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Pyrrolidine dithiocarbamate inhibits immunostimulant-induced tetrahydrobiopterin synthesis in rat vascular smooth muscle

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Abstract

GTP cyclohydrolase I is the first and rate-limiting enzyme in the synthesis of tetrahydrobiopterin, a cofactor of nitric oxide (NO) synthase. Immunostimulants increase NO and tetrahydrobiopterin synthesis in vascular smooth muscle cells by coinducing NO synthase and GTP cyclohydrolase I gene expression. Given that nuclear factor κB mediates the induction of NO synthase gene expression by lipopolysaccharide (LPS), the role of nuclear factor κB in the induction of GTP cyclohydrolase I in LPS-stimulated rat vascular smooth muscle cells was assessed by examining the effects of pyrrolidine dithiocarbamate, an inhibitor of the activation of nuclear factor κB, on the abundance of GTP cyclohydrolase I mRNA and biopterin synthesis. Pyrrolidine dithiocarbamate inhibited both NO and biopterin synthesis induced by LPS in a dose-dependent manner with similar half-maximal inhibitory concentrations, 12 μM for NO and 17 μM for biopterin, respectively. At a concentration of 25 μM, which inhibited NO and biopterin synthesis but caused no cytotoxicity, pyrrolidine dithiocarbamate substantially reduced the LPS-induced increase in the abundance of NO synthase and GTP cyclohydrolase I mRNAs. These results suggest that pyrrolidine dithiocarbamate inhibits LPS-induced NO and biopterin synthesis by inhibiting the expression of NO synthase and GTP cyclohydrolase I genes. Thus, the induction of both genes necessary for cellular NO synthesis in vascular smooth muscle appears to be regulated, at least in part, by a common mechanism: nuclear factor κB activation.

Keywords: Nuclear factor κB; Tetrahydrobiopterin; GTP cyclohydrolase I; Nitric oxide (NO); Smooth muscle cell, vascular; (Rat)

1. Introduction

Tetrahydrobiopterin is an essential cofactor of all isoforms of nitric oxide (NO) synthase (Nathan, 1992). Bacterial lipopolysaccharide (LPS) and other immunostimulants induce an isoform of NO synthase in vascular smooth muscle (Busse and Mulsch, 1991; Gross and Levi, 1992). The induction of the NO synthase and the overproduction of NO in vascular smooth muscle have been implicated in the genesis of septic and cytokine-induced circulatory shock (Thiemermann and Vane, 1990; Kilbourn and Griffith, 1992). Whereas the induction of NO synthase is necessary for immunostimulant-mediated NO overproduction, de novo tetrahydrobiopterin synthesis is also elicited in vascular smooth muscle by immunostimulants and is essential for the NO synthase activity (Gross and Levi, 1992). We recently showed that immunostimulants coinduce the expression of NO synthase and GTP cyclohydrolase I genes in vascular smooth muscle; GTP cyclohydrolase I is the enzyme that is normally rate-limiting for the synthesis of tetrahydrobiopterin (Hattori and Gross, 1993). Because both events are necessary for the induction of NO synthesis in vascular smooth muscle, we questioned whether a common mechanism underlies the coinduction of NO synthase and GTP cyclohydrolase I gene expression by immunostimulants. The control of transcription in response to immunostimulants is mediated by several ubiquitous transcription factors in many different cellular systems. Prominent among these factors is nuclear factor κB, which has been suggested to mediate induction of NO synthase (Sherman et al., 1993; Mulsch et al., 1993; Eberhardt et al.,

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1994; Xie et al., 1994). We have now examined whether nuclear factor κB also participates in the induction of GTP cyclohydrolase I in LPS-stimulated vascular smooth muscle by assessing the effects of pyrrolidine dithiocarbamate, an inhibitor of nuclear factor κB activation (Schreck et al., 1992), on GTP cyclohydrolase I mRNA abundance and biopterin synthesis.

2. Materials and methods

2.1. Cell culture

Vascular smooth muscle cells were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats as previously described (Gross and Levi, 1992). Cells from the 10th to 15th passage were seeded onto 96-well plates for nitrite assay or onto 10-cm dishes for biopterin measurement and RNA preparation.

2.2. Analysis of NO synthase and GTP cyclohydrolase I mRNA abundance

RNA was extracted from vascular smooth muscle cells by a modified guanidinium isothiocyanate method (RNAzol; Cinna/Biotecx, Houston, TX, USA). Reverse transcription-polymerase chain reaction analysis was performed as previously described (Hattori and Gross, 1993). Briefly, first-strand cDNA was synthesized from random primers with murine Moloney leukemia virus reverse transcriptase (Promega, Madison, WI, USA) and was then amplified by polymerase chain reaction with synthetic gene-specific primers for rat GTP cyclohydrolase I (Hatakeyama et al., 1991) or mouse inducible isoform of NO synthase (Lyons et al., 1992). Primers used were: NO synthase forward primer, 5′-CTGCAGGTCTTTGACGCTCGG-3′; NO synthase reverse primer, 5′-GTGGAACACAGGGGTGATG-CT-3′; GTP cyclohydrolase I forward primer, 5′-GGA-TACCAGGAGACCATCTCA-3′; and GTP cyclohydrolase I reverse primer, 5′-TAGCATGGTGCTAGT-GACAGT-3′. PCR amplification was performed with a kit (Perkin Elmer Cetus, Norwalk, CT, USA) for 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. To ensure that equal amounts of reverse-transcribed RNA were added to the polymerase chain reaction, we subjected glyceraldehyde-3-phosphate dehydrogenase cDNA to amplification in parallel as a reference, with primers described (Terada et al., 1992). Polymerase chain reaction products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and were visualized by ultraviolet light-induced fluorescence. The polymerase chain reactions resulted in a single product of the predicted size for NO synthase (807 bp) and GTP cyclohydrolase I (372 bp). The identity of polymerase chain reaction products as those corresponding to rat inducible NO synthase (Nunokawa et al., 1993) and rat GTP cyclohydrolase I (Hatakeyama et al., 1991) was confirmed by subcloning and sequencing as previously described (Hattori and Gross, 1993).

2.3. Nitrite assay

Nitrite production, an indicator of NO synthesis, was measured in the vascular smooth muscle cell culture medium (Gross and Levi, 1992). Nitrite was quantified colorimetrically after adding 100 μl of Griess regent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 μl samples. Absorbance at 550 nm was determined with a microplate reader (Molecular Devices, Richmond, CA, USA). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

2.4. Cell respiration

Cell respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mosmann, 1983). Cells in 96-well plates were incubated at 37°C with MTT (0.4 mg/ml) for an additional 60 min following the 24 h incubation for nitrite determination. The culture medium was removed by aspiration and the cells were solubilized in 100 μl of dimethylsulfoxide. The extent of reduction of MTT to formazan within cells was quantitated by measurement of absorbance at 550 nm. Formazan production was expressed as a percentage of the values obtained from control cells.

2.5. Biopterin and neopterin assay

Biopterin (tetrahydrobiopterin and more oxidized species) and neopterin were measured essentially as described by Fukushima and Nixon (Fukushima and Nixon, 1980). Culture medium and cells were treated separately with 0.2 M perchloric acid and oxidized by exposure to 0.2 M perchloric acid containing 0.2% I_2 and 0.4% KI for 1 h at room temperature in the dark. Ascorbate (2%) was added to remove residual free I_2 and the mixture was centrifuged for 10 min at 10000 x *g*. Biopterin and neopterin in the supernatant were quantitated by C_{18} reversed-phase high-performance liquid chromatography with fluorescence detection, with authentic biopterin or neopterin used as standards.

2.6. Statistical evaluation

Values are expressed as means ± S.E.M. of three observations. Student's unpaired *t*-tests were used to assess the statistical significance of differences. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of pyrrolidine dithiocarbamate on the synthesis of NO

To assess the effects of pyrrolidine dithiocarbamate on NO synthase induction, we incubated vascular smooth muscle cells with a combination of LPS (30 μg/ml) and interferon-γ (50 ng/ml) in the presence of pyrrolidine dithiocarbamate (2.5–100 μM). After 24 h, the accumulation of nitrite, a stable oxidation product of the unstable free radical NO, was determined as a measure of NO synthesis. Induction of NO synthesis by LPS/interferon-γ was inhibited in a dose-dependent manner by pyrrolidine dithiocarbamate at concentrations up to 25 μM with a half-maximal inhibitory concentration of ~ 12 μM; at concentrations of > 25 μM, pyrrolidine dithiocarbamate completely inhibited NO production (Fig. 1). In parallel with the nitrite assay, the cytotoxic effect of pyrrolidine dithiocarbamate on LPS/interferon-γ-treated cells was assessed by the mitochondria-dependent reduction of MTT to formazan. Cell respiration was significantly inhibited

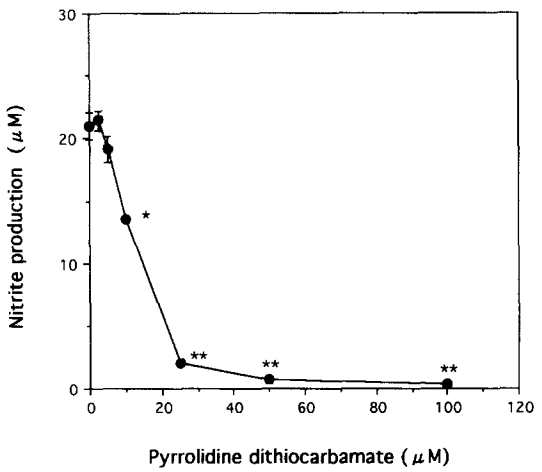

Fig. 1. Effect of pyrrolidine dithiocarbamate on nitrite production in LPS/interferon-γ-stimulated rat aortic vascular smooth muscle cells. Cells were treated with a combination of LPS (30 μg/ml) and interferon-γ (50 ng/ml) in the presence of various concentrations of pyrrolidine dithiocarbamate for 24 h, after which nitrite accumulation in the culture medium was measured. Data are means ± S.E.M. ($n = 9$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus LPS/interferon-γ in the absence of pyrrolidine dithiocarbamate. Nitrite was undetected in the medium from unstimulated cells.

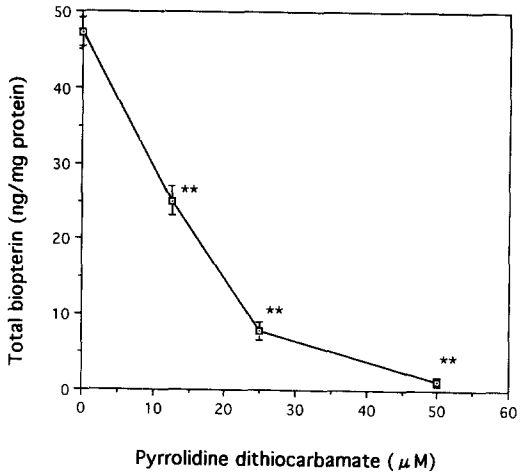

Fig. 2. Effect of pyrrolidine dithiocarbamate on biopterin synthesis in LPS/interferon-γ-stimulated rat aortic vascular smooth muscle cells. Cells were treated with a combination of LPS (30 μg/ml) and interferon-γ (50 ng/ml) in the presence of various concentrations of pyrrolidine dithiocarbamate for 24 h, after which total biopterin (cellular contents plus accumulation in supernatants) was measured. Data are means ± S.E.M. ($n = 9$), expressed as total biopterin per mg cell protein. For calculation of biopterin in cell supernatants, background biopterin present in fetal bovine serum was subtracted. $^{**}P < 0.01$ versus LPS/interferon-γ in the absence of pyrrolidine dithiocarbamate.

by pyrrolidine dithiocarbamate at a concentration of 100 μM (data not shown).

3.2. Effect of pyrrolidine dithiocarbamate on the synthesis of biopterin and neopterin

Total (cellular plus extracellular) biopterin synthesis was markedly induced in vascular smooth muscle cells by incubation with LPS/interferon-γ for 24 h and this increase was inhibited by pyrrolidine dithiocarbamate in a dose-dependent manner with a half-maximal inhibitory concentration of ~ 17 μM (Fig. 2). Table 1 shows cellular biopterin contents and the ratio of cellular to total biopterin. Up to 94% of the newly synthesized biopterin was released into the culture medium

Table 1
Effect of pyrrolidine dithiocarbamate on cellular biopterin content and ratio of cellular to total biopterin 24 h after LPS/interferon-γ

Treatment	Cellular biopterin (ng/mg protein)	Cellular/total biopterin (%)
Basal	0.16 ± 0.03	1.46
LPS/IFN	1.88 ± 0.14	6.36
LPS/IFN + PDTC 25 μM	1.34 ± 0.09	11.84
LPS/IFN + PDTC 50 μM	1.74 ± 0.19	12.98

PDTC = pyrrolidine dithiocarbamate. Cells were treated with a combination of LPS (30 μg/ml) and interferon-γ (50 ng/ml) in the absence and presence of pyrrolidine dithiocarbamate for 24 h. Cellular biopterin contents and the ratio of cellular to total biopterin are shown. Data are means ± S.E.M. ($n = 9$).

in LPS/interferon-γ-treated cells (see Table 1); changes in intracellular biopterin concentrations therefore appeared relatively small. On the other hand, neopterin, an oxidized intermediate in the de novo biosynthesis of tetrahydrobiopterin, was at very low level both in the cellular extract and in the cell culture medium, and remained at low level even after stimulation of the cells with LPS/interferon-γ (data not shown).

3.3. Effect of pyrrolidine dithiocarbamate on the abundance of GTP cyclohydrolase I and NO synthase mRNA

To evaluate the basis for the inhibition by pyrrolidine dithiocarbamate of induction of tetrahydrobiopterin and NO synthesis, we investigated the effect of pyrrolidine dithiocarbamate on the LPS-induced increase in GTP cyclohydrolase I and NO synthase mRNA abundance by reverse transcription-polymerase chain reaction. Whereas the abundance of GTP cyclohydrolase I and NO synthase mRNA is low in untreated vascular smooth muscle cells, LPS/interferon-γ markedly increased the amounts of both transcripts after 8 h (Fig. 3). Pyrrolidine dithiocarbamate (25 μM) markedly inhibited the LPS/interferon-γ-induced increase in both GTP cyclohydrolase I and NO synthase mRNA. Control polymerase chain reaction experiments demonstrated equivalent amounts of glyceraldehyde-3-phosphate dehydrogenase mRNA in all samples.

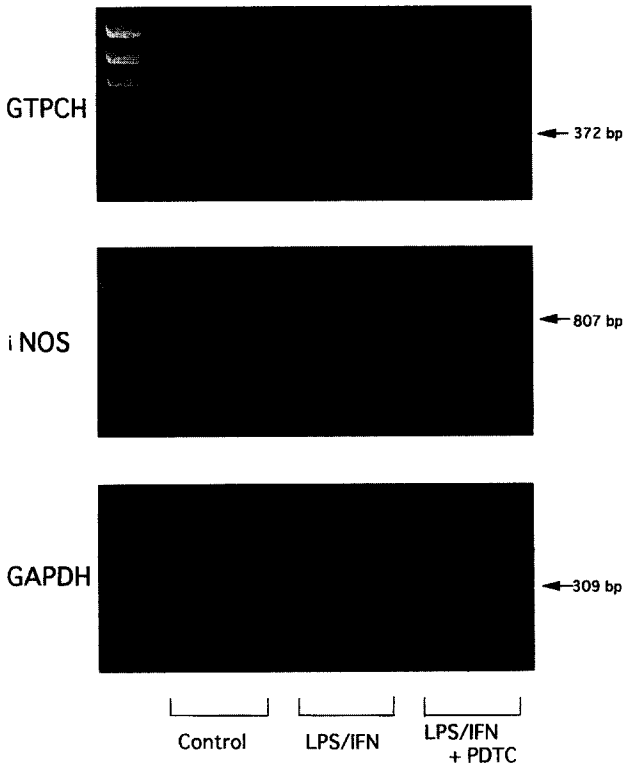

Fig. 3. Effect of pyrrolidine dithiocarbamate on the LPS/interferon-γ-induced increase in GTP cyclohydrolase I and NO synthase mRNA abundance. Rat aortic vascular smooth muscle cells were incubated, in triplicate, for 8 h in the absence (control) or presence of either a combination of LPS (30 μg/ml) and interferon-γ (50 ng/ml) alone (LPS/IFN) or LPS/IFN plus 25 μM pyrrolidine dithiocarbamate (PDTC). Cells were harvested, and RNA was prepared and assayed by reverse transcription-polymerase chain reaction with gene-specific primers. Results with primers specific for glyceraldehyde-3-phosphate dehydrogenase are shown for comparison. DNA size markers in the left lanes correspond to: 2000, 1200, 800, 400, 200, and 100 bp. GTPCH: GTP cyclohydrolase I, iNOS: inducible NO synthase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Arrows indicate the predicted size of polymerase chain reaction products.

4. Discussion

Nuclear factor κB appears to be involved in LPS signaling (Schreck and Baeuerle, 1991). We previously observed that a nuclear protein from LPS-stimulated rat vascular smooth muscle cells bound to a nuclear factor κB oligonucleotide probe, and that the binding was apparant within 30 min after stimulation with LPS and remained at high levels for at least 24 h (Gross, unpublished data). These observations suggest that nuclear factor κB plays a role in the control of transcription in response to LPS in rat vascular smooth muscle. We have shown that pyrrolidine dithiocarbamate, an inhibitor of the nuclear factor κB activation, at noncytotoxic concentrations inhibits GTP cyclohydrolase I gene expression and tetrahydrobiopterin synthesis as well as NO synthase gene expression and NO synthesis in LPS-stimulated rat vascular smooth muscle cells. This inhibition is unlikely due to a direct effect of pyrrolidine dithiocarbamate on the assays of nitrite and biopterin because: (1) the standards of these assays were unaffected by the presence of pyrrolidine dithiocarbamate, (2) when pyrrolidine dithiocarbamate was added at increasing intervals after the stimulation of vascular smooth muscle cells with LPS, the inhibition of production of nitrite and biopterin significantly decreased as the interval lengthened. These observations also suggest that pyrrolidine dithiocarbamate inhibits the induction of NO synthase and GTP cyclohydrolase I rather than their catalytic activity. Moreover, another inhibitor of the activation of nucleasr factor kB, pentamethyl-hydroxychromane which is a vitamin E derivative (Suzuki and Packer, 1993) and shown to be an inhibitor of NO synthase induction (Hattori et al., 1995a), also inhibited GTP cyclohydrolase I gene expression and tetrahydrobiopterin synthesis in LPS-stimulated rat vascular smooth muscle cells (data not shown).

Nuclear factor κB has been suggested to mediate NO synthase induction in LPS-activated macrophages (Sherman et al., 1993; Mulsch et al., 1993) and inter-

leukin-1-activated mesangial cells (Eberhardt et al., 1994), on the basis of studies with inhibitors of nuclear factor κB. Furthermore, two nuclear factor κB binding sites have been identified in the promotor region of the inducible NO synthase gene and have been shown to be required for inducibility by LPS in mouse macrophages (Xie et al., 1993, 1994). However, a role for nuclear factor κB has not previously been demonstrated in the induction of GTP cyclohydrolase I gene expression or synthesis of tetrahydrobiopterin, an essential cofactor of all isoforms of NO synthases. We previously showed using inhibitors for tetrahydrobiopterin synthesis that increased tetrahydrobiopterin synthesis is depending on increased GTP cyclohydrolase I activity in rat vascular smooth muscle cells (Gross and Levi, 1992). It has also been shown in LPS-treated rats that increased activity of GTP cyclohydrolase I parallels increased tetrahydrobiopterin levels in various tissues (Werner-Felmayer et al., 1993). In some cases, neopterin could be an indicator for GTP cyclohydrolase I activity since it is cleaved from the first intermediate in the de novo biosynthesis of tetrahydrobiopterin, i.e. 7,8-dihydroneopterin triphosphate. However, GTP cyclohydrolase activity in LPS-activated rat vascular smooth muscle cells is not reflected by this oxidised intermediate because both intracellular and extracelluar levels of neopterin were very low, consistent with the finding that GTP cyclohydrolase I activity is lower than the subsequent 6-pyruvoyltetrahydropterin synthase activity in the tetrahydrobiopterin synthetic pathway in rodent cells (Werner et al., 1991), unlike human macrophages. Thus, pyrrolidine dithiocarbamate appears to inhibit tetrahydrobiopterin synthesis largely through inhibition of GTP cyclohydrolase I induction by preventing GTP cyclohydrolase I gene expression.

We previously showed that immunostimulant-evoked tetrahydrobioptein synthesis occurs with a time course that parallels that for coinduced NO synthesis and is preceded by an increase in GTP cyclohydrolase I mRNA (Gross and Levi, 1992; Hattori and Gross, 1993). In addition, we very recently reported that LPS treatment in vivo induces GTP cyclohydrolase I mRNA in the rat tissues (Hattori et al., 1995b). The intracellular concentration of tetrahydrobiopterin appears to be rate-limiting for NO synthesis. Indeed, a significant up-regulation of NO synthesis in vascular smooth muscle is observed with administration of excess tetrahydrobiopterin, and NO synthesis can be prevented by inhibitors of enzymes, such as GTP cyclohydrolase I, that participate in tetrahydrobiopterin synthesis. Our observation that immunostimulant-activated vascular smooth muscle cells released the majority of newly synthesized tetrahydrobiopterin suggests that tetrahydrobiopterin synthesized in vascular smooth muscle cells may serve as a cofactor for NO synthase not only in vascular smooth muscle cells themselves but also in adjacent tissues or cells.

Our study does not identify the site at which nuclear factor κB participates in the signal transduction cascade between LPS stimulation of vascular smooth muscle and GTP cyclohydrolase I induction. However, LPS increases GTP cyclohydrolase I mRNA abundance in the presence of a protein synthesis inhibitor, indicating no requirement for intermediary protein synthesis (Hattori and Gross, 1993). Furthermore, cloning of the 5′ flanking region of the rat GTP cyclohydrolase I gene by polymerase chain reaction revealed the presence of several nuclear factor κB binding sites (Ishii et al., 1993) although these have not yet been shown to control gene expression. Together with the present data, these observations suggest that nuclear factor κB participates in the regulation of GTP cyclohydrolase I at the transcriptional level in LPS-treated vascular smooth muscle. Thus, we conclude that immunostimulants coinduce NO synthase and GTP cyclohydrolase I gene expression; both events are necessary for activation of cellular NO synthesis and are regulated, at least in part, by a common mechanism.

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