Volume 320, number 2, 135–139 FEBS 12279 April 1993

Phorbol esters induce nitric oxide synthase and increase arginine influx in cultured peritoneal macrophages

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Received 22 January 1993; revised version received 17 February 1993

Incubation of peritoneal macrophages with β-phorbol 12,13-dibutyrate promotes a time-dependent release of NO to the incubation medium. This effect was antagonized by LPS, a well known inducer of nitric oxide synthase (NOS) expression in macrophages, and was inhibited by N^G-methyl-L-arginine and N^ω-nitro-L-arginine. An increase in intracellular cGMP and NOS activity was observed in parallel with NO release. The induction of NOS was accompanied by a stimulation of arginine influx within the cell. These results suggest that activation of protein kinase C by phorbol esters is sufficient to promote NOS induction in macrophages.

Nitric oxide; Phorbol ester; Protein kinase C; Macrophage

1. INTRODUCTION

Nitric oxide has emerged as an important intra- and intercellular regulatory molecule with functions as diverse as vasodilatation, neural communication or host defense [1–3]. Nitric oxide synthase is the enzyme involved in the production of nitric oxide from molecular O_2 and arginine, and appears to be a growing family of isoenzymes [1,2]. At least three different isoforms have been identified in mammalian tissues: neural and endothelial cells contain two different, although related, constitutive isoenzymes that require Ca^{2+} and calmodulin to be active, whereas the isoenzyme expressed in macrophages after stimulation with cytokines and endotoxins does not require these cofactors [2,3].

The expression of the Ca^{2+}-independent cytokine-inducible NOS has been described in various cell types, in addition to macrophages [4–6]. In hepatocytes, a form of NOS induced by phorbol esters has been reported [7], although the correspondence of this NOS with the macrophage isoenzyme remains to be established in view of the calmodulin requirement described for the hepatic NOS induced in response to LPS [8]. Macrophage NOS is by far the best characterized isoenzyme among the cytokine-inducible forms, both from a biological and chemical point of view, and the presence of a complex regulatory mechanism in the control of NOS expression by cytokines and endotoxins has been shown [9,10]. Therefore we decided to investigate whether activation of macrophage PKC with phorbol esters or with Ca^{2+} and 1,2-diacylglycerol promotes the expression of NOS.

In this work we show that PKC activation by phorbol esters is sufficient to promote the expression of inducible NOS in macrophages and elicits an increase in the arginine influx to the cell. A clear antagonism exists between phorbol esters and LPS in promoting NOS expression.

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Abbreviations: 1,2-DOG, *sn*-1,2-dioctanoylglycerol; EGF, epidermal growth factor; β-TGF, transforming growth factor β; INF-γ, interferon-γ; D-NMA, N^G-methyl-D-arginine; L-NMA, N^G-methyl-L-arginine; L-NNA, N^ω-nitro-L-arginine; LPS, lipopolysaccharide; NOS, nitric oxide synthase; PDBu, β-phorbol 12,13-dibutyrate; α-PDD, α-phorbol 12,13-didecanoate; PMA, β-phorbol 12-myristate, 13-acetate.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[U-^{14}C]arginine and a cGMP assay kit were from Amersham. 2′,5′-ADP-Sepharose was from Pharmacia. LPS was from Difco, and Dowex AG50W-X8 was from Bio-Rad. Nitrate reductase from *Aspergillus* and other enzymes were from Boehringer Mannheim. (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4) was from Dr. B. Schircks Laboratories (Jona, Switzerland). Arginine derivatives and other chemicals and biochemicals were from Sigma or Merck.

2.2. *Cell cultures*

Peritoneal macrophages were prepared following previous protocols [11]. Briefly, anesthetized 2-month-old male rats were injected intraperitoneally with 50 ml of sterile PBS and after 10 min the ascitic liquid was removed. Rats suffering hemorrhaging were discarded. The ascitic fluid was centrifuged (10 min; $200 \times g$) and the cell pellet washed twice with PBS. Cells were seeded at 10^6/cm^2 in RPMI 1640 supplemented with 10% of heat-inactivated FCS, and adherent cells were maintained overnight in this medium. Experiments were carried out in Phenol red-free DMEM medium supplemented with 1 mM arginine and 10% heat-inactivated FCS.

2.3. *NO assay*

Release of NO by the cell culture was determined by the accumulation of nitrite and nitrate (total NO) as follows: 200 μl of culture medium were incubated with 0.5 U of nitrate reductase (Boehringer) in the presence of 50 μM NADPH and 5 μM FAD [12]. Excess NADPH, which interferes with the chemical determination of nitrite, was oxidized with 0.2 mM pyruvate and 1 μg of lactate dehydrogenase. Nitrite was determined with Greiss reagent [13] by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration). After incubation for 5 min the tubes were centrifuged in an Eppendorf centrifuge and 150 μl of supernatant were transferred to a 96-well microplate. After a first reading of the absorbance at 548 nm, 50 μl of naphthylenediamine (1 mM in the assay) were added. The reaction was completed after 15 min of incubation and the absorbance at 548 nm was compared with a standard of $NaNO_2$. The absorbance corresponding at 0 time was subtracted. Solutions of NO were prepared by bubbling the gas through a helium-treated phosphate-buffered saline (PBS) solution. The NO concentration in the medium was determined with Greiss reagent.

2.4. *NOS activity determination*

NOS activity was followed by the release of [U-^{14}C]citrulline from [U-^{14}C]arginine in the presence of 1 μM BH_4 and 0.5 mM NADPH. Cell pellets were homogenized as previously described and the enzyme activity was measured after partial purification by a 2′,5′-ADP-Sepharose chromatography column as described [7,14]. One unit of NOS was defined as producing 1 nmol of NO per minute.

2.5. *cGMP determination*

To measure the intracellular concentration of cGMP the cell layer was washed with 1 ml of ice-cold PBS, followed by the addition of 0.5 ml of a mixture of ethanol/water (2:1). After freezing and thawing to favor cell disruption, the cells were homogenized, centrifuged in an Eppendorf centrifuge for 10 min and the resulting supernatants were speed-vacuum dried and resuspended in the buffer recommended by the supplier of the cGMP assay kit (Amersham). The protein concentration was measured in the pellet after ethanolic precipitation.

2.6. *Measurement of arginine influx*

Arginine influx in macrophages was measured after washing the cell layer twice with PBS at 37°C followed by incubation of the macrophages with [U-^{14}C]arginine (100 mCi/mol) for 30 and 60 s. The dishes were rapidly washed twice with 1 ml of ice-cold PBS and the radioactivity incorporated was measured. Linearity was observed at least during the first 30 s of incubation.

2.7. *Protein assay*

Protein was assayed according to Bradford [15] using bovine serum albumin as a standard.

3. RESULTS

3.1. *PDBu induces NO release in cultured peritoneal macrophages*

Primary cultures of adherent macrophages were challenged with 100 nM PDBu and NO release was measured at various times by the method of Greiss (nitrite plus nitrate). As Fig. 1A shows, the half-maximal effect was observed after 4 h of exposure of the culture to PDBu. The production of NO by cells incubated with LPS was lower than after exposure to phorbol esters (53% after 4 h of incubation). Fig. 1B shows the dose dependence for NO release after stimulation with PDBu, and the absence of effect when macrophages were incubated with the biologically inactive phorbol ester isomer 4-α-PDD. The release of NO elicited by PDBu fits with the range of concentration required to activate PKC by phorbol esters, and taken together these results suggest that PKC activation is involved and may be sufficient to induce NO release by cultured macrophages.

Macrophages release NO after stimulation with cytokines such as LPS or INF-γ, and a synergism has been described for the effect of these cytokines on NOS in-

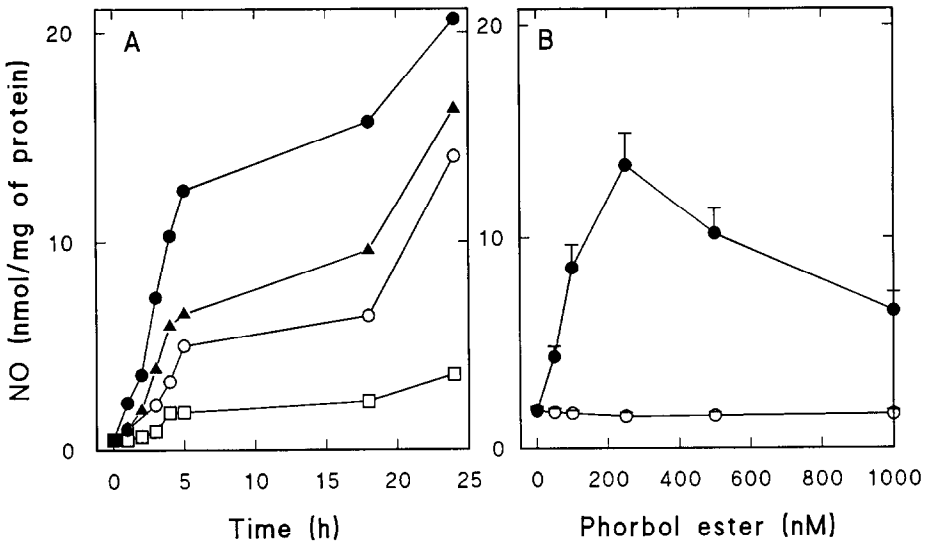

Fig. 1. NO release by cultured peritoneal macrophages incubated with phorbol esters and LPS. Macrophages (7×10^5) were incubated for 18 h in RPMI 1640 medium containing 10% heat-inactivated FCS and after extensive washing with PBS, the medium was replaced by phenol red-free DMEM medium containing 1 mM arginine and NO was measured using the Greiss reagent. Cells were challenged without (□) or with 100 nM PDBu (●), 50 μg/ml LPS (▲) or 100 nM PDBu plus 50 μg/ml of LPS (○) (panel A). The dose-dependent effect of PDBu (●) and α-PDD (○) were assayed after 4 h of incubation (panel B). Results show a representative experiment (panel A) or the means ± S.E.M. of 4 independent cell preparations, assayed per duplicate (panel B).

Table I

NOS induction by phorbol esters

Cultured macrophages (10^7 cells) were incubated with the indicated substances for 4 h. At the end of the incubation period the cell layers were homogenized and NOS was partially purified by 2',5'-ADP-Sepharose chromatography. The enzyme activity was followed by the release of [U-^{14}C]citrulline. The intracellular concentration of cGMP was measured using a commercial assay kit. Results are the mean of two (NOS) or the mean ± S.E.M. of three (cGMP) experiments.

Ligand	NOS (units/mg protein)	cGMP (pmol/mg protein)
None	6.3	1.3 ± 0.2
PDBu, 100 nM	43.9	5.9 ± 0.4
LPS, 50 μg/ml	14.7	2.4 ± 0.2
PDBu + LPS	ND	2.1 ± 0.3
PDBu + L-NNA, 100 μM	0.8	1.3 ± 0.2
NO, 3 μM	ND	9.3 ± 1.2

ND, not determined.

duction and NO release [9,10]. In contrast, a clear antagonism between PDBu and LPS in NO release was evidenced in macrophages (Fig. 1A). This response was absent in cells incubated with LPS and α-PDD (not shown).

3.2. *PKC activation is involved in NOS induction*

The release of NO by macrophages elicited by PDBu correlates well with the expression of NOS activity (Table I) as suggested both by the time-course profile of NO accumulation (Fig. 1A), and by the fact that NO release was inhibited in cells treated with cycloheximide (Table II), indicating that the integrity of the protein synthesis machinery was necessary for the effect to be observed. Moreover, the presence in the incubation medium of the NOS inhibitor L-NMA, but not the inactive isomer D-NMA, efficiently blocked the NO release by the cells (Table II).

To further analyze the mechanism by which PDBu promotes NO release in cultured macrophages, the effect of some putative PKC activity modulators was assayed. As Table II shows, the incubation of the macrophages with the PKC inhibitors calphostine C (81% inhibition) and the isoquinoline H-7 (60% inhibition) abolishes the effect of PDBu on NO release. However, such is not the case for staurosporine, also a well known inhibitor of PKC, that only inhibits 45% of the PDBu effect. The biochemical basis for these different responses to the PKC inhibitors are unknown but may be related both to the fate of PKC after prolonged incubation of the macrophages with phorbol esters (proteolysis), and to a differential substrate-specificity for the PKC inhibitors used, in the sense described in an earlier work [16].

The observation that β-TGF, insulin and INF-γ block the stimulatory effect of PBDu is also interesting. Conversely, incubation with EGF even potentiates the action of PDBu (Table II), suggesting that a complex modulation of NOS expression exists between the cytokines.

3.3. *PKC activators increase cGMP in macrophages*

One of the intracellular targets of NO is the activation of soluble guanylate cyclase [17], and accordingly, the intracellular concentration of cGMP will increase in macrophages stimulated with phorbol esters or cytokines. As Table I shows, cGMP increased in cells stimulated with either PDBu (4.5-fold increase) or LPS (1.8-fold increase), in agreement with their effects on the NO concentration. However, when both PDBu and LPS are simultaneously added only a 1.6-fold increase in cGMP is observed. Moreover, in the presence of the NOS inhibitor L-NNA, PDBu fails to produce changes in cGMP, which suggests that NO release is required to promote guanylate cyclase activation. The changes in cGMP produced by PDBu are in the range of those reached in cells incubated with a solution of NO (3 μM).

3.4. *Arginine transport is stimulated by phorbol esters and cytokines*

Since macrophage NOS appears not to be regulated by enzyme effectors as occurs for the Ca^{2+}/calmodulin-dependent constitutive isoenzyme of neural and endothelial tissues, the arginine influx to the cell was studied as a possible relevant factor in the control of enzyme activity. As shown in Fig. 2A, at arginine concentrations in the low micromolar range, similar to those prevailing in plasma, treatment of the cells with LPS or PDBu increases arginine transport by 5.0- and 5.7-fold,

Table II

Modulation of NO release in cultured peritoneal macrophages

Macrophages (7×10^5) were incubated for 4 h with the indicated cytokines and substances and NO production was measured using the Greiss reagent. Results are the means ± S.E.M. of three independent cell preparations.

Ligand(s)	NO release (nmol/mg protein)		
	None	PDBu (100 nM)	LPS (50 μg/ml)
None	1.6 ± 0.2	12.2 ± 1.7	6.5 ± 0.8
Cycloheximide, 1 μM	1.7 ± 0.2	1.6 ± 0.1	1.5 ± 0.1
Staurosporine, 500 nM	1.6 ± 0.1	7.4 ± 0.4	4.9 ± 0.6
Calphostine C, 500 nM	1.9 ± 0.3	3.9 ± 0.5	ND
H-7, 10 μM	ND	5.8 ± 0.3	ND
1,2-DOG, 2 μg/ml	6.3 ± 0.5	17.0 ± 0.3	2.7 ± 0.1
L-NMA, 100 μM	1.6 ± 0.3	1.5 ± 0.5	1.4 ± 0.3
D-NMA, 250 μM	1.7 ± 0.2	9.9 ± 0.6	5.9 ± 0.4
β-TGF, 0.1 ng/ml	3.3 ± 0.2	1.7 ± 0.1	ND
EGF, 100 ng/ml	10.0 ± 0.5	19.8 ± 1.1	6.0 ± 1.0
Insulin, 20 nM	2.0 ± 0.1	4.6 ± 0.4	ND
IFN-γ	2.4 ± 0.3	3.8 ± 0.5	4.3 ± 0.2

ND, not determined.

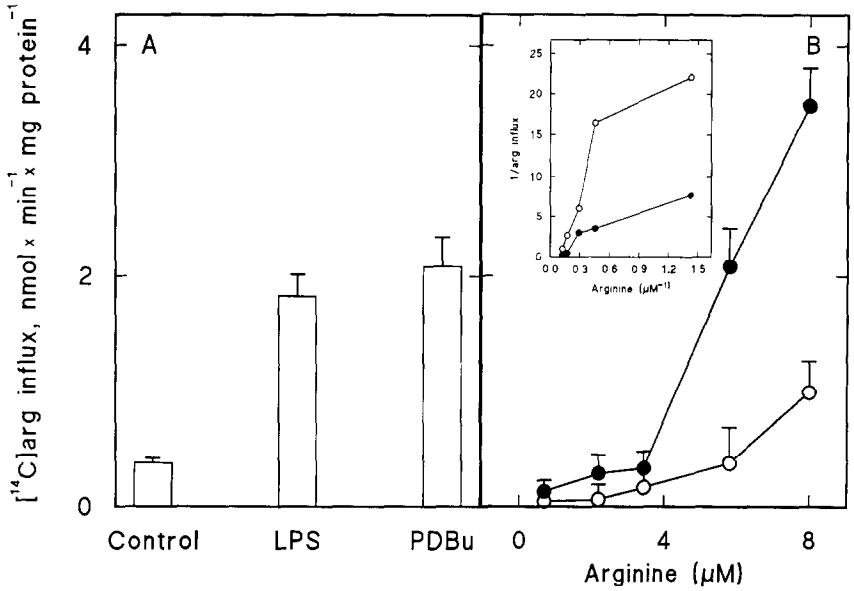

Fig. 2. Effect of PDBu on arginine influx. Macrophages (2×10^6) were incubated with 100 nM PDBu or 50 μg/ml of LPS for 4 h and the transport of [U-^{14}C]arginine (5 μM) was measured (panel A). The rate of arginine influx was calculated by the measurement of the radioactivity incorporated at 30 and 60 s. The dose-dependent influx was assayed in unstimulated (○) or 4-h treated cells with 100 nM PDBu (●) (panel B). The inset shows the Lineweaver–Burk plot for the arginine uptake corresponding to the data given in panel B. Results are means ± S.E.M. of 3 independent determinations.

respectively. The Lineweaver–Burk plot for arginine influx (inset) shows a complex behavior that may be interpreted either by the presence of two transport systems or as a process with negative cooperativity [18]. The observation that both LPS and PDBu stimulate arginine influx suggests the existence of common pathways in NOS expression and regulation (via substrate availability) by both factors.

4. DISCUSSION

Expression of the calcium-independent NOS in macrophages by cytokines such as INF-γ, α-TNF, LPS or by bacterial endotoxins is probably the best characterized system for the study of the expression of the inducible isoenzyme [2,3,9,10]. However, the intracellular signals involved in the control of the expression of this NOS isoenzyme are unknown. Our results provide an additional mechanism to induce NOS in macrophages after activation of PKC by phorbol esters, and extend our initial observation in cultured hepatocytes [7], supporting the view that NOS induction by phorbol esters might be a middle-term (hours) response to PKC activation in different cell systems. This involvement of PKC as the initial event in the expression of NOS in macrophages, and in the absence of other cooperative signals (i.e. calcium mobilization), is supported by the fact that this kinase is the biological receptor of phorbol esters in non-neural tissues, in which the amount of *n*-chimaerine, the other known phorbol ester receptor, is negligible [19]. Besides, the possibility of the involvement of PKC in the mechanism of NOS induction triggered by some cytokines cannot be excluded as suggested by the effect of INF-γ [20].

The molecular basis for the antagonism between phorbol esters and LPS in promoting NOS induction deserves further study. In LPS-activated macrophages, it has been shown that PKC stimulation is an early target as result of the release of metabolites (i.e. arachidonic acid) following exposure of the cells either to LPS or its derivatives, the biologically active moieties lipid A and lipid X [21,22]. Indeed, both lipids A and X activate PKC in vitro [22,23]. However, it is possible that the PKC subspecies activated by LPS through its intracellular active metabolites, might be different from those elicited by phorbol esters, which, besides promoting PKC activation also trigger the proteolytic degradation of the kinase, and one of its fragments exhibits an unregulated protein kinase catalytic activity (also termed PKM), which in turn may initiate an additional phosphorylation cascade [16,24]. Alternatively, phorbol esters may alter the transmembrane signaling pathway of LPS, causing a blockage of the biological response to this molecule, for example the inhibition by phorbol esters of the differentiation and clonal expansion of LPS-activated B-cells [25].

The antagonism between PDBu and LPS on NOS induction may also be interpreted in the light of the cross-regulatory effects observed among different cytokines. Incubation of LPS-activated peritoneal neutrophils with IL-8 blocks both the release of NO and NOS induction at the transcriptional level [26], and the same

antagonistic response is observed between β-TGF and IFN-γ in the NO release in macrophages [27]. Therefore, because the enzymatic activity of NOS is not regulated at the post-transcriptional level, it seems that a complex regulation of the control of NOS expression by cytokines operates.

Finally, the assessment of sustained PKC activation as a self-sufficient signal to elicit NO release from macrophages, and the characterization of some of the transcriptional factors stimulated after PKC activation (AP-1 transcriptional complex), may help to unravel the mechanism involved in the expression of inducible NOS.

Acknowledgements: We are indebted to E. Lundin for his help in the preparation of the manuscript and to Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) for the financial support to A.M.G. This work was funded by grants from Comunidad de Madrid (C183/91) and from CICYT (PB91-134), Spain.

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