Volume 321, number 2,3, 215–218 FEBS 12361 April 1993

Diethyldithiocarbamate inhibits induction of macrophage NO synthase

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Received 8 March 1993

We investigated whether sodium diethyldithiocarbamate (DETC), an inhibitor of the nuclear transcription factor kappa B (NFkappaB), modulates induction of NO synthase (NOS) in murine bone marrow-derived macrophages. A short exposure (between 1 and 16 h) of L929-cell medium-preconditioned macrophages to *E. coli* lipopolysaccharide (LPS) significantly increased the level of NOS mRNA, and elicited NO formation as detected by electron spin resonance spectroscopy and by the release of nitrite. DETC (0.1–1 mM) present during stimulation with LPS prevented the increase in NOS mRNA and the expression of NOS activity. These findings suggest that NFkappaB is involved in the signal transduction pathway linking stimulation of macrophages by LPS with transcription of the gene encoding inducible NOS.

NO synthase; Transcription; NFkappaB; Diethyldithiocarbamate; Bone marrow-derived macrophage

1. INTRODUCTION

Intra- and extracellular pathogens, their breakdown products, several cytokines and a plethora of other noxious agents and conditions induce the expression of NO synthase in mammalian cells (for review see [1–3]). NO generated from L-arginine by the inducible NO synthase (iNOS) is regarded as a defense effector molecule with cytotoxic/cytostatic and microbicidal/microbiostatic activity [1,2]. To date little or nothing is known about the transduction cascade that unifies all these pathogenic signals translating them into activation of the iNOS gene. Eukaryotic gene expression is controlled by promotor and enhancer DNA sequences, which are activated by specific protein factors. One of the nuclear transcription factors initially identified in B-cells and monocytes, the multiprotein complex NFkappaB [4], is rapidly activated upon perturbation of cells by conditions similar to those known to induce NOS. NFkappaB is known to mediate the immediate-early gene response by enhancing the transcription of a multitude of genes encoding defense and signalling proteins, such as cytokines and cytokine receptors [4]. Activation of NFkappaB can be specifically prevented by antioxidants, thiols and iron chelators, and especially by dithiocarbamates [4]. To clarify whether NFkappaB participates in the induction of NOS, we assessed the effect of diethyldithiocarbamate (DETC) on iNOS mRNA levels and expression of NOS activity in lipopolysaccharide (LPS)-stimulated murine bone marrow-derived macrophages.

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2. MATERIALS AND METHODS

Bone marrow cells from the femurs of Balb/c mice were isolated and cultured in L929-cell conditioned medium as described recently [5]. Cells grown on culture dishes (3.5 cm diameter; 10^7 cells/dish) were exposed to LPS (0.1 μg/ml; *E. coli* serotype 055:B5), DETC (0.01 to 1 mM; both from Sigma, Deisenhofen, Germany) and hemin (Normosang, Leiras, Turku, Finland) according to the protocols described in section 3.

Messenger RNA encoding iNOS was assessed by Northern blot technique using a *Hin*cII/SSP I fragment of the mouse iNOS [6] as a cDNA probe. The cDNA probe was a generous gift of Drs. Q. Xie and C.F. Nathan, Cornell University Medical College, New York. Total RNA was extracted from macrophages by guanidinium isothiocyanate extraction [7]. For most experiments, 10 μg total RNA was size-fractionated by electrophoresis on 1% agarose gels in 20 mM MOPS; pH 7.0, 5 mM sodium acetate, 1 mM EDTA and 3% formaldehyde. RNA was transferred to nylon membranes (Amersham-Buchler, Braunschweig, Germany) and fixed by baking at 80°C. Northern blots were prehybridized at 42°C in a solution containing 50% formamide, 5 × Denhardt's, 5 × standard saline buffer (SSC), 0.2% SDS and 250 μg/ml denatured salmon sperm DNA. Hybridization was accomplished in the same solution containing 0.5 μCi/ml labeled iNOS cDNA probe by incubating overnight at 42°C. The cDNA probe was labeled with ^{32}P to a specific activity of 50 μCi/μg using a labelling kit obtained from Pharmacia (Freiburg, Germany). As a control filters were also hybridized with cDNA encoding 18 S ribosomal RNA.

Accumulation of nitrite was measured by the Griess diazotation reaction as described [8]. Absorbance readings at 540 nm were corrected for medium blank values. NO was measured by electron spin resonance (ESR) spectrometry [9]. In these experiments macrophages were incubated for 1 h with DETC (5 mM) at the end of each incubation protocol (see Table I). The cells were then harvested and frozen for ESR analysis. DETC forms a complex with intracellular ferrous iron which efficiently traps NO, thereby generating a paramagnetic mononitrosyl-ferrous iron complex. The amount of NO trapped was calculated by double integration of the derivative ESR signal and comparison with a standard [9].

Viability of the cells was assessed by Trypan blue exclusion. Protein biosynthesis was quantified by [^{3}H]leucine incorporation as described [8].

3. RESULTS

3.1. *DETC inhibits NO and nitrite formation by LPS-stimulated macrophages*

To assess the effect of DETC on expression of iNOS activity, pre-conditioned macrophages (10^7/2 ml) were incubated with LPS (0.1 μg/ml) in the absence or presence of DETC (0.01–1 mM) according to the incubation protocols listed in Table I. At the end of each incubation, medium was removed for determination of nitrite and the cells were incubated for one hour more in fresh medium containing 5 mM DETC and 1 μM superoxide dismutase. Cells were then harvested and frozen for recording of ESR spectra. Exposure to LPS between 2.5 and 16 h (protocols 1 to 4, respectively) lead at least to a fourfold increase in the accumulation of nitrite in the culture medium during 16 h (Table I), compared to that released by non-stimulated macrophages during the same period (protocol 2). Accumulation of nitrite reflected NO production, which was directly determined at the end of each incubation by quantifying intracellular paramagnetic $NOFe(DETC)_2$ complexes (Table I). It should be mentioned that, in accordance with previous findings [10], there is a discrepancy between the values of NO formation as calculated by ESR spectroscopy (3 to 4 nmol/h) and the total NO formation (8 to 12 nmol/h). The latter value was estimated from the average rate of nitrite accumulation (100 nmol/16 h) assuming that nitrite and nitrate, the stable metabolites of NO, to be formed in equal quantities.

LPS-induced stimulation of nitrite and NO formation in macrophages was inhibited by DETC in a concentration-dependent fashion. When present during long-term exposure to LPS (16 h), 0.1 mM DETC was more effective than 1 mM, an effect not observed with shorter incubation periods (protocols 2–4). Preincubation of macrophages with DETC prior to exposure to LPS did not significantly increase the efficiency of DETC in preventing NO synthesis (cp. protocols 1 and 2, Table I). The inhibition by DETC was not due to a direct inhibition of NOS activity, since DETC does not affect NO formation in homogenates from LPS-stimulated macrophages [11].

To exclude that inhibition of NOS induction by DETC resulted from depletion of intracellular iron and interference with the synthesis of heme required for ex-

Table I

DETC attenuates LPS-induced NO and nitrite formation by murine bone marrow derived macrophages

Protocol	Periods of exposure			Periods of accumulation	
	To LPS (μg/ml)	To DETC (mM)	To hemin (μM)	Of NO (nmol/h)	Of NO_2^- (μM)
1	1.5–4 h	0–4 h		20–21 h	4–20 h
	0.1	0		2.7	30.0
	0.1	0.1		1.9	16.9
	0.1	1.0		1.1	14.0
2	0–4 h	0–4 h	0–4 h	20–21 h	4–20 h
	0	0		0.4	6.5
	0.1	0		3.4	37.0
	0.1	0.1		1.3	20.0
	0.1	1.0		0.3	6.7
	0.1	0	1	3.9	47.0
	0.1	1.0	1	0.3	6.4
3	0–6 h	0–6 h		22–23 h	6–22 h
	0.1	0		2.5	27.4
	0.1	1.0		1.5	11.2
4	0–16 h	0–16 h		16–17 h	0–16 h
	0	0		0.2	3.0
	0.1	0		4.0	50.5
	0.1	0.01		4.1	50.0
	0.1	0.1		1.6	12.3
	0.1	1.0		2.0	18.1

Data obtained from 2 sets of experiments performed in triplicate with different cell batches. S.E.M. fell within 10% of absolute values in all experiments. Incubations (10^7 macrophages/2 ml medium) started at 0 h and proceeded for up to 23 h. Time periods indicate duration of incubation with LPS, DETC and hemin and the sampling periods for nitrite and NO accumulation.

pression of NOS activity [12], hemin (1 μM) was included into the incubates during exposure of macrophages to LPS and DETC. Inhibition of nitrite and NO formation by DETC was not prevented by hemin (protocol 2, Table I). This finding argues against the possibility that DETC acts by impairing heme synthesis.

Furthermore, we excluded an unspecific toxic effect of DETC by testing the viability of DETC-exposed macrophages with trypan blue, and by incorporation of [^{3}H]leucine which can be taken as a measure of protein biosynthesis. In experiments carried out according to protocol 4, 80% of the cells remained viable with the highest dose of DETC tested (1 mM). The rate of [^{3}H]leucine incorporation was not affected by up to 1 mM DETC. Furthermore, the total amounts of RNA isolated from DETC-treated and untreated cells as measured by absorbance at 260 nm were not significantly different (data not shown).

3.2. *DETC inhibits the LPS-induced increase in iNOS mRNA level*

The iNOS mRNA levels in LPS-stimulated and unstimulated macrophages were analyzed by Northern blot technique. As shown in Fig. 1 exposure of macrophages to LPS according to protocol 4 (4 h with and then 16 h without LPS) significantly increased the level of iNOS mRNA (lane 2) compared with that of unstimulated macrophages incubated for the same period (lane 1). However, the 4.4 kb sized iNOS mRNA was also detected in non-stimulated macrophages, which may account for the low levels of NO and nitrite generated under these conditions (Table I). Preactivation of the macrophages by L929 cell-conditioned medium was apparently sufficient to weakly induce NOS. Exposure to LPS for only 1 h significantly increased the amount of iNOS mRNA within the next 4 h (Fig. 1, lane 3). If DETC (1 mM) was present 30 min before, during and 1 h after stimulation with LPS the amount of iNOS mRNA was reduced to unstimulated levels (Fig. 1, lane 4). Similar results were obtained with a macrophage cell line, JJ774, exposed to LPS and interferon γ (data not shown).

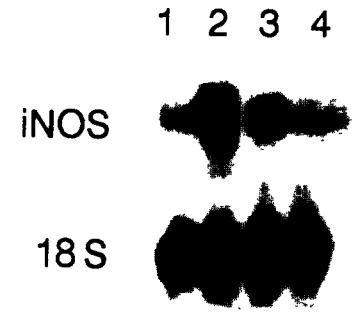

Fig. 1. DETC inhibits the increase in iNOS mRNA levels induced by LPS. Macrophages (10^7/2 ml) were incubated as follows: Lane 1, 20 h without LPS; lane 2, 4 h with LPS (0.1 μg/ml) followed by 16 h without LPS; lane 3, 0.5 h without, followed by 1 h with and 4 h without LPS; lane 4, as described for lane 3, but with inclusion of DETC (1 mM) for the first 2.5 h. Total RNA was then isolated, and the levels of iNOS mRNA and 18 S RNA were assessed by Northern blot technique as described in section 2. Identical results were obtained in 3 further experiments.

4. DISCUSSION

Our results suggest that NFkappaB is involved in regulation of NOS induction at the transcriptional level in LPS-stimulated murine bone marrow derived macrophages. This conclusion derives from our finding that the increase in iNOS mRNA levels and expression of NOS activity was inhibited by DETC, a compound recently characterized as selective inhibitor of NFkappaB [4]. In some cells this factor is cytosolic and inactive due to binding to an inhibitory subunit, IkappaB [4]. Activation is elicited by many agents which dissociate the NFkappaB–IkappaB complex by an unknown mechanism, presumably involving phosphorylation of IkappaB by a protein kinase that is sensitive to oxidative stress [4]. DETC specifically interferes with this activation process at a yet unidentified step, while it does not affect the activation of other nuclear transcription factors [4].

Our study does not identify the true site where NFkappaB participates in the signal transduction cascade between LPS stimulation of macrophages and iNOS transcription. LPS presumably does not directly activate the iNOS gene since iNOS mRNA is detected at the earliest 2 h after stimulation and its transcription is inhibited by cycloheximide, indicating a requirement for protein synthesis [13,14]. LPS is known to elicit a multitude of intracellular signals in macrophages (TNFα, IL-1β, PGE_2, cyclic AMP [15]), which all enhance induction of LPS-elicited NOS when applied exogenously [1,15]. Enhancement is also observed with interferon γ [1] and phorbol esters [15], indicating that at least 2 independent pathways are required for maximal activation of NOS gene transcription in macrophages. NFkappaB may be involved in the control of transcription of secondary LPS signals, or could be activated by these signals and then bind to enhancer sequences activating the iNOS encoding DNA. Clarification of this issue will await the identification of the promotor and enhancer sequences regulating the iNOS gene.

Acknowledgements: We are indebted to Mrs. C. Herzog and Mr. F. Zimmermann for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Bu 436/4-2).

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