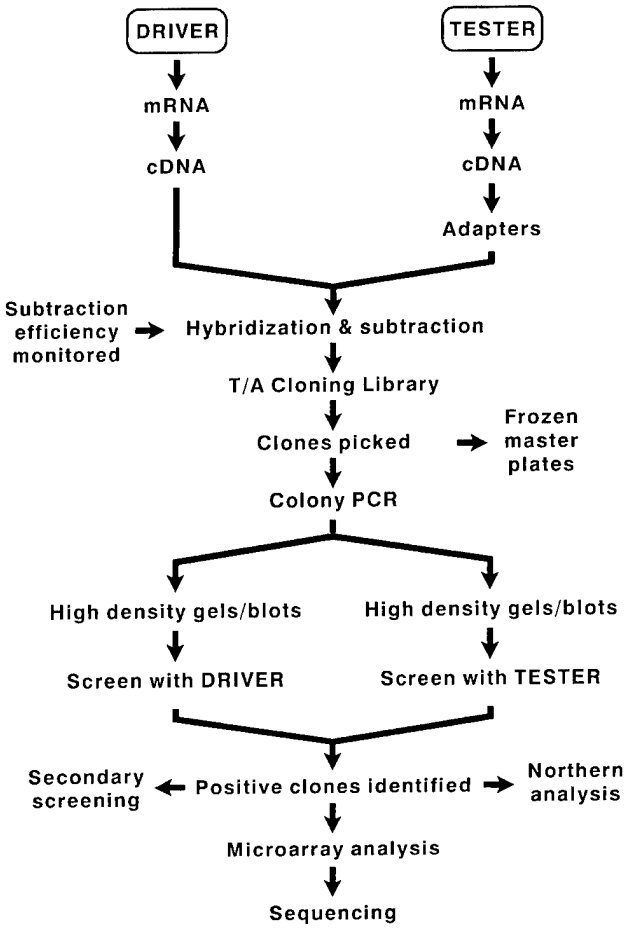

Fig. 2. Schematic overview of subtractive suppression hybridization (SSH) strategy. The major steps for making SSH cDNA libraries, using the Clontech PCR-Select methods are outlined. If brief, two populations of cDNA, termed 'driver' and 'tester' are co-hybridized. The cDNA sequences that are enriched in the 'tester' PC12 cells exposed population are retained in the SSH library, and the abundant cDNA sequences that are present equally in both population are excluded from the library.

sequences that are common between the two pools, are not amplified in subsequent steps and thereby 'subtracted out'. The remaining unhybridized cDNAs are unique to the tester (in this case, the hypoxia-exposed) pool. These cDNAs represent the 'transcriptome' for the experimental conditions, and therefore the specific genes whose expression is induced by hypoxia. This entire procedure can be performed in reverse to identify the genes that are *repressed* by exposure to hypoxia. A schematic flow diagram summarizing the overall approach used to make subtractive cDNA libraries is shown in Fig. 2.

We recently constructed a subtractive cDNA library to characterize the effects of hypoxia (6 h, 1% O_2) on PC12 cells. This library is specifically enriched in cDNA sequences from genes that are more highly expressed after exposure to hypoxia. We found that a number of the resulting clones corresponded to genes that others and we have previously found to be regulated by hypoxia in PC12 and other cell types. These include tyrosine hydroxylase, *junB*, and VEGF [9–11,33–35]. The subtractive library has relatively little redundancy; of 20 clones sequenced initially, only one sequence (tyrosine hydroxylase) was identified in more than one clone (two clones); the remainder of the genes sequenced were represented only once in this pool. This library yielded approximately 800 'hypoxia-regulated' cDNA clones. Thus, there are many more genes regulated by hypoxia than have been previously identified. However, this result is not surprising, considering the complex nature of the cellular response to hypoxia, which involves both chemoreceptor functions and cellular tolerance to low O_2.

In addition to known hypoxia-regulated genes, this library also contains a number of known genes that have not previously been reported to be regulated by hypoxia. These sequences encode a wide range of proteins, including signaling molecules, structural proteins, and transcription factors. Interestingly, we also found a number of putative novel hypoxia-regulated genes in this library, in that they share little or no significant homology with any of the known sequences in the public databases.

To verify the differential expression of various cDNAs in the subtracted library, we performed 'virtual' Northern blots. Virtual Northern blots are made with cDNA generated by reverse transcription from RNA, and yield information similar to that provided by standard Northern blots [36,37]. Fig. 3A is a virtual Northern blot hybridized with a ^{32}P-labeled VEGF cDNA probe. VEGF expression has been shown to be dramatically induced by hypoxia in PC12 and other cell types [33]. It can be seen that VEGF levels are higher in the hypoxia-exposed (unsubtracted) sample compared to the control (unsubtracted) sample. Furthermore, VEGF expression is greatly enriched in the forward-subtracted library. Importantly, this technique also *excludes* abundant sequences that are not differentially expressed between the two mRNA populations from the libraries. Thus, highly expressed genes that are not regulated (i.e. housekeeping genes), such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH) should be excluded from the subtracted pool. In Fig. 3B, G3PDH levels were analyzed by PCR in cDNA samples from the final (subtracted) cDNA library and in the original (unsubtracted) cDNA pool. Samples were subjected to various numbers of PCR cycles, as indicated in Fig. 3B. It can be seen that G3PDH is readily detectable in the original (unsubtracted) cDNA sample, but is completely absent in the subtracted sample (even after 33 PCR cycles). G3PDH is a highly abundant gene that we have previously shown is *not* regulated in PC12 cells by exposure to hypoxia [38]. Thus, this gene was accurately excluded from the subtracted cDNA library.

As illustrated above, the subtractive cDNA library approach has enabled us to isolate bona fide hypoxia-regulated genes. This technology also provides a powerful tool to identify novel gene targets regulated by hypoxia. The cDNAs isolated with this method are subsequently used to construct custom cDNA microarrays.

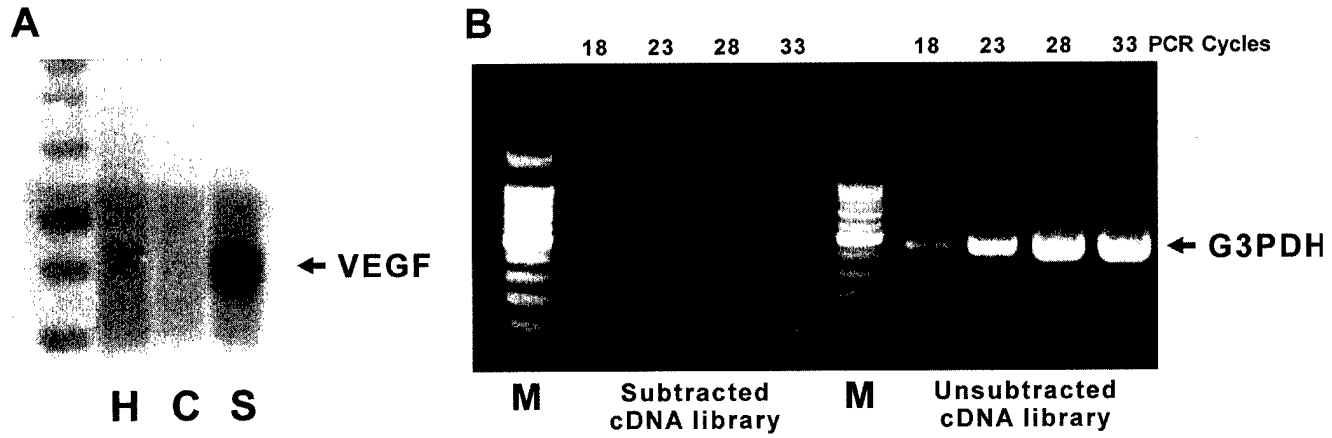

Fig. 3. Vascular endothelial growth factor (VEGF) is enriched in the SSH library and G3PDH is excluded. (A) Pooled cDNA samples from to hypoxia (H, 1% O_2 for 6 h), or control conditions (C), and a cDNA sample from the subtracted library (S) were subjected to 'virtual Northern blot' analysis using a 32P-labeled probe specific for VEGF. (B) cDNA samples from the subtracted and unsubtracted cDNA libraries were subjected to 18, 23, 28 or 33 cycles of PCR using primers specific for G3PDH, as indicated.

4. Use of functional genomics and proteomics to study global gene and protein expression patterns

Research into the role of specific genes in mediating the response to an environmental stimulus has historically been restricted to the study of single genes and/or proteins. It is highly simplistic to predict that complex physiological and pathological processes are mediated by regulation of a single gene. Rather, hundreds or, perhaps, thousands of genes and proteins would be expected to be regulated in these physiological responses [39–43]. Thus, a comprehensive understanding of complex biological processes at the cellular level requires a more global view of gene expression. Two recent technological advances have made this possible: (1) comprehensive DNA databases which have resulted from the various genome-sequencing projects; and (2) development of high-density nucleic acid microarrays. These new advances permit the analysis of global gene expression patterns, and facilitate a better understanding of complex physiological traits. This line of investigation has been termed 'functional genomics'. This term is broadly used to refer to mRNA (transcription) expression profile analysis. The complement of mRNAs transcribed from the genome is also referred to as the 'transcriptome'.

With this new technology, it is now possible to simultaneously monitor expression levels of thousands of genes, using cDNA microarrays. In this approach, a robotic 'arrayer' is used to spot individual DNA sequences, in the form of either cDNAs or oligonucleotides in closely spaced grids on either glass slides or nylon filters. Fig. 4 illustrates a schematic summary of the general methodology of this approach. In keeping with accepted microarray parlance, the DNA that is spotted or printed onto coated glass slide is called the 'probe' and the labeled DNA (from control and experimental RNA samples) is called the 'target'. Briefly, the cDNAs of interest are amplified by PCR from unique custom cDNA libraries, as described above. Following purification and quality control, small aliquots (5 nl) are printed on poly-L-lysine coated glass slides using a computer-controlled precision microarrayer robot (Omnigrid, GeneMachines, San Carlos, CA, USA).

The "targets" that are used to interrogate the cDNA

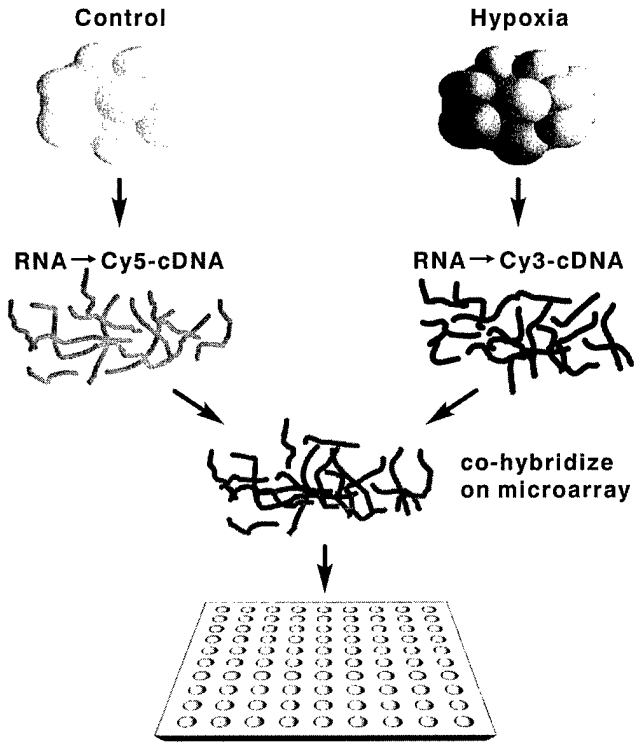

Fig. 4. Gene expression analysis using a DNA microarray. Deoxy-Gy5 (red fluorphore, shown as light grey)- and deoxy-Gy3 (green fluorphore, shown as dark grey)-labeled dNTPs and reverse transcriptase are used to convert RNA to fluorescently labeled cDNA. The two samples are mixed and hybridized with a cDNA microarray. The relative abundance in the hypoxia-exposed sample (Cy3, green) as compared to the control sample (Cy5, red) is reflected by the ratio of green to red fluorescence measured at the individual array elements representing each gene.

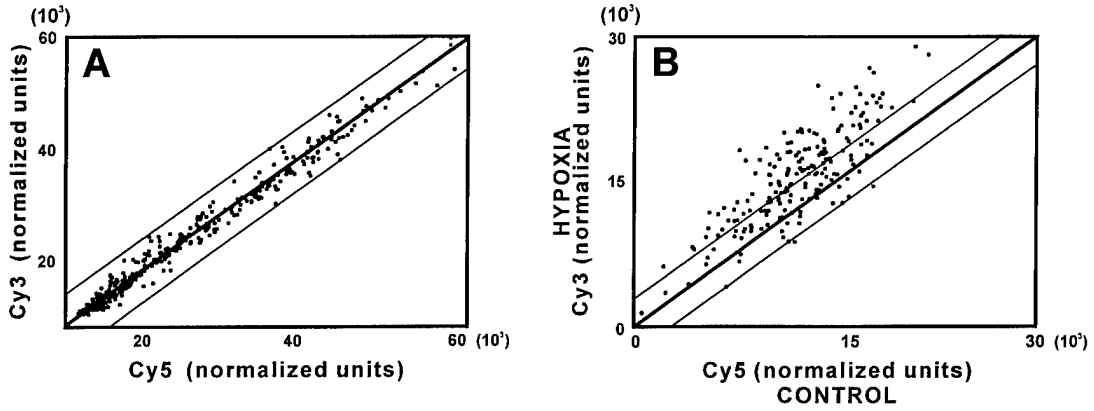

Fig. 5. Effect of hypoxia on expression of genes in the SSH library. Analysis of data from an experiment such as that described in Fig. 4. Background-subtracted fluorescence intensities were plotted for each spot. Equal amounts of control RNA was used to generate both Cy3 (green) and Cy5 (red) labeled cDNA. Co-hybridization showed spots with fluorescence intensities centering around the 1:1 ratio line. RNA from hypoxia treated cells was used to generate Cy3-labeled cDNA; control RNA was used to generate Cy5-labeled cDNA. The scatter plot shows spots with intensities shifted towards the green fluorescence, indicating that the majority of the genes on the microarray were unregulated.

templates printed on glass slides are generated from two RNA populations (control versus experimental, in this case, RNA from cells exposed to either normoxia or hypoxia). These two RNA populations are separately converted to fluorescently labeled cDNA with either green (Cy3) or red (Cy5) fluorophore-labeled deoxynucleotides using reverse transcriptase. Using this approach, changes in mRNAs that are differentially expressed by a minimum of approximately 2-fold can be detected. In a previous study, cDNA microarrays were used to identify and study the entire repertoire of glucose-regulated yeast genes [39]. In these experiments a glass slide microarray containing 6100 discrete cDNA probes corresponding to all genes of the yeast genome was used to identify the transcriptome responsible for regulating sugar metabolism in a single experiment. A study of this type using traditional hybridization techniques (e.g. Northern blot analysis of single genes) would require years to complete. Over the past year or two, a rapidly growing number of studies have confirmed the validity of using cDNA microarrays for mRNA profile analysis (for selected examples see [41,44–51]). An excellent example of the power of this approach comes from a recent study from the Brown laboratory at Stanford University, which examined the transcriptional response of more that 8000 human genes in fibroblasts to stimulation with serum over a detailed time course [41]. These studies described a previously unknown, highly complex level of coordinated gene regulation. We are using a similar approach to identify hypoxia-responsive genes in the PC12 cell line [52].

5. Analysis of gene expression patterns using cDNA microarrays and custom hypoxia-regulated subtractive libraries

We have microarrayed our subtractive PC12 cDNA library (6 h, 1% O_2) onto glass slides. The results from an example experiment are shown in Fig. 5. In this experiment, total RNA was isolated from PC12 cells and converted into fluorescently labeled cDNA, using reverse transcriptase. Panel A shows the results when a single RNA sample was divided into two reactions, one labeled with the Cy5 fluorophore (red), and the other labeled with the Cy3 fluorophore (green). Both labeled 'target' cDNA samples were then simultaneously hybridized to the 'probe' that was immobilized on the glass slides. Because both cDNA samples were derived from the same sample of total RNA, equal fluorescent intensities (i.e. control vs control; one sample labeled with Cy3 and the other with Cy5) should be equal for each spot. This is exactly what we found as indicated by the in the Cy5 vs Cy3 plot in Fig. 5A. The line of identity in the plot represents equal Cy5 and Cy3 hybridization (1:1 ratio). Importantly, the cDNA probes from hypoxia-exposed cells had a much greater level of hybridization (higher intensity Cy3 labeling) than did the cDNA probe from the control cells, as shown in Fig. 5B. This result was expected, since the cDNA sequences spotted on the slides are enriched in genes that display increased expression levels in response to hypoxia.

6. Confirmation of regulation of clones

Northern blots and RT-PCR are used to confirm the regulation of each selected target gene by hypoxia. Many of the hypoxia regulated genes may not be detectable by Northern blot, as a large fraction of mammalian mRNA consists of species that are expressed at relatively low levels [53,54]. Quantitative real time PCR (QRT-PCR) provides highly sensitive and accurate quantitation of levels of specific mRNAs present in small samples (for recent reviews see [53,54]. This method permits rapid detection and quantification by using fluorescent oligonucleotide probes with a 5′ reporter dye and a downstream 3′ quencher dye. Using this method, as little as a single molecule of DNA has been

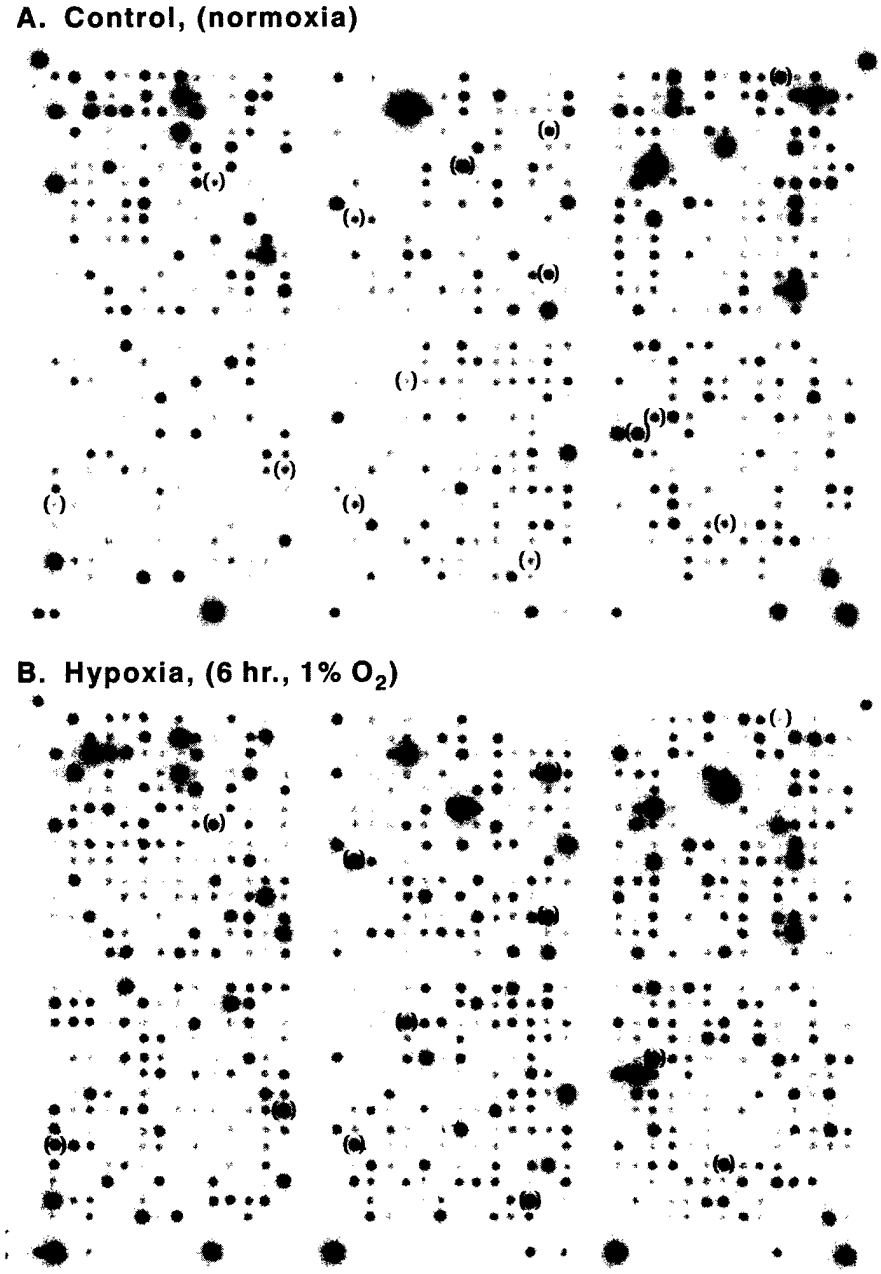

Fig. 6. Hypoxia regulates the overall gene expression pattern in PC12 cells. 10 g of total RNA from either control (A) or hypoxia = exposed (6 h 1% 0_2) (B) cells was converted to cDNA using reverse transcriptase and 32P-dATP, as described. Radiolabeled cDNA was then hybridized to filter-based cDNA microarrays containing 1176 cDNA sequences corresponding to various rat genes (Rat 1.2 AtlasArrays, Clontech, Palo Alto, CA, USA). Bracketed spots designate some of the genes that displayed large reproducible differences in gene expression levels between the two conditions.

detected from within a DNA mixture containing a high background of total genomic DNA [56].

7. Identification of hypoxia-regulated known genes using filter-based cDNA microarrays

Custom subtractive cDNA libraries facilitate the identification of novel genes. It is both an asset and a limitation of this approach that many of the cDNA sequences obtained may be previously unknown. Because of this, at least initially, our ability to assign functions to hypoxia-regulated genes may be limited. To quickly screen the effects of hypoxia on a pool of known genes, we have analyzed the effects of hypoxia on gene expression patterns using commercial filter-based microarrays (Clontech Atlas Rat 1.2 array). In these experiments, we again exposed PC12 cells to hypoxia (1% O2) for 6 h. Duplicate filters were separately hybridized with probes derived either from control or hypoxia-exposed cells. Representative arrays from such an experiment are shown in Fig. 6.

The data were analyzed using Atlas Image software (v. 1.0.1, Clontech). The data were then expressed as the ratio of normalized expression levels in control vs hypoxia. In these experiments, 105 out of 1176 genes (or 8.9%) were consistently regulated by greater than 2-fold by hypoxia in two separate experiments. Importantly, six of these are genes that others and we have previously shown by Northern blot or RT-PCR to be regulated by hypoxia in PC12 cells. The reproducibility between experiments was quite high; 105 out of 126, or 83% of the genes that were found to be regulated by hypoxia in the first experiment were similarly regulated in the second experiment. Moreover, all of the previously confirmed hypoxia-regulated genes were similarly regulated by hypoxia in both microarray experiments. Thus, cDNA microarray methodology can be used to accurately identify genes that are both up-regulated and down-regulated by hypoxia. Brackets in Fig. 6 indicate the fourteen genes that were most strongly regulated by hypoxia.

There are two major drawbacks to the commercial filter-based cDNA microarray technology described above. First, the researcher is limited to the pre-arrayed generic set of clones on the filter, which is relatively small in number and may contain even fewer target genes that are relevant to the specific problem being investigated. Thus, this approach provides no opportunity to discover novel genes. Second, the ^{32}P-labeling technology precludes simultaneous (competitive) hybridization. That is, control and experimental samples must be hybridized on separate filters or sequentially. Arraying custom libraries on glass slides effectively circumvents both of these problems, enabling researchers to simultaneously monitor the expression levels of thousands of genes, both known and unknown.

8. Summary

Transplantation of dopamine-secreting cells from fetal mesencephalon directly into the striatum has been successful in alleviating PD-associated motor dysfunction, both in patients and animal models. Unfortunately, the majority of these cells die within a short time following transplantation. It is likely that the hypoxic environment in the brain parenchyma is a primary factor in the death of these transplanted cells. This is supported by the work of Lopez-Barneo and associates who showed that the dopaminergic cells of the carotid body lead to long-term amelioration of PD-related symptoms [7,8]. This is most likely due to the high levels of tolerance these cells have for hypoxia. Thus, tolerance to hypoxia is a beneficial property, which may enhance the survivability of transplanted cells. We have used subtractive cDNA libraries and microarray analysis to begin identifying the genes that mediate hypoxia tolerance in the dopaminergic PC12 cells. Our findings indicate that the hypoxia-tolerant phenotype is mediated by an expression profile involving hundreds of genes. These genes are involved in virtually all aspects of cell function including membrane function, signal transduction, transactivation, and neurotransmitter release. Our goal is to identify and characterize the gene expression profile that mediates the hypoxia-tolerant phenotype and thus enhanced survivability of transplanted cells.

Acknowledgements

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