AD-A286 074

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting b... per response, including the time for reviewing instructions, searching existing data sources, gathering and ma... information. Send comments regarding this burden estimate or any other aspect of this collection of infor... Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Su... Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY U[SE ONLY]

2. REPORT DATE: 1994

3. REPORT TYPE AND DATES COVERED: Journal article

4. TITLE AND SUBTITLE
Protein kinase C is a mediator of lipopolysaccharide-induced vascular suppression in the rat aorta

5. FUNDING NUMBERS
PE - 62233N
PR - MM33C30
TA - 01
WU -1001

6. AUTHOR(S)
McKenna TM; Clegg JM; Williams TJ

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

8. PERFORMING ORGANIZATION REPORT NUMBER
NMRI 94-52

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Medical Research and Development Command
National Naval Medical Center
Building 1, Tower 12
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
DN246558

11. SUPPLEMENTARY NOTES
Reprinted from: Shock 1994 August; Vol.2 No.2 pp. 84-89

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

14. SUBJECT TERMS
kinase C; lipopolysaccharide-induced; vascular, rat, aorta

15. NUMBER OF PAGES
6

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

SHOCK, Vol. 2, No. 2, pp. 84–89, 1994

PROTEIN KINASE C IS A MEDIATOR OF LIPOPOLYSACCHARIDE-INDUCED VASCULAR SUPPRESSION IN THE RAT AORTA

Thomas M. McKenna, Joanne M. Clegg, and Taffy J. Williams

Septic Shock Research Program, Naval Medical Research Institute, Bethesda, Maryland 20889

Received 1/10/94; accepted in the final form 5/11/94.

ABSTRACT—Treatment of vascular tissue with lipopolysaccharide (LPS) *in vitro* induces hyporesponsiveness to contractile agonists. We investigated whether protein kinase C (PKC) transduces the LPS signal into contractile dysfunction. Rat aortic tissue was incubated .5–18 h with LPS (10 or 30 ng/mL) or α- and β-phorbol 12,13-dibutyrate (PDB, .1 or 1 μM), either alone or combined with cycloheximide (50 μM) or the kinase inhibitors sphingosine (20 μM), H7 (1-(5-isoquinolinylsulfonyl)-2-methyl piperazine, 25 μM), and HA1004 (*N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide, 25 μM). LPS and β-PDB induced a sustained translocation of PKC activity from the cytosol to the membrane, an increased protein synthesis-dependent expression of nitric oxide synthase (NOS) activity, and an impaired contractility that could be partially reversed by treatment with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester. Incubation with α-PDB, an inactive isomer of β-PDB, did not alter any of the tissue functions. Sphingosine blocked LPS- and β-PDB-induced NOS activity and LPS-induced impairments in tissue contractility and PKC translocation. Incubation with H7 also protected against LPS-induced vasoplegia, while HA1004, used as a negative control for H7, provided little protection against LPS. These data indicate that PKC plays a role as an intracellular mediator of LPS-induced NOS activity and vascular suppression.

INTRODUCTION

Cardiovascular tissue from septic animals shows diminished contractile function when tested *in vitro*, indicating that the contractile defect is intrinsic to the tissue and does not require the continuous presence of circulating sepsis-associated factors. Contractile dysfunction can also be induced by *in vitro* treatment of vascular smooth muscle (VSM) with lipopolysaccharide (LPS), interleukin-1, or tumor necrosis factor. The contractile impairments require *de novo* protein synthesis (1), and an inducible, Ca^{2+}-independent nitric oxide synthase (NOS) has been shown to be a proximal intracellular mediator for hypocontractility (2). The augmented NOS activity produces nitric oxide that in turn activates soluble guanylate cyclase to generate cGMP which then inhibits vascular contraction. The mechanism by which sepsis-associated extracellular mediators are coupled intracellularly to the enhanced NOS expression is unclear.

Protein kinase C (PKC) activity resides in a family of isozymes which may act as intracellular intermediaries to transmit LPS and cytokine signals into *de novo* synthesis of NOS (3) or cofactors that enhance NOS activity (4, 5). Vascular smooth muscle is rich in PKC, manifesting isozymes distributed among calcium and phospholipid-dependent species which include PKC_{α} and $PKC_{\beta II}$ (6, 7), and Ca^{2+}-independent species such as PKC_{ϵ} and PKC_{ζ} (8). One of the consequences of activation of PKC is enhanced gene transcription. Treatment with the PKC activator phorbol 12-myristate 13-acetate, or exposure to 20% serum (under growth-arrested conditions) stimulates PKC-dependent protooncogene expression in rat VSM cells (9). In rat macrophages, activation of PKC by phorbol 12,13-dibutyrate induces NOS expression (10).

Although to our knowledge it has not been shown whether LPS can activate PKC in VSM cells, lipid A (the toxic lipid portion of LPS) and LPS itself cause PKC translocation and activation in B lymphocyte membranes (11) and in *in vitro* models for PKC activation (11–13). In the present study, we present evidence that LPS activates rat aortic VSM PKC, that activated PKC in turn elevates NOS activity, and that treatment of VSM with PKC inhibitors blocks LPS-induced NOS activity and vascular tissue contractile dysfunction.

MATERIALS AND METHODS

Tissue preparation and incubation

Male Sprague-Dawley rats (250–400 g, Taconic Farms, Germantown, NY; or Charles River, Wilmington, MA) were used in these studies. The rats were decapitated, the thoracic aorta dissected out, and four to seven 3.5 mm long rings were prepared from each rat. Most aortas were de-endothelialized before sectioning into rings by perfusion with phosphate-buffered saline (PBS) containing 1% sodium deoxycholate for 30 s followed by a PBS and bubble rinse for 5 min. The efficacy of this technique in removing the endothelium has been documented (1). Rings were incubated individually in 2 ml of Dulbecco's modified Eagle's medium (DME) supplemented with 1% fetal calf serum (FCS), 100 μg/mL streptomycin, and 100 U/mL penicillin, and were distributed so that tissue isolated from each aorta was allocated to every combination of treatments within an experiment. During incubation, the rings were exposed to LPS (10 or 30 ng/mL), α- or β-PDB (.1 or 1 μM), either alone or combined with cycloheximide (50 μM), sphingosine (20 μM), H7 (25 μM), or HA1004 (25 μM). The media and rings were rotated gently for 18 h in an incubator at 37°C under a 95% O_2-5% CO_2 atmosphere. For measures of PKC activity, six rings were sectioned from each aorta, and allocated in duplicate to different treatments. The rings were incubated .5 to 18 h in 2 ml media supplemented as detailed above.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, Department of Health and Human Services, Publication (NIH)86-23 (1985).

Address correspondence and reprint requests to Dr. Thomas McKenna, Septic Shock Research Program, Code 623, Naval Medical Research Institute, 8901 Wisconsin Ave., Bethesda, MD 20889-5607.

Protein kinase C isolation and assay

PKC was isolated from de-endothelialized aortic rings essentially as described by Thomas et al. (14). After incubation, two identically treated rings were washed and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 15,000 × g for 15 min, and the soluble fraction retained for measures of cytosolic PKC. The pellet was rehomogenized in fresh buffer, and Nonidet P-40 was added to the particulate suspension to a final concentration of 1% to solubilize any PKC tightly associated with the membrane fraction. Soluble and particulate preparations were subjected to ion-exchange chromatography on DEAE-cellulose, with leupeptin (25 μg/mL final concentration) immediately added to all eluted sample fractions. PKC activity in the column eluates was measured immediately following elution with a commercially available kit (Amersham, Arlington Heights, IL). PKC activity was assayed using the manufacturer's protocol. Phosphorylation of the peptide substrate by calcium- and phospholipid-independent kinase activity was corrected for by subtracting the radioactivity transferred in a lipid-free assay system containing EGTA. The transferred radioactivity was quantitated by scintillation spectrophotometry. Protein content of the soluble and particulate fractions was measured by bicinchoninic acid assay (Pierce, Rockford, IL) and used to normalize PKC values which are expressed as pmol of P transferred mg^{-1} protein min^{-1}.

Assay for conversion of [L-^{3}H]arginine to [L-^{3}H]citrulline

[L-^{3}H]Citrulline formation was measured by a modification of the technique of Bredt and Snyder (15). After incubation, aortic rings were washed for 1 h in 1 L of Krebs-Ringer bicarbonate buffer (KRB; millimolar composition: NaCl 118; KCl 4.7; $CaCl_2$ 1.3; $MgSO_4 \cdot 7H_2O$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 11.7) at pH 7.4 and 37°C while continually bubbled with 95% O_2-5% CO_2. The rings were then incubated for 15 min in 250 μL of KRB at 37°C containing [L-^{3}H]arginine (3 μCi/mL). The assay was terminated by treating the rings for 2 min with 750 μL of ice-cold KRB supplemented with 5 mM arginine and 4 mM EDTA. Thereafter, the rings were blotted, dropped into 1 mL of ice-cold trichloroacetic acid, homogenized with a Polytron (Brinkman, 30,000 rpm), and centrifuged (2500 × g for 15 min at 0°C). The supernatant was recovered and extracted three times with 2 mL of water-saturated diethyl ether, and .5 mL aliquots of the aqueous fraction were neutralized with 2 mL of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 6.0). The 2.5 mL samples were applied to 2 mL columns of Dowex AG50W-X8 (Na^+ form) and eluted with 2 mL of H_2O. [L-^{3}H]Citrulline content in the eluant was quantified by liquid scintillation spectroscopy. The protein content of the pellet was determined as detailed above. [L-^{3}H]Citrulline production was expressed as fmol mg^{-1} of protein min^{-1}.

Measurement of aortic ring contraction

Because rings with an intact endothelium are more sensitive to LPS-induced suppression than are de-endothelialized rings (1), contractions by endothelium-intact rings were measured after incubation with 10 ng/mL LPS, while de-endothelialized ring contractility was measured after incubation with 30 ng/mL LPS. After incubation, rings were mounted between two stainless steel hooks in 10 ml jacketed tissue baths. One hook was fixed in the bath, and the other hook was attached to a strain gauge (Kulite Semiconductor, Ridgefield, NJ) for measures of ring tension. Contractions by up to eight rings were measured simultaneously and recorded on multichannel strip chart recorders. The rings were maintained in KRB at a resting tension of 2.4 g and allowed to equilibrate for 30 min. The rings were then contracted by two applications of norepinephrine (1 μM); acetylcholine (1 μM) was added to the baths at maximal contraction to the first dose of NE to test for the functional integrity of the vascular endothelium (16). The tissue baths were flushed with fresh KRB between applications of NE until ring tension returned to resting values. These procedures required approximately 90 min and served to equilibrate the rings to resting tension and to wash LPS and PKC inhibitors from the tissues. Rings were then contracted by withdrawing KRB from the tissue baths and adding increasing volumes of a high KCl KRB (120 mM, made by equimolar substitution of KCl for NaCl) to the baths to yield final KCl concentrations of 10 to 80 mM. In another set of experiments, rings were repetitively precontracted with 80 mM KCl and then washed, until maximum contractions to KCl stabilized. After further washing and attainment of a stable baseline tension, rings were treated with the NOS inhibitor L-NAME (*N*-nitro-L-arginine methyl ester) or the inactive isomer D-NAME (.1 mM) for 15 min, and then contracted with 80 mM KCl. At maximum contraction, all rings were treated with .1 μM PDB, and any further increments in tension were recorded. All rings were blotted and weighed after experiments were completed.

Statistics

Aortic ring contractile performance to KCl was characterized by deriving E_{max} (maximum generated tension) and EC_{50} values (concentration of agonist causing a half-maximal contraction) with a nonlinear regression analysis program (17) that fitted second or third-order polynomials to ring dose-responses. Examination of differences among treatment means in measures of aortic ring PKC activity, [^{3}H]citrulline production, and contractile performance was by the appropriate one-way, Kruskall-Wallis, or randomized blocks analysis of variance; if significant differences were present, then pairs of means were tested by Student-Newman-Keuls *a posteriori* tests (18). Data were subjected to logarithmic transformation prior to analysis if means were positively correlated with variances. Comparisons between two treatment effects were by paired *t* tests. Differences with probabilities of .05 or less were accepted as significant. All data are expressed as means ± S.E.

Materials

DME was purchased from GIBCO, Grand Island, NY. Defined FCS was obtained from Hyclone Laboratories, Logan, UT. The lot used contained less than 25 pg/mL LPS. Penicillin/streptomycin and PBS were obtained from Quality Biological, Gaithersburg, MD. 4α- and 4β-PDB, and L- and D-NAME were purchased from LC Services, Woburn, MA. Norepinephrine bitartrate, leupeptin, and D-sphingosine were bought from Sigma Chemical Co., St. Louis, MO. H7, HA1004, and cycloheximide were purchased from Calbiochem-Behring Corp., San Diego, CA. Nonidet P-40 was obtained from BDH Chemicals Ltd, Poole, England. DEAE-cellulose was bought from Pierce, Rockford, IL. Dowex AG50W-X8 was acquired from Bio-Rad, Richmond, CA. [L-2,3-^{3}H]Arginine (56 Ci/mmol) and [γ-^{32}P]ATP (triethylammonium salt, 3000 Ci/mmol) were purchased from DuPont New England Nuclear, Boston, MA. LPS (*Escherichia coli* LPS serotype 055:B5) was obtained from Difco, Detroit, MI. The LPS was stored as a stock preparation in DME (1 mg/mL). For experiments, the LPS stock was diluted into DME and sonicated for 3 min at maximum power in a cup horn sonicator (Cole-Parmer Instruments, 4710 series, Chicago, IL). D-Sphingosine was prepared by modification of the technique of Merrill et al. (19). The lipid was solubilized in absolute ethanol to a concentration of 33 mM; this solution was then added in a ratio of 1:100 (v/v) to FCS, incubated at 37°C for 1 h, and stored at −20°C. H7 and HA1004 were dissolved in PBS at 1 mg/mL and stored at 4°C in the dark. Phorbols were diluted in dimethyl sulfoxide (2.5 mg/mL) and stored at −80°C.

RESULTS

PKC activation by LPS and PDB

Incubation with 10 ng/mL LPS caused PKC to translocate from the cytosol to the membrane, resulting in significant elevations in membrane-associated activity after 1 and 4 h, while cytosolic PKC levels declined nonsignificantly (Fig. 1A). The PKC distribution returned to normal after 18 h despite continuous LPS exposure. Directly activating PKC with 1 μM PDB also caused a cytosol to membrane translocation (Fig. 1B). PDB-induced PKC translocation was significant after 4 h exposure and was associated with an apparent, although nonsignificant decrease in both PKC pools at 18 h. To test for nonspecific effects of PDB, rings were treated for 4 h with 1 μM α-PDB, the inactive isomer of PDB. PKC distribution was not altered in these rings (control and α-PDB-treated ring cytosolic PKC activities were 700 ± 292 and 764 ± 170 pmol mg^{-1} protein min^{-1}, respectively; membrane-associated PKC activities were 406 ± 186 and 517 ± 144, respectively, N = 6 rings per group). The ability of the PKC inhibitors sphingosine (19) and H7 (20) to prevent LPS-induced PKC translocation was also examined. A LPS dose of 30 ng/mL induced a 33-fold increase

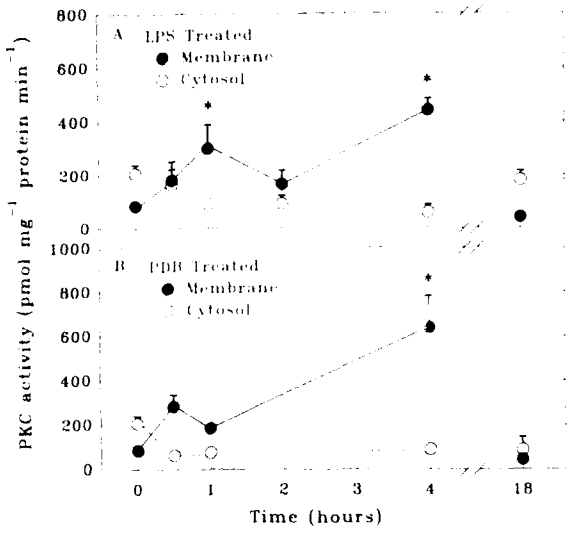

FIG. 1. **Aortic ring membrane and cytosol PKC activities after incubation in LPS- and PDB-treated media.** *A*, PKC activity in rings incubated in control medium (0 time), or in medium containing 10 ng/mL LPS for .5 to 18 h. *B*, PKC activity in rings incubated with 1 μM PDB as described above. *, significant increases in membrane-associated PKC activity in LPS and PDB-incubated rings in comparison to control (0-time) rings. The data are plotted as means ± S.E.; small SEM do not extend beyond the symbols. N = 15 rats; values are based on 5 to 15 pairs of rings per data point.

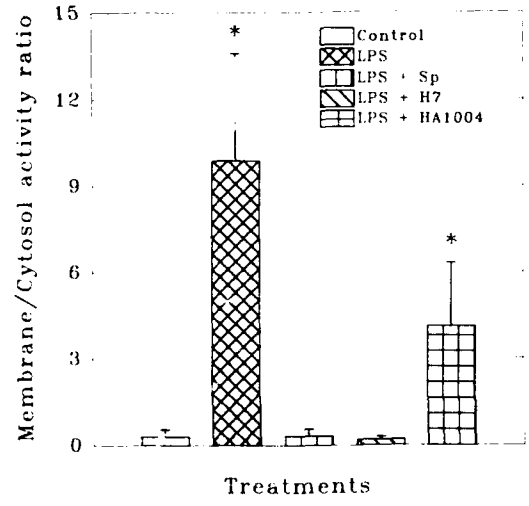

FIG. 2. **Blockade of LPS-induced cytosol to membrane translocation by PKC inhibitors.** Rings were incubated in control medium (*open bar*), medium supplemented with 30 ng/mL LPS (*crosshatched*), or LPS-supplemented media with sphingosine (*Sp*, 20 μM, vertical), H7 (25 μM, diagonal), or HA1004 (25 μM, squares). *, a significant increase in membrane to cytosol ratio in comparison to control. The data are plotted as means ± S.E. N = 10 rats; values are based on five pairs of rings per data point.

in the PKC membrane-to-cytosol ratio in treated compared to control rings (Fig. 2). Coincubation of rings with LPS and 20 μM sphingosine or 25 μM H7 completely blocked LPS-induced PKC translocation, while 25 μM HA1004, which has less PKC inhibitory capacity than H7 (20), did not prevent a significant LPS-induced PKC translocation (Fig. 2).

Nitric oxide synthase activity

NOS activity, as reflected by an enhanced conversion of [^{3}H]arginine into [^{3}H]citrulline (15), was significantly increased in rings incubated with 30 ng/mL LPS and .1 or 1 μM PDB (Fig. 3). PDB-stimulated NOS activity was apparently maximal at .1 μM, because incubation with 1 μM PDB did not yield additional [^{3}H]citrulline. Blockade of PKC with 20 μM sphingosine effectively barred induction of NOS activity by both LPS and PDB. Cycloheximide (50 μM) significantly suppressed control, LPS, and PDB-incubated ring [^{3}H]citrulline production (Table 1).

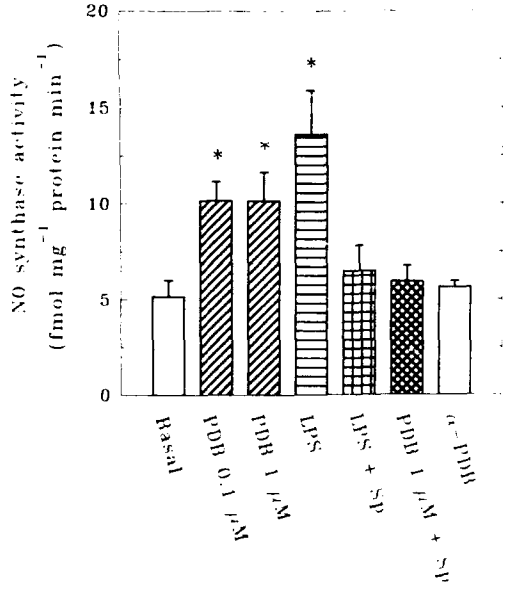

FIG. 3. **NOS activities in aortic rings incubated 18 h with PDB (.1 and 1 μM), α-PDB (1 μM), and LPS (30 ng/mL).** Rings were also incubated with combinations of LPS and 20 μM sphingosine (*Sp*), and 1 μM PDB and sphingosine. *, significant increases in NOS activities compared to untreated rings (basal). The data are plotted as means ± S.E. N = 5 rats.

Nitric oxide contributes to LPS and PDB-induced contractile dysfunction

Rings incubated with 30 ng/mL LPS or .1 μM PDB showed significantly depressed contractions when stimulated with KCl (Fig. 4). Treatment with L-NAME, but not D-NAME, significantly increased (by 42 and 36%, respectively) LPS- and PDB-incubated ring contractions to KCl, a finding consistent with blockade of the enhanced NOS activity shown in Fig. 3. Rings incubated with α-PDB did not show depressed contraction. Rings incubated with PDB did not contract normally to a subsequent stimulation with PDB, in contrast to control, and α-PDB- and LPS-incubated tissue.

PKC blockade protects against LPS-induced contractile dysfunction

Because the endothelium significantly modulates vascular contractility, the capacity of H7 to protect against LPS-induced contractile dysfunction was examined in both endothelium-intact and de-endothelialized rings. Endothelium-intact aortic rings incubated with 10 ng/mL LPS exhibited significant decreases in maximal contraction and sensitivity to KCl in com-

TABLE 1. **Influence of cycloheximide treatment on conversion of [^{3}H]arginine to [^{3}H]citrulline in rat aortic rings incubated 18 h in control medium or media supplemented with LPS or PDB**

Treatment	[^{3}H]Citrulline (fmol mg^{-1} protein min^{-1})
Control*	7.7 + 1.2
LPS (30 ng/ml)	24.9 + 6.0†
PDB (1 μM)	13.8 + 2.5†
Control + Cycloheximide (50 μM)	2.7 + 0.3§
LPS (30 ng/ml) + Cycloheximide (50 μM)	2.0 + 0.1§
PDB (1 μM) + Cycloheximide (50 μM)	1.8 + 0.2§

* Rings from individual rats were allocated to each treatment; N = 3 rats.
† [^{3}H]Citrulline formation significantly greater than control ($p < 0.05$).
§ [^{3}H]Citrulline formation significantly less than control ($p < 0.05$).

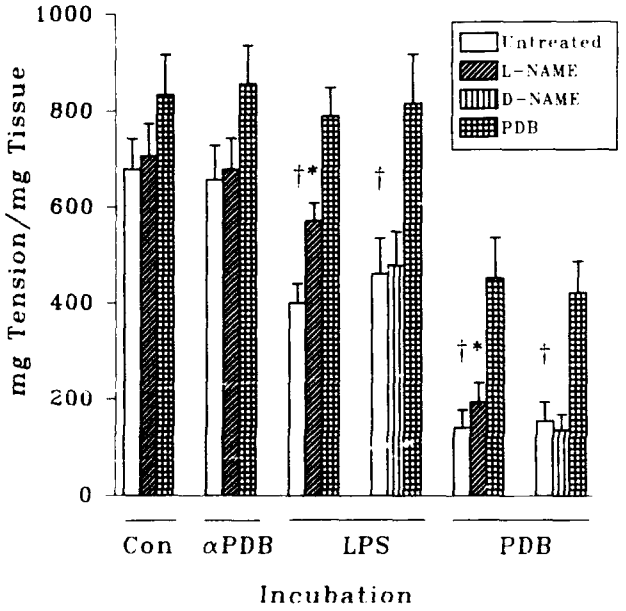

FIG. 4. **Contractions by aortic rings incubated 18 h in control media (*Con*), and media containing α-PDB (.1 μM), LPS (30 ng/mL), and PDB (.1 μM).** The rings were contracted with 80 mM KCl (*open bars*), washed, and contracted again with 80 mM KCl after 15 min treatment with .1 mM L-NAME (*striated*) or D-NAME (*vertical lines*). Increases in tension in response to PDB (.1 μM) added at maximum KCl-induced contraction are denoted by checked bars. †, a significantly reduced initial contraction compared to control; *, a significant increase compared to initial contraction. The data are plotted as means ± S.E. N = 6 rats.

parison to control rings (Fig. 5*A*, Table 2). In contrast, rings coincubated with LPS and H7 retained normal contractile function. HA1004, used as a negative control for H7, did not block the suppressive effects of LPS on the tissue (Fig. 5*B* and Table 2). De-endothelialized ring maximal contraction and sensitivity to KCl were depressed after incubation with 30 ng/mL LPS (Fig. 5*C* and Table 2). The LPS-induced dysfunctions were blocked in rings coincubated with H7, while HA1004-treated rings showed nonsignificant improvements in maximal contraction and normal sensitivity to KCl (Fig. 5*D* and Table 2).

The ability of PKC blockade to ameliorate LPS-induced contractile dysfunction was further explored in de-endothelialized aortic rings coincubated with 20 μM sphingosine. Sphingosine by itself suppressed control ring contractions. Despite the suppression due to sphingosine, rings incubated with combined

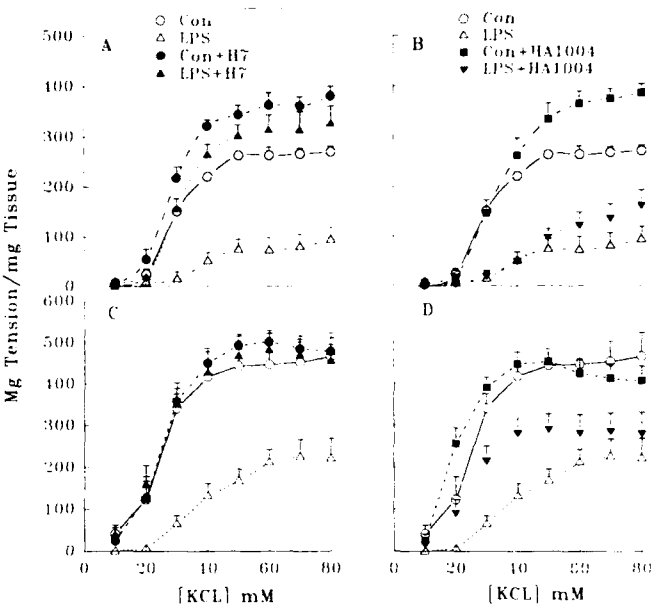

FIG. 5. **Contraction to KCl by endothelium-intact (*A* and *B*) and de-endothelialized (*C* and *D*) aortic rings incubated 18 h in combinations of control, LPS-, H7-, and HA1004-treated media.** *A*, responses by endothelium-intact rings incubated in medium only, medium plus H7 (25 μM), medium containing LPS (10 ng/mL), and medium containing LPS plus H7. *B*, for clarity in presentation, responses by rings incubated in medium only and LPS-treated medium are reproduced from *A*. Additional rings were incubated in medium plus HA1004 (25 μM), and medium containing LPS plus HA1004. *C*, responses by de-endothelialized rings incubated in medium only, medium plus H7 (25 μM), medium containing LPS (30 ng/mL), and medium containing LPS plus H7. *D*, for clarity in presentation, responses by rings incubated in medium only and LPS-treated medium are reproduced from *C*. Additional rings were incubated in medium plus HA1004 (25 μM), and medium containing LPS plus HA1004. The data are presented as in Fig. 1. N = 7 rats (intact endothelium) and 9 rats (de-endothelialized).

TABLE 2. **Maximum generated tension (E_{max}) and sensitivity (EC_{50}) values for KCl-stimulated rat aortic rings incubated 18 h in control media or media supplemented with LPS, or LPS and PKC inhibitors**

Treatment	E_{max} (mg tension/mg tissue)		EC_{50} (mM)	
	Control	LPS‡	Control	LPS‡
	Rings with intact endothelium			
None*	280 ± 11	98 ± 31§	30 ± 1	41 ± 4§
H7 (25 μM)	382 ± 18	348 ± 31	28 ± 1	32 ± 2
HA1004 (25 μM)	404 ± 16	185 ± 36§	36 ± 3	49 ± 4§
	De-Endothelialized rings			
None**	514 ± 56	256 ± 51§	25 ± 2	38 ± 4§
H7 (25 μM)	522 ± 30	510 ± 43	25 ± 2	26 ± 3
HA1004 (25 μM)	456 ± 30	315 ± 43§	18 ± 2	24 ± 2
None*	522 ± 37	214 ± 31§	25 ± 2	34 ± 2§
Sphingosine (20 μM)	417 ± 21	380 ± 39	24 ± 2	27 ± 1

‡ Lipopolysaccharide, 10 ng/ml (rings with intact endothelium) or 30 ng/ml (de-endothelialized rings).
* N = 7 rats, ** N = 9 rats.
§ $p < 0.05$ in comparison to equivalently treated control.

sphingosine and LPS contracted significantly better than rings incubated only with LPS (Fig. 6). Sphingosine also shielded the rings against LPS-induced loss of sensitivity to KCl (Table 2).

DISCUSSION

It is not known how LPS initiates cellular responses in vascular smooth muscle. Although LPS receptors have not been demonstrated in this tissue, numerous candidate LPS receptors have been identified in other cell types (see Ref. 21 for review). These receptors may be coupled to intracellular second messenger systems that activate PKC, or alternatively, LPS may activate PKC by a mechanism in which cell surface molecules bind LPS and facilitate a subsequent insertion of the LPS into the cell membrane (22). Because the lipid A moiety of LPS has been shown to activate PKC *in vitro* (12), it is possible that cell membrane-incorporated LPS could directly activate PKC.

PKC activity resides in a family of at least nine isozymes which exhibit differences in tissue and cellular distribution and cofactor requirements (see Ref. 23 for review). These differences suggest that PKC isozymes may be differentially regulated and functionally distinct, and evidence exists for individual PKC isotype behavior in vascular smooth muscle. $PKC_{\beta II}$ isozyme levels increase in streptozotocin-induced diabetic rat aortas, while the α-form remains unchanged (7). In the ferret aorta, activated PKC_ζ translocates to the intranuclear compartment rather than to the sarcolemma as does PKC_ϵ. The authors of that study suggest that the intranuclear localization of PKC_ζ makes it a candidate for transmitting agonist signals into gene expression and cell proliferation (8).

While the current study cannot identify which isotype(s) of PKC may be responsible for transmitting the LPS signal into enhanced NOS activity and vascular contractile depression, we found that inhibition of PKC activity by agents directed at either the active site (H7 (20)) or the regulatory site (sphingosine (19)) blocked LPS-induced PKC translocation, NOS expression, and contractile dysfunction. H7 is not a specific PKC inhibitor, because it works at the catalytic site of the enzyme, which is highly conserved among serine/threonine kinases, and consequently

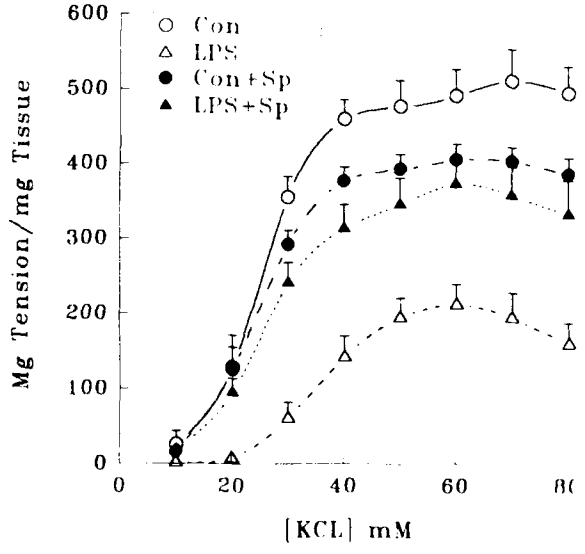

FIG. 6. **Contraction to KCl by de-endothelialized aortic rings incubated for 18 h in medium only (*Con*), medium plus sphingosine (*Sp*, 20 μM), medium containing LPS (30 ng/mL), and medium containing LPS plus sphingosine**. The data are presented as in Fig. 1. N = 7 rats.

H7 inhibits other kinases including cAMP and cGMP-dependent, myosin light chain, and casein I and II kinases (20). PKC-dependent processes are highlighted by comparison with responses to treatment with HA1004, which has a kinase inhibitory profile similar to H7 but inhibits PKC to a lesser extent (20). In our system, HA1004 clearly did not protect ring contractile function against LPS as well as H7. Sphingosine, a PKC inhibitor which acts by competing with diacylglycerol, phosphatidylserine, or phorbols at the PKC regulatory site also inhibits calmodulin-dependent enzymes, including myosin light chain kinase (24). In addition, sphingosine has cytotoxic actions, probably as a consequence of PKC inhibition (25). It is likely that these sphingosine influences persisted in rings during testing of contractile performance, because rings incubated only with sphingosine showed significantly diminished maximum contractions. This inhibition was not so great, however, that it masked the protection accorded by PKC inhibition during LPS exposure. While H7 and sphingosine are not specific PKC inhibitors, the compounds are chemically dissimilar and, with the exception of PKC, show different inhibitory targets. Therefore, it is unlikely that H7 and sphingosine would share the same nonspecific side effects, and the use of these two different agents supports a role for PKC in transducing the LPS signal into vascular suppression since both inhibitors protected vascular contractile function.

PDB was not as effective as LPS at inducing NOS activity. It is possible that PDB may not stimulate an appropriate isotype of PKC (26) as well as does the LPS-initiated mechanism or that PDB may downregulate PKC to such a large extent that it actually blocks transduction of the suppressive signal (Fig. 4). Alternatively PKC activation may be necessary but not sufficient to cause a LPS-like vascular suppression; i.e., a supplemental LPS-associated stimulus is required for full impairment of contractile function to occur. This type of relationship is exemplified in a study by Kawahara et al. (27) in which stimulation of VSM cells with both a phorbol and the calcium ionophore A23187 essentially doubled cellular *c-fos* mRNA levels in comparison to levels resulting from phorbol stimulation only. A recent study in aortic smooth muscle cells has also shown LPS-stimulated tyrosine kinase activity to be a likely candidate for such an additional stimulatory pathway (28). A possible requirement for multiple LPS-stimulated mediators to fully induce NOS activity in VSM can explain the apparent paradox that, while PKC is intimately involved in promoting contraction, under LPS stimulation PKC is also linked to the induction of NOS activity and subsequent vascular suppression. An alternative explanation for the different roles of PKC is that distinct PKC isozymes mediate contraction and LPS signals.

Control-incubated aortic rings expressed low levels of inducible NOS activity (Table 1). This NOS activity could be induced in response to trauma associated with ring preparation and incubation, to LPS contamination in the medium, or to combined stimuli. We have measured control medium LPS by Limulus amebocyte lysate chromogenic assay and found levels ranging from .1 to .4 ng/mL. It is possible that this quantity of LPS could induce a basal NOS activity. The variable increases

in control ring contractility that occurred after treatment with L-NAME, H7, and HA1004 may have resulted from inhibition of the basal NOS activity.

In conclusion, exposure of rat aortas to LPS results in translocation of PKC to vascular cell membranes, induction of NOS activity, and impaired vascular contraction. All of these responses are also provoked by directly activating PKC with PDB. PKC blockade protects against LPS-induced augmentation of NOS activity and contractile dysfunction. These observations implicate PKC as an intracellular mediator of LPS action in vascular smooth muscle cells. Because PKC also mediates vascular endothelial cell pro-inflammatory responses such as increased cell layer permeability, expression of adhesion molecules, and platelet-activating factor and cyclooxygenase synthesis (29–32), as well as VSM contractility defects, it may be possible to ameliorate several aspects of the vascular dysfunction characteristic of sepsis or endotoxemia by development of inhibitors specifically directed at PKC isotypes mediating the altered vascular functions.

ACKNOWLEDGMENTS

We thank Drs. Louis Homer and Michael Falk for review of the manuscript. The expert technical assistance of David Wood and John Fassoth is gratefully acknowledged.

This work was supported by Research Task 62233N MM33C30.001-1001. The opinions and assertions contained herein are the private ones of the authors and should not be construed as reflecting the views of the U.S. Navy, the naval service at large, or the Department of Defense.

REFERENCES

1. McKenna TM: Prolonged exposure of rat aorta to low levels of endotoxin *in vitro* results in impaired contractility. Association with vascular cytokine release. *J Clin Invest* 86:160–168, 1990.
2. Knowles RG, Salter M, Brooks SL, Moncada S: Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem Biophys Res Commun* 172:1042–1048, 1990.
3. Nakayama DK, Geller DA, Lowenstein CJ, Davies P, Pitt BR, Simmons RL, Billiar TR: Cytokines and lipopolysaccharide induce nitric oxide synthase in cultured rat pulmonary artery smooth muscle. *Am J Respir Cell Mol Biol* 7:471–476, 1992.
4. Gross SS, Levi R: Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J Biol Chem* 267:25722–25729, 1992.
5. Hattori Y, Gross SS: GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle: Characterization, sequence and relationship to nitric oxide synthase. *Biochem Biophys Res Commun* 195:435–441, 1993.
6. Singer HA, Schworer CM, Sweeley C, Benscoter H: Activation of protein kinase C isozymes by contractile stimuli in arterial smooth muscle. *Arch Biochem Biophys* 299:320–329, 1992.
7. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL: Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: Differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci USA* 89:11059–11063, 1992.
8. Khalil RA, Lajoie C, Resnick MS, Morgan KG: Ca(2+)-independent isoforms of protein kinase C differentially translocate in smooth muscle. *Am J Physiol* 263:C714–C719, 1992.
9. Pukac LA, Ottlinger ME, Karnovsky MJ: Heparin suppresses specific second messenger pathways for protooncogene expression in rat vascular smooth muscle cells. *J Biol Chem* 267:3707–3711, 1992.
10. Hortelano S, Genaro AM, Bosca L: Phorbol esters induce nitric oxide synthase and increase arginine influx in cultured peritoneal macrophages. *FEBS Lett* 320:135–139, 1993.
11. Bosca L, Diaz Guerra MJ: Activation of protein kinase C from B lymphocytes by lipid A. *Biochem Biophys Res Commun* 152:149–154, 1988.
12. Romano M, Hawiger J: Interaction of endotoxic lipid A and lipid X with purified human platelet protein kinase C. *J Biol Chem* 265:1765–1770, 1990.
13. Wightman PD, Raetz CR: The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. *J Biol Chem* 259:10048–10052, 1984.
14. Thomas TP, Gopalakrishna R, Anderson WB: Hormone- and tumor promoter-induced activation or membrane association of protein kinase C in intact cells. *Methods Enzymol* 141:399–411, 1987.
15. Bredt DS, Snyder SH: Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc Natl Acad Sci USA* 86:9030–9033, 1989.
16. Furchgott RF: Role of endothelium in responses of vascular smooth muscle. *Circ Res* 53:557–573, 1983.
17. Nelson DP, Homer LD: Non-linear regression analysis: a general program for data modeling using personal microcomputers. Report No. 83-5. San Diego, CA: Naval Health Research Center, 1983.
18. Sokal RR, Rohlf FJ: *Biometry*. San Francisco: W. H. Freeman, 1969, pp. 239–246.
19. Merrill AHJ, Nimkar S, Menaldino D, Hannun YA, Loomis C, Bell RM, Tyagi SR, Lambeth JD, Stevens VL, Hunter R, Liotta DC: Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C *in vitro* and for the cellular effects of these compounds. *Biochemistry* 28:3138–3145, 1989.
20. Hidaka H, Inagaki M, Kawamoto S, Sasaki Y: Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* 23:5036–5041, 1984.
21. Morrison DC, Lei MG, Chen TY, Flebbe LM, Halling J, Field S: Identification and characterization of mammalian cell membrane receptors for LPS-endotoxin. *Adv Exp Med Biol* 319:23–29, 1992.
22. Morrison DC: The case for specific lipopolysaccharide receptors expressed on mammalian cells. *Microb Pathog* 7:389–398, 1989.
23. Nishizuka Y: The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334:661–665, 1988.
24. Jefferson AB, Schulman H: Sphingosine inhibits calmodulin-dependent enzymes. *J Biol Chem* 263:15241–15244, 1988.
25. Stevens VL, Nimkar S, Jamison WC, Liotta DC, Merrill AHJ: Characteristics of the growth inhibition and cytotoxicity of long-chain (sphingoid) bases for Chinese hamster ovary cells: Evidence for an involvement of protein kinase C. *Biochim Biophys Acta* 1051:37–45, 1990.
26. Marquez C, Martinez C, Kroemer G, Bosca L: Protein kinase C isoenzymes display differential affinity for phorbol esters. Analysis of phorbol ester receptors in B cell differentiation. *J Immunol* 149:2560–2568, 1992.
27. Kawahara Y, Sunako M, Tsuda T, Fukuzaki H, Fukumoto Y, Takai Y: Angiotensin II induces expression of the c-*fos* gene through protein kinase C activation and calcium ion mobilization in cultured vascular smooth muscle cells. *Biochem Biophys Res Commun* 150:52–59, 1988.
28. Marczin N, Papapetropoulos A, Catravas JD: Tyrosine kinase inhibitors suppress endotoxin- and IL-1β-induced NO synthesis in aortic smooth muscle cells. *Am J Physiol* 265:H1014-H1018, 1993.
29. Stasek JE, Jr, Patterson CE, Garcia JG: Protein kinase C phosphorylates caldesmon77 and vimentin and enhances albumin permeability across cultured bovine pulmonary artery endothelial cell monolayers. *J Cell Physiol* 153:62–75, 1992.
30. Mattila P, Majuri ML, Mattila PS, Renkonen R: TNF α-induced expression of endothelial adhesion molecules, ICAM-1 and VCAM-1, is linked to protein kinase C activation. *Scand J Immunol* 36:159–165, 1992.
31. Heller R, Bussolino F, Ghigo D, Garbarino G, Schroder H, Pescarmona G, Till U, Bosia A: Protein kinase C and cyclic AMP modulate thrombin-induced platelet-activating factor synthesis in human endothelial cells. *Biochim Biophys Acta* 1093:55–64, 1991.
32. Maier JA, Ragnotti G: An oligomer targeted against protein kinase C alpha prevents interleukin-1 α induction of cyclooxygenase expression in human endothelial cells. *Exp Cell Res* 205:52–58, 1993.