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Evidence that inducible nitric oxide synthase is involved in LPS-induced plasma leakage in rat skin through the activation of nuclear factor-κB

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1 Rats challenged with lipopolysaccharide (LPS) produce large amounts of nitric oxide (NO) following the induction of the inducible NO-synthase (iNOS) in several tissues and organs. Recent studies have shown that the expression of iNOS is regulated at the transcriptional level by a transcription nuclear factor-κB (NF-κB). In this study we investigated the role of NO in a model of LPS-induced plasma-leakage in rat skin and the involvement of NF-κB.

2 Plasma leakage in the rat skin was measured over a period of 30 min to 2 h as the local accumulation of intravenous (i.v.) injection of [^{125}I]-human serum albumin ([^{125}I]-HSA) in response to intradermal (i.d.) injection of LPS. LPS (1, 10, 100 μg/site) produced a dose-related increase in plasma extravasation (18.2±3.2, 27.2±2.9, 40.4±9.6 μl/site) as compared to saline control (11.4±2.2 μl/site). This increase was maximal after 2 h; therefore this time point and the dose of LPS 10 μg/site was used in all the successive experiments.

3 To investigate the role of NO in LPS-induced plasma leakage in rat skin, the non-selective NOS inhibitor N^{G}nitro-L-arginine-methyl ester (L-NAME) or the more selective iNOS inhibitor S-methyl-isothiourea (SMT) was injected i.d. with LPS. L-NAME and SMT (0.01, 0.1 and 1 μmol/site) inhibited LPS-induced plasma leakage in a dose-related fashion (L-NAME: 26.0±5.5, 20.2±1.6, 18.0±2.0 μl/site; SMT: 19.5±1.5, 17.0±1.6, 15.0±2.6 μl/site) as compared to LPS alone (27.2±2.9 μl/site). At the lowest concentration used (0.01 μmol/site), SMT significantly reduced plasma leakage by 30%±0.7 while L-NAME (0.01 μmol/site) was not effective.

4 Treatment with increasing concentrations of pyrrolidinedithyocarbamate (PDTC) (0.01, 0.1, 1 μmol/site), an inhibitor of NF-κB activation, injected i.d. 30 min before LPS challenge, inhibited in a concentration-dependent fashion LPS-induced plasma leakage by 9.0±0.6, 33±4.0, 51±2.0% respectively. Moreover, PDTC (0.1, 1 μmol/site) suppressed LPS-induced NF-κB DNA-binding.

5 Western blot analysis showed significant levels of iNOS proteins in the skin samples of LPS-treated rats, as compared to basal levels present in saline-injected rat skin. PDTC (0.1, 1.0 μmol/site) dose-dependently decreased the amount of iNOS protein expression induced by LPS.

6 Our results indicate that LPS-induced plasma leakage in rat skin is modulated by NO mainly produced by the inducible isoform of NOS. Furthermore, the suppression of plasma leakage by PDTC, an inhibitor of NF-κB activation, is correlated to the inhibition of iNOS protein expression.

Keywords: LPS-induced plasma leakage; nitric oxide; iNOS; L-NAME; nuclear factor-κB.

Introduction

Lipopolysaccharide (LPS) from Gram-negative bacteria when given intradermally causes an inflammatory reaction characterized by hyperaemia, haemorrage, leukocyte infiltration and increased plasma leakage in rabbit (Kopaniak *et al*., 1980; Issekutz & Bhimji, 1982) and mouse skin (Fujii *et al*., 1996). In addition, LPS administration leads to the production of several pro-inflammatory mediators including cytokines (Kielian & Blecha, 1995), prostaglandins (Lee *et al*., 1992) and nitric oxide (NO) (Wright *et al*., 1992) which maintain and modulate the inflammatory response. Production of NO from the guanidino nitrogen of L-arginine and molecular oxygen is catalyzed by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS are known to exist and can be classified into two categories, namely constitutive (cNOS) and inducible (iNOS) (Knowles & Moncada, 1994). Both enzymes are inhibited, *in vivo* and *in vitro*, by several L-arginine analogues including N^G-nitro-L-arginine-methyl ester (L-NAME) (Moncada *et al*., 1991). Non-isoform selective inhibition of NO formation may lead to side effects by inhibiting several physiological actions of NO. Recently, aminoguanidine and S-methyl-isothiourea (SMT) have been demonstrated to inhibit selectively the inducible NOS isoform *in vitro* and *in vivo* (Misko *et al*., 1993; Southan *et al*., 1995) and have been used succesfully in some inflammatory models where iNOS seems to play a predominant role (Szabò *et al*., 1995; Wu *et al*., 1995; Iuvone *et al*., 1997).

The expression of several genes involved in inflammatory responses, including that coding for iNOS, is regulated at the transcriptional level by the nuclear factor NF-κB (Xie *et al*., 1994; Wulczyn *et al*., 1996). NF-κB is an inducible transcription factor that mediates signal transduction between the cytoplasm and nucleus in many cell types (Baeuerle & Henkel, 1994). In resting cells, NF-κB is localized in the cytoplasm as an heterodimer composed of two polypeptides of

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50 kDa (p50) and 65 kDa (p65), which are associated with cytoplasmic inhibitory proteins, including IkB-α (Grimm & Baeuerle, 1993). Following activation by several stimuli, including LPS, the p50/p65 complex migrates to the nucleus where it promotes gene transcription. Although, recent observations support the hypothesis that NF-κB activation occurs in the inflammatory process *in vivo* (Blackwell *et al*., 1996; Essani *et al*., 1996), a direct relationship between iNOS induction and NF-κB activation *in vivo* has not yet been demonstrated.

Therefore, in this study we investigated the role of NO in LPS-induced plasma leakage in rat skin by use of the non selective NOS inhibitor L-NAME and the more selective iNOS inhibitor SMT. In addition, we studied the involvement of NF-κB by use of the antioxidant pyrrolidinedithyocarbamate (PDTC), an inhibitor of NF-κB activation.

Part of this work has been presented previously to the British Pharmacological Society Meeting in Edinburgh, September 1997.

Methods

Plasma leakage measurement in rat skin

The formation of local plasma leakage in the skin was measured in male Wistar rats (250–300 g). The rats were anaesthetized with Nembutal (60 mg kg^{-1}, i.p.), the back of the rats was shaved. ^{125}I-labelled human serum albumin ([^{125}I]-HSA) (2 mCi kg^{-1} of body weight in 1% of Evans blue solution) was injected via the tail vein. LPS (1–10 μg/site), L-NAME (0.01–1.0 μmol/site), SMT (0.01–1.0 μmol/site) or saline were injected intradermally (100 μl) according to a balanced site injection plan, in duplicate immediately before [^{125}I]-HSA administration. PDTC (0.01–1.0 μmol/site) was injected locally 30 min before stimulus. After 30 min to 2 h blood samples were taken by cardiac puncture and the animals were killed. The injection sites were punched out and samples were counted in an automatic γ-counter (Cobra5005, Packard). Plasma leakage at each site was expressed as μl of plasma by dividing skin sample ^{125}I counts by ^{125}I counts in 1 μl of plasma (Williams, 1979).

Preparation of skin nuclear mini-extracts

In some experiments, performed without injection of [^{125}I]-HSA, the skin punches were taken and nuclear mini-extracts were prepared from the isolated skin according to the procedure of Schreiber *et al*. (1989). Briefly, the skin was suspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)) and homogenized at the highest setting for 15 s in a Polytron PT 3000 (Kinematica AG) tissue homogenizer. The homogenates were chilled on ice for 15 min; 25 μl of 10% Nonidet P-40 was added and vigorously vortexed for 10 s. The nuclear fraction was precipited by centrifugation at $1500 \times g$ for 5 min and suspended in 50–100 μl of buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM PMSF). The mixture was left on ice for 15 min with frequent agitation. Nuclear extracts were prepared by centrifugation at $1500 \times g$ for 5 min and stored at −80°C. Protein concentration was determined by the Biorad protein assay kit.

Electrophoretic mobility shift assay (EMSA)

Double stranded NF-κB consensus oligonucleotide probe (5′ AGTTGAGGGGATTTTCCCAGGC 3′; synthesized by CEINGE, Naples, Italy) was end-labelled with [^{32}P]-γATP (Amersham). Nuclear extracts (15 μg) were incubated for 30 min with radiolabelled oligonucleotides ($2.5–5.0 \times 10^4$ c.p.m.) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg μl^{-1} bovine serum albumin, 10% (v/v) glycerol. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 5% non denaturing polyacrylamide gel in $0.25 \times$ TBE buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at −80°C for 20 h.

Western blot analysis

Cytosolic proteins from rat skin homogenates were boiled (3 min) in gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg ml^{-1} bromophenol) in a ratio of 1 : 1 and centrifuged at $10{,}000 \times g$ for 10 min. Protein concentrations were determined and equivalent amounts (70 μg) for each sample were electrophoresed in a 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membrane, according to the manufacturer's instructions (Biorad). The membrane was saturated by incubation at 4°C overnight with 10% dry skimmed milk in PBS and then incubated with anti-iNOS antibody (Santa Cruz) for 1 h at room temperature. The membrane was washed three times with 1% Triton-X100 in PBS and then incubated with anti-rabbit immunoglobulins coupled to peroxidase (Amersham). The immune complexes were visualized by use of chemiluminescence Western blotting detection reagents (ECL Plus; Amersham).

Materials

LPS (from *E. Coli*) and all the compounds, unless otherwise stated, were purchased by Sigma Chemical Company. PDTC was from Biomedicals ICN. [^{125}I]-HSA was obtained from Amersham.

Statistics

Results are expressed as the mean ± s.e.mean of n animals where each value is the average of responses in duplicate sites. Statistical comparisons were made by one way-ANOVA followed by Bonferroni's test for multiple comparisons; $^{**}P < 0.01$; $^{*}P < 0.05$.

Results

Plasma leakage induced by LPS

Time-course studies revealed that following i.d. injection of LPS (10 μg/site), plasma leakage did not change as compared to saline control in the first 30 min. Plasma leakage started to increase 1 h later, then reached a maximum and significant ($P < 0.05$ vs saline) increase at 2 h and decreased 4 h after LPS challenge (Figure 1). Therefore the 2 h time point was chosen for the next experiments.

Figure 2 shows that the i.d. injection of LPS (1, 10, 100 μg/site) produced a dose-dependent increase in plasma leakage

(18.2 ± 3.2, 27.2 ± 3.6, 40.4 ± 9.6 μl/site; $P<0.05$) as compared to saline control (11.4 ± 2.2 μl/site). All the other experiments were performed with 10 μg/site of LPS.

Effect of NOS inhibitors on LPS-induced plasma leakage

To study the role of NO on LPS-induced plasma leakage, we investigated the effect of the non-selective NOS inhibitor L-NAME and the more selective iNOS inibitor SMT. Both compounds (0.01, 0.1, 1.0 μmol/site) injected with LPS (10 μg/site) reduced plasma leakage in a dose-related fashion (L-NAME: 26.0 ± 5.5, 20.2 ± 1.6, 18.0 ± 2.0 μl/site; SMT: 19.5 ± 1.5, 17.0 ± 1.6, 15.0 ± 2.6 μl/site) as compared to LPS alone (27.2 ± 2.9 μl/site) (Figure 3). At the lowest concentration used (0.01 μmol/site), SMT significantly reduced plasma leakage by $30\pm0.7\%$ ($P<0.01$) while L-NAME (0.01 μmol/site) was not effective (Figure 3).

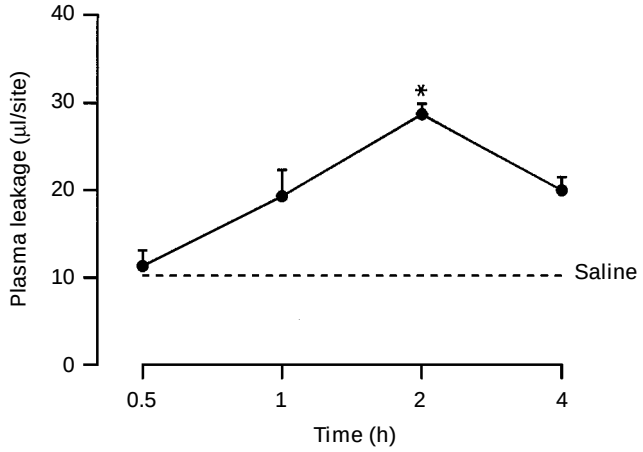

Figure 1 Time-course of LPS-induced plasma leakage in rat skin. LPS (10 μg/site) or saline were injected i.d. immediately before injection of [^{125}I]-HSA. At the indicated time points after LPS or saline injection plasma leakage was measured as local accumulation of i.v. injected [^{125}I]-HSA. Each point represents the mean of $n=5$ experiments in duplicate; vertical lines show s.e.mean. *$P<0.05$ vs saline.

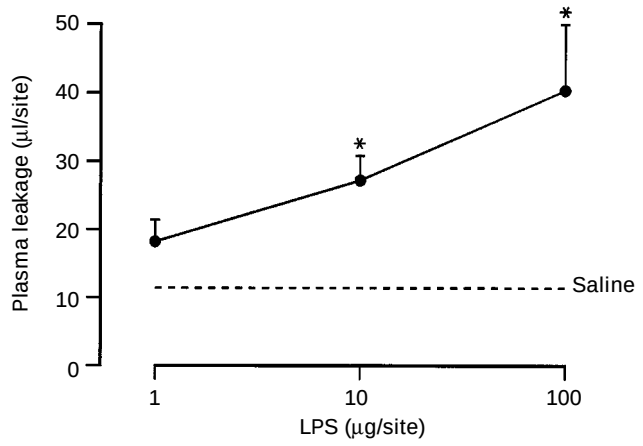

Figure 2 Dose-dependent effect of LPS on plasma leakage in rat skin. Saline or LPS (1, 10, 100 μg/site) was injected i.d. in rat skin immediately before injection of [^{125}I]-HSA. Plasma leakage was measured over a period of 2 h as local accumulation of i.v. injected [^{125}I]-HSA. Each point represents the mean of $n=5$ experiments in duplicate; vertical lines show s.e.mean. *$P<0.05$ vs saline.

Effect of PDTC on LPS-induced plasma leakage

Treatment of increasing doses of PDTC (0.01, 0.1, 1.0 μmol/site), an antioxidant inhibitor of NF-κB activation, injected i.d. 30 min before LPS (10 μg/site), resulted in a dose-dependent inhibition of plasma leakage (24.6 ± 6.0 μl/site; 18.2 ± 4.6 μl/site, $P<0.05$; 12.8 ± 1.9 μl/site, $P<0.01$) as compared to LPS alone (27.2 ± 2.9 μl/site) (Figure 4).

Effect of PDTC on LPS-induced NF-κB activation

To detect NF-κB DNA-binding activity, an electrophoretic mobility shift assay (EMSA) was performed with nuclear

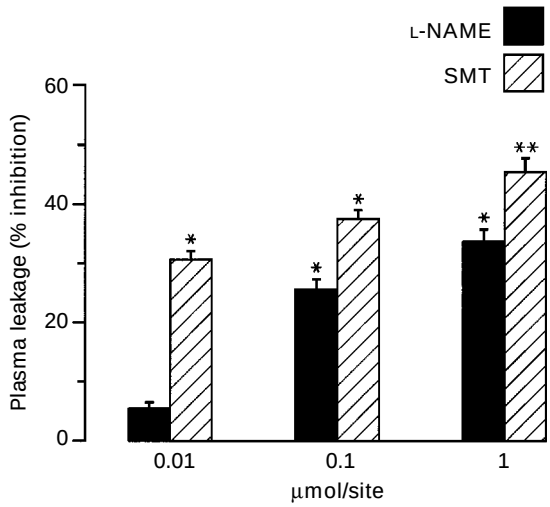

Figure 3 Dose-dependent effect of L-NAME and SMT on LPS-induced plasma leakage in rat skin. L-NAME and SMT (0.01, 0.1, 1.0 μmol/site) were injected i.d. with LPS (10 μg/site) immediately before injection of [^{125}I]-HSA. Plasma leakage was measured over a period of 2 h as local accumulation of i.v. injected [^{125}I]-HSA. Results are expressed as percentage inhibition of LPS-induced plasma leakage. Each column represents the mean$\pm$s.e. of $n=6$ experiments in duplicate. *$P<0.05$, **$P<0.01$ vs LPS.

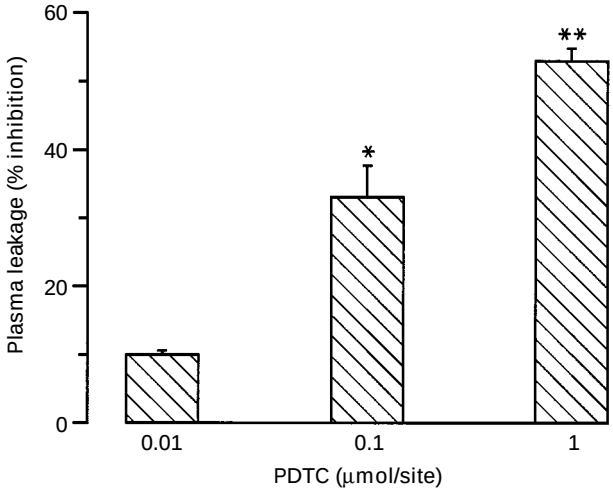

Figure 4 Dose-dependent effect of PDTC on LPS-induced plasma leakage in rat skin. PDTC (0.01, 0.1, 1.0 μmol/site) was injected i.d. 30 min before LPS (10 μg/site). Plasma leakage was measured over a period of 2 h as local accumulation of i.v. injected [^{125}I]-HSA. Results are expressed as percentage inhibition of LPS-induced plasma leakage. Each column represents the mean$\pm$s.e. of $n=6$ experiments in duplicate. *$P<0.05$, **$P<0.01$ vs LPS.

extracts from LPS-treated rat skin and a labelled oligonucleotide containing the NF-κB binding site. As shown in Figure 5a, DNA-protein complexes were slightly detectable in nuclear extracts from saline-treated rat skin (lane 1), whereas a retarded band was clearly detected at 2 h after LPS injection (10 μg site) (lane 3). Treatment with PDTC (0.1, 1.0 μmol/site) injected 30 min before LPS challenge caused a dose-dependent inhibition of LPS-induced NF-κB activation (lane 5 and 7, respectively). The specificity of the retarded complex was demonstrated by the fact that it was abrogated in the presence of a 100 fold molar excess of the same unlabelled oligonucleotide included in the reaction mixture (lane 2, 4, 6, 8).

Effect of PDTC on LPS-induced iNOS protein expression

The iNOS protein expression in skin homogenates of LPS-treated rats was investigated by Western blot analysis. Low levels of iNOS protein expression were detectable in saline-treated rats (lane 1). The injection of LPS (10 μg/site) in rat skin resulted in an increase of iNOS protein expression (lane 3) which was prevented dose-dependently by treatment with PDTC (0.1, 1.0 μmol/site) (lanes 5 and 7 respectively) (Figure 5b).

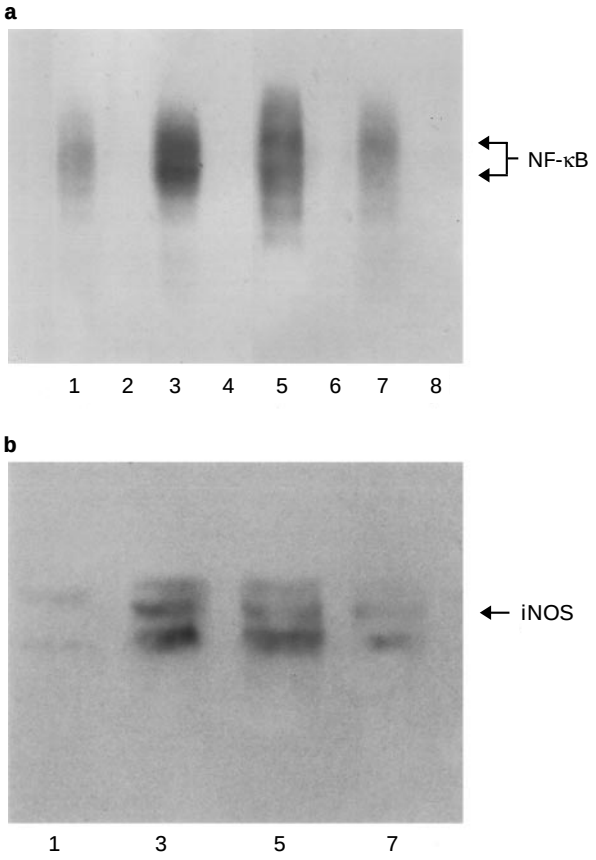

Figure 5 (a) Inhibition of NF-κB DNA-binding by PDTC. The EMSA shows the effect of PDTC on LPS-induced NF-κB DNA-binding in nuclear extracts from rat skin. NF-κB DNA-binding from saline injected animals (lane 1). Increase of NF-κB DNA-binding from rats injected i.d. with LPS (10 μg/site) (lane 3). Inhibition of NF-κB DNA-binding from rats injected with PDTC (0.1, 1.0 μmol/site) 30 min before LPS (lane 5 and 7, respectively). Specificity of retarded complexes was demonstrated by the addition of an excess of the same unlabelled oligonucleotide used as probe (lanes 2, 4, 6, 8). (b) Effect of PDTC on LPS-induced iNOS protein expression. Western blot analysis shows the effect of PDTC (0.1, 1.0 μmol/site) on iNOS protein expression in LPS (10 μg/site) injected rat skin. Lane 1: saline-injected rats; lane 2: LPS-injected rats; lane 3: LPS plus PDTC (0.1 μmol/site); lane 4: LPS plus PDTC (1.0 μmol/site). Data illustrated are from a single experiment and are representative of a total of three separate experiments.

Discussion

The oedema formation at sites of injury is a hallmark of inflammation that is sustained by the release of several pro-inflammatory mediators. In our study we demonstrated that, following LPS injection in rat skin plasma leakage dose-dependently increased and reached a peak 2 h after endotoxin injection. In addition, our results show that LPS-induced plasma leakage in rat skin is sustained, at least in part, by the production of NO.

Previous studies showed that NO generated by the constitutive NOS isoform in the vascular endothelium is involved in the regulation of blood pressure and organ blood flow distribution and inhibits the adhesion of leukocytes to the endothelium (Kubes *et al*., 1991). In contrast, NO produced in large amounts by the inducible NOS accounts for its pro-inflammatory effect, as demonstrated in dextran- and carrageenin-induced rat paw oedema (Ialenti *et al*., 1992). In spite of the fact that both cNOS and iNOS may be involved in inflammation, in the present study we suggested that LPS-induced plasma leakage in rat skin is sustained by NO mainly produced by the inducible NOS isoform. In fact, when rats were treated with SMT a more selective iNOS inhibitor (Southan *et al*., 1995), plasma leakage was reduced at all the concentrations used to a greater extent than L-NAME, a non isoform selective NOS inhibitor (Rees *et al*., 1990; Moncada *et al*., 1991). Moreover, at the lowest concentration used, SMT still significantly reduced plasma leakage whereas L-NAME was no longer effective. SMT has been shown to have a relative selectivity towards iNOS; in fact, SMT appears to be at least 10 to 30 fold more potent as an inhibitor of the inducible NOS in LPS-stimulated J774 macrophages as compared to the L-arginine substitute inhibitors (Szabò *et al*., 1994). Furthermore, SMT has been shown to be less potent than methylarginine as a cNOS inhibitor in anaesthetized rats (Southan *et al*., 1995). Whereas, L-NAME exerts its inhibitory activity both on cNOS and on iNOS (Rees *et al*., 1990; Moncada *et al*., 1991). Furthermore, L-NAME, depending upon the concentration used, may increase mean arterial blood pressure (Gardiner *et al*., 1990; Bryant *et al*., 1995) resulting in a local vasoconstriction while SMT, as shown by Szabò *et al*. (1994) does not change local blood flow. Taken together our results indicate that NO produced by iNOS plays an important role in LPS-induced plasma leakage in rat skin.

Our data are in agreement with other findings showing that the selective iNOS inhibitor aminoguanidine reduced plasma leakage in mouse skin (Fujii *et al*., 1996). Furthermore, recently, it has also been shown that LPS administration to rats leads to the induction of a Ca^{2+} independent NOS in several organs (Làszlò *et al*., 1995). In addition, in a model of acute inflammation induced by carrageenin the inducible NOS isoform is predominant (Tomlinson *et al*., 1994; Salvemini *et al*., 1996). It follows therefore, that, the selective inhibition of the expression of this enzyme may represent an important therapeutic goal.

Changes in NO levels are usually correlated with similar changes in iNOS mRNA levels, indicating that iNOS regulation mostly occurs at the transcriptional level. A growing body of evidence suggests a role for NF-κB in the expression of several genes involved in the immune and

inflammatory response, including that coding for iNOS (Wulczyn *et al*., 1996). In fact, activation of NF-κB has been shown to mediate the enhanced expression of the iNOS gene in LPS-challenged macrophages (Xie *et al*., 1994). Recently, NF-κB activation has been demonstrated to occur in rat lung (Blackwell *et al*., 1996) and liver (Essani *et al*., 1996) following systemic endotoxaemia.

Our results show that the antioxidant PDTC, an inhibitor of NF-κB activation, dose-dependently inhibited LPS-induced plasma leakage in the rat skin. Moreover, PDTC was able to inhibit both NF-κB DNA binding activity in the nuclear extracts of LPS-treated rat skin and, similarly, decreased iNOS protein expression in the skin.

The antioxidant PDTC has been shown to inhibit potently NF-κB activation in intact cells (Schreck *et al*., 1992) as well as to inhibit iNOS expression in LPS-stimulated macrophages (Mulsch *et al*., 1993), but the mechanism of action remains obscure. A likely target for the antioxidants seems to be the signal transduction cascade leading to the activation of NF-κB and in many cell type this is the crucial step in the initiation of iNOS gene-expression (Adcock *et al*., 1994). Indeed, NF-κB activation by many stimuli has been proposed to be mediated by an increased formation of reactive oxygen species: antioxidants inhibit NF-κB activation while hydrogen peroxide and superoxide anions activate NF-κB in many cell lines (Schreck *et al*., 1991).

In conclusion, our results indicate that LPS induces iNOS protein expression in the skin and increases plasma leakage through enhanced NO production. Moreover, we showed that the activation of NF-κB occurs *in vivo* and it is required to promote the transcription of iNOS protein at the inflamed site. Therefore, in view of the potential deleterious effects of high amounts of NO produced by iNOS in inflammation, our data suggest that selective iNOS inhibitors and/or inhibitors of NF-κB activation may represent a new therapeutic tool for the pharmacological control of inflammation.

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