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Allopurinol Pretreatment Improves Evoked Response Recovery Following Global Cerebral Ischemia in Dogs

Richard B. Mink, MD; Andrew J. Dutka, MD; and John M. Hallenbeck, MD

The reperfusion of previously ischemic tissue may lead to the formation of highly reactive free radicals that promote tissue injury. Xanthine oxidase has been implicated as one source of these free radicals. We examined the role of xanthine oxidase in brain injury using a cerebrospinal fluid compression model of global cerebral ischemia with 15 minutes of ischemia and 4 hours of reperfusion. Seven dogs were pretreated with the xanthine oxidase inhibitor allopurinol (50 mg/kg for 5 days). Neurophysiological recovery was monitored with cortical somatosensory evoked potentials. As an attempt to correlate brain recovery with the mechanism of protection, free brain malondialdehyde was measured at the end of reperfusion by high-performance liquid chromatography. Brain water content was measured by wet-dry weights. Compared with seven untreated control dogs, allopurinol pretreatment significantly improved recovery of somatosensory evoked potentials after 4 hours of reperfusion. However, the amount of free malondialdehyde in the allopurinol-treated dogs was 32% greater than that in the controls. Brain water content was similar in the two groups. These results suggest that xanthine oxidase contributes to brain injury after ischemia and reperfusion. However, tissue damage caused by xanthine oxidase may be mediated through mechanisms other than free radical production. (*Stroke* 1991;22:660-665)

The reperfusion of previously ischemic cerebral tissue is essential to the recovery of brain function. However, reperfusion may paradoxically lead to the formation of highly reactive oxygen free radicals that damage tissue.[1] Xanthine oxidase (XO), an enzyme that converts hypoxanthine to xanthine and xanthine to uric acid, may be one source of these free radicals. In normal brain, XO exists mainly as the non–free radical generating xanthine dehydrogenase (XD),[2] although a small amount of oxidase is found in the cerebral endothelium.[3] During ischemia, XD may be converted to XO which then generates free radicals during reperfusion.[4-6] Irreversible XO is formed from proteolytic cleavage of XD whereas sulfhydryl oxidation results in the formation of reversible XO.[7,8]

If XO contributes to tissue damage in cerebral ischemia/reperfusion injury, then inhibiting the enzyme should improve cerebral recovery after ischemia and reperfusion. Furthermore, if XO causes damage by generating free radicals, improved recovery should be accompanied by lower levels of lipid peroxidation, an index of free radical damage. We tested these hypotheses in a canine model of global cerebral ischemia after pretreatment with allopurinol, an inhibitor of both XD and XO.

Materials and Methods

The experimental protocol was reviewed by the institutional animal care and use committee and certified as conforming to the principles described in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (Department of Health and Human Services publication No. 86-23). Details of this preparation have been published previously.[9] Sixteen adult male mongrel dogs weighing 11.3–13.2 kg were preanesthetized with 2 mg/kg i.m. xylazine and 0.05 mg/kg i.m. atropine and were

From the Diving Medicine Department (R.B.M., A.J.D.), Naval Medical Research Institute, Bethesda, Md., the Department of Critical Care Medicine (R.B.M.), Children's National Medical Center, Washington, D.C., and the Department of Neurology (J.M.H.), Bethesda Naval Hospital, Bethesda, Md.

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Address for correspondence: Richard B. Mink, MD, National Naval Medical Center, Naval Medical Research Institute, Mail stop 5a, Bethesda, MD 20814-5055.

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maintained on intravenous α-chloralose (80 mg kg initially, then 20 mg kg every 20 minutes). The dogs were intubated and mechanically ventilated and received 0.1 mg kg i.v. pancuronium bromide every hour for muscle relaxation. Intravenous and intra-arterial femoral lines were placed for the administration of fluids and drugs, monitoring of blood pressure, and sampling of blood for the measurement of pH, Pao_2, $Paco_2$, and hematocrit. Rectal temperature was maintained at 38.0±0.5°C. Somatosensory evoked potentials (SEPs) were measured (CA 1000, Nicolet Instruments Corp., Madison, Wis.) over the right cerebral cortex with stimulation of the left median nerve (stimulus 17–19 mA, duration 100 msec, 1.7 repetitions sec^{-1}, bandpass filter 30–3000 Hz, average of 40 repetitions). Prior to ischemia, baseline SEPs were obtained and the P1–N1 amplitudes averaged; SEP recovery is expressed as a percentage of this baseline amplitude. An 18-gauge spinal needle was placed percutaneously in the cisterna magna to monitor intracranial pressure and infuse Elliott's B solution, a mock cerebrospinal fluid (CSF) solution.[10]

The allopurinol-treated dogs were randomly selected and received 300 mg allopurinol (Zyloprim, Burroughs Wellcome Co., Research Triangle Park, N.C.) orally twice per day for 4 days preceding the experiment. On the day of the investigation, an additional 50 mg/kg was given intravenously. The control dogs received the vehicle (normal saline, pH 10.2) intravenously. Animals were subjected to 15 minutes of cerebral ischemia by elevating the intracranial pressure to equal the mean arterial pressure with the infusion of warmed (38°C) Elliott's B solution. Proper placement of the spinal needle in the cisternal magna was verified by an unmeasurable SEP during ischemia. In previous experiments with this model, [^{14}C]iodoantipyrine was used to confirm the complete absence of cerebral blood flow during ischemia.[2,9] To combat the systemic hypertension that occurs with intracranial hypertension, aliquots of blood were withdrawn and 2.5–10 mg i.v. phentolamine was administered as necessary. Reperfusion was initiated by allowing CSF to drain until the intracranial pressure was <20 mm Hg. A cerebral perfusion pressure of at least 60 mm Hg was maintained during reperfusion by infusing previously withdrawn blood, administering fluids, and/or infusing norepinephrine. At the conclusion of the 4-hour reperfusion period, the brains were rapidly removed and frozen in liquid nitrogen for assays of the amount of free malondialdehyde, the water content, and the XO and XD+XO activities. Gray and white matter water contents were determined by drying at 110°C to constant weight.

Free malondialdehyde, an index of lipid peroxidation, was measured in the right parietal cerebral cortex by the method of Esterbauer et al.[11] Tissue was homogenized on ice in 0.1 M Tris buffer (pH 7.40). An aqueous solution was prepared by precipitation with acetonitrile followed by centrifugation at 3°C. Malondialdehyde in the solution was separated by a high-performance liquid chromatograph equipped with a carbohydrate analysis column (Waters Chromatography Division, Millipore Corp., Milford, Mass.) and a 270 nm ultraviolet detector. A standard malondialdehyde solution was made by hydrolyzing malonaldehyde-bis(diethylacetal) (Aldrich Chemical Co., Milwaukee, Wisc.) in 1% sulfuric acid. The malondialdehyde concentration in the standard solution was confirmed by measuring the ultraviolet absorbance at 245 nm (ϵ=13,700). The amount of malondialdehyde in the sample was calculated by comparing the peak height of the sample with that of the standard solution.

The XO and XD+XO activities were determined by a modification of the method of Mousson et al.[12] Brain tissue was homogenized and centrifuged, and the supernatant was removed. The supernatant was not passed through a column to remove endogenous inhibitors since this also removes the administered allopurinol. Aliquots were incubated at 37°C with 8-[^{14}C]hypoxanthine (*ICN Biomedicals, Inc., Costa* Mesa, Calif.) in the presence (XD+XO activity) or absence (XO activity) of oxidized nicotinamide adenine dinucleotide. At the conclusion of the 1-hour incubation, the reaction was terminated by the addition of 1.7 M perchloric acid. After centrifugation, hypoxanthine was separated from xanthine and uric acid by thin-layer chromatography. Radioactivity was determined by direct counting, and enzyme activity was calculated by the ratio of the radioactivity of xanthine and uric acid to that of hypoxanthine plus xanthine plus uric acid. The limit of detection of this assay is 0.3 nmol xanthine and uric acid/min/g protein for both XO and XD+XO.

We used both parametric and nonparametric methods of statistical analysis. Wilcoxon's rank sum test was used for between-group comparisons. Repeated-measures analysis of variance was used to test the effects of treatment over the time course of the experiment. Data are presented as mean±SD; $p<0.05$ was considered significant.

Results

A total of 16 experiments were performed. Two experiments were terminated prematurely. In one, the dog died during reperfusion and in the other, there was difficulty placing the spinal needle in the cisterna magna. One control animal underwent 240 minutes of reperfusion, but due to technical difficulties, the last measurements were obtained at 210 minutes of reperfusion. Thus, seven dogs were studied in each group, but physiological data at 225 and 240 minutes in the control group represent six experiments.

Selected physiological variables are listed in Table 1. Group measurements were compared before and after ischemia and at 60, 120, 180, and 240 minutes of reperfusion. Data are similar in the two groups except for a lower CSF pressure and a lower $Paco_2$ in

TABLE 1. Selected Physiological Variables in Dogs Subjected to Global Cerebral Ischemia

Variable	Baseline		End ischemia		End reperfusion	
	Control	Allopurinol	Control	Allopurinol	Control	Allopurinol
Mean blood pressure (mm Hg)	131±11	125±17	155±38	114±42	135±42	138±20
Cerebrospinal fluid pressure (mm Hg)*	6±3	5±1	11±6	8±3	12±3	8±3
Cerebral perfusion pressure (mm Hg)	125±11	120±17	142±37	106±32	126±50	130±20
Temperature (°C)	38.65±0.45	38.80±0.50	38.66±0.45	38.70±0.25	38.71±0.48	39.17±0.23
Hematocrit (%)	42±4	45±4	41±7	44±3	47±4	47±4
pH	7.40±0.06	7.41±0.03	7.35±0.07	7.35±0.05	7.40±0.05	7.40±0.03
Pao_2 (mm Hg)	94±6	92±7	84±9	87±5	90±10	91±11
$Paco_2$ (mm Hg)*	35±2	32±3	38±3	36±5	35±2	31±4

Data are mean±SD, $n=7$ in each group (except $n=6$ in control group at end reperfusion).
*$p<0.05$ different from control by repeated measures analysis of variance.

the allopurinol group. Mean cerebral perfusion pressure at the end of ischemia in the allopurinol group was lower than, but not significantly different from, that in the control group. Peak mean arterial pressure was similar ($p>0.05$) in the two groups (237±36 mm Hg in the allopurinol group versus 251±43 mm Hg in the control group). The cerebral perfusion pressure was at least 60 mm Hg in all dogs.

The time from sacrifice of the dog to placement of the brain in liquid nitrogen did not differ significantly between groups. These times were 4.7±0.37 minutes for the allopurinol group and 5.3±0.47 minutes for the control group.

Reproducibility of the SEPs was excellent. The coefficient of variation for the baseline SEPs averaged 3.9% in the allopurinol group and 3.2% in the control group. Recovery of SEP during the 240 minutes of reperfusion is shown in Figure 1. Allopurinol-treated dogs had significantly greater recovery of SEP than the untreated controls. Final recovery was 17.3±13.7% in the allopurinol group compared with 5.4±5.5% in the control group ($p<0.05$).

The amount of free malondialdehyde measured at the end of reperfusion was higher ($p<0.01$) in the allopurinol group than in the control group (Figure 2). Cerebral gray and white matter water contents were similar in the two groups (Figure 3).

Brain XO and XD+XO activities were undetectable in all allopurinol-treated dogs. In the control group, these activities were 3.38±1.60 nmol xanthine and uric acid/min/g protein for XO and 4.71±2.69 nmol xanthine and uric acid/min/g protein for XD+XO.

Discussion

A pathological role for XO in ischemia/reperfusion injury was first suggested by Granger et al.[4] They proposed that XD was irreversibly converted to XO during ischemia. Then, when oxygen became available during reperfusion, superoxide radicals were generated by XO as the accumulated hypoxanthine was oxidized to xanthine and uric acid. We have

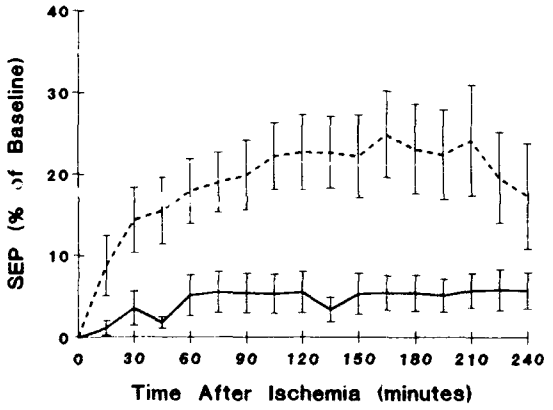

FIGURE 1. *Graph [mean±SD] of percent recovery of baseline somatosensory evoked potential (SEP) during 4 hours of reperfusion in dogs. Recovery in the allopurinol group (broken line) is significantly greater (p<0.05) than that in the control group (solid line)*

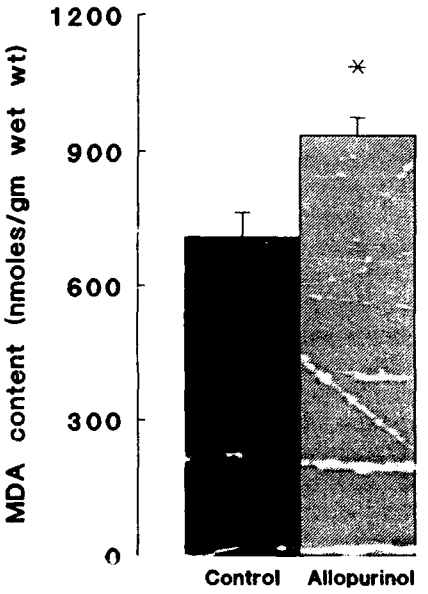

FIGURE 2. *Bar graph [mean±SD] of free malondialdehyde (MDA) content in cortical brain tissue of dogs in the allopurinol and control groups after 15 minutes of ischemia followed by 4 hours of reperfusion. *p<0.01 different from control by Wilcoxon's rank sum test.*

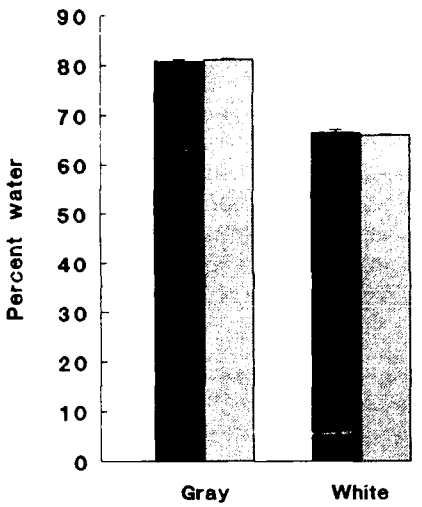

FIGURE 3. *Bar graph [mean±SD] of brain gray and white matter water contents of dogs in the allopurinol (hatched bars) and control (solid bars) groups.*

recently shown that irreversible conversion of XD to XO does not occur in cerebral ischemia/reperfusion injury.[2] However, a role for XO in brain injury cannot be excluded. Reversible conversion of XD to XO has been demonstrated in some tissues during ischemia, and this XO is a potential source of free radicals during reperfusion.[7,13] In addition, XO accounts for 20% of the total XD+XO activity in the uninjured dog brain.[2] Since cerebral XO is found principally in the endothelium,[3] even a small amount of the oxidase may cause tissue injury by damaging the blood–brain barrier.

Pretreatment of dogs with allopurinol improved SEP recovery after 4 hours of reperfusion. Recovery was associated with inhibition of both XO and XD activities. The $Paco_2$ in the allopurinol group was slightly lower than that in the control group, but this difference was small and probably not of physiologic significance. Mean cerebral perfusion pressure at the end of ischemia in the allopurinol-treated dogs was lower than, but not significantly different from, that in the controls. Even so, both a lower $Paco_2$ and a lower cerebral perfusion pressure would decrease cerebral blood flow[14] and likely inhibit the recovery of SEP in the allopurinol group. Although rectal, and not brain, temperature was measured, care was taken to avoid brain cooling by warming the Elliott's B solution to 38°C prior to infusion. Furthermore, unlike in small mammals, a significant effort is needed to cool the dog brain by even 1°C.[15,16] In investigations in which the dog brain was selectively cooled, rectal temperature also decreased.[15,16] We did not observe such a decrease in either group.

We used the SEP amplitude in our study since it has been suggested that this measurement correlates better with cortical ischemia than do changes in latency.[17,18] The SEP amplitude also has been observed to parallel changes in brain oxygen utilization.[19]

Allopurinol pretreatment has been shown to reduce tissue injury after ischemia/reperfusion in the heart, intestine, and liver.[20-22] Itoh et al[23] examined the effect of allopurinol on cerebral recovery after 4 hours of bilateral common carotid artery occlusion in spontaneously hypertensive rats. Rats pretreated with the drug had a significantly lower mortality and a better neurological outcome at 72 hours than untreated animals.[23] In a rat model of continuous partial ischemia, allopurinol reduced brain infarction at 3 and 24 hours.[24] However, in a gerbil model employing unilateral carotid artery ligation, allopurinol pretreatment improved outcome only at 2–4 hours, not at 24 hours.[25] These studies did not examine the mechanism by which allopurinol was protective.

We used high-performance liquid chromatography to measure the malondialdehyde content because the more conventional assay, the thiobarbituric acid test, gives artifactually high levels of "malondialdehyde" by measuring non-malondialdehyde species.[26,27] Our finding of an increased brain malondialdehyde content in the allopurinol-treated dogs was unexpected. This result was not due to a dilutional effect since brain water content was similar in the two groups. In addition, allopurinol has not been reported to increase lipid peroxidation in any in vitro or in vivo system. Since this assay measures the amount of non–tissue-bound malondialdehyde, allopurinol could have altered the binding of malondialdehyde, increasing the free amount without altering the total amount. Vitamin E deficiency has been shown to alter the proportion of free malondialdehyde in rat liver, with the percentage of unbound malondialdehyde increasing from 8% to 60% when rats are fed a vitamin E–deficient diet.[27] Another possibility is that allopurinol inhibited the metabolism of malondialdehyde. Experiments using liver and kidney demonstrate that malondialdehyde is first oxidized to malonic semialdehyde, and then decarboxylated to form acetaldehyde. Acetaldehyde is oxidized to acetate predominantly by aldehyde dehydrogenase.[28,29] However, acetaldehyde also can be oxidized by XO.[30] Inhibiting XO with allopurinol may have led to higher in vivo malondialdehyde levels by altering acetaldehyde elimination. Malondialdehyde may also be produced when prostaglandins are synthesized since arachidonic acid metabolism is associated with free radical intermediates.[31] If allopurinol protected cells so that arachidonic acid metabolism continued, SEP recovery would then be associated with an increase in brain malondialdehyde content.

In addition to inhibiting the formation of free radicals, other mechanisms have been proposed to account for the protective effect of allopurinol. During ischemia, energy stores are depleted as adenosine triphosphate is degraded to the purines inosine and hypoxanthine. Hypoxanthine can be further metabolized to xanthine and uric acid by XD or XO. Upon reperfusion, the energy state is restored by either de novo synthesis of purine bases

or by reutilization of inosine and hypoxanthine through salvage pathways. The latter method is significantly more energy-efficient. One mechanism by which allopurinol may be protective in ischemia/reperfusion injury is by inhibiting XD and XO, preventing the metabolism of hypoxanthine to the nonsalvageable xanthine and uric acid, and thereby limiting loss of the purine bases. This leads to greater salvage of hypoxanthine, improved restoration of the energy state, and better recovery.[25,32] A role for allopurinol as a direct free radical scavenger has also been proposed,[33,34] although one investigation discounts this mechanism.[35] Allopurinol has also been shown to have vasodilatory effects that could promote blood flow to previously ischemic areas.[36]

Our study provides evidence for the participation of XO in cerebral ischemia/reperfusion injury. Documented inhibition of XO and XD by allopurinol was associated with improved neurophysiological brain recovery. Rather than presume a mechanism by which allopurinol was protective, we attempted to correlate improved recovery with an index of free radical damage, lipid peroxidation. Our data suggest that the detrimental effects of XO are not due to the generation of free radicals, although interpretation of the malondialdehyde results is complicated by an unpredicted effect of allopurinol on measurement of malondialdehyde. Since the presence of XD and XO in the human brain has been confirmed,[37] further study of the improved cerebral recovery with allopurinol is warranted. These studies should incorporate an evaluation of the mechanism by which allopurinol is protective in their design.

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