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SPECIAL REPORT



# Inhibition by spermine of the induction of nitric oxide synthase in J774.2 macrophages: requirement of a serum factor

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Polyamines are endogenous regulators of various cell functions. Nitric oxide (NO) is a cytostatic and cytotoxic free radical which is produced by the inducible NO synthase (iNOS) in immuno-stimulated macrophages. We tested whether spermine modulates the induction of iNOS in J774.2 macrophages. Stimulation of macrophages by bacterial lipopolysaccharide (LPS) or γ-interferon increased the accumulation of nitrite in the culture medium. Spermine ($10^{-6}$–$10^{-4}$ M) inhibited nitrite production without causing cytotoxicity. This inhibition of NO formation by spermine was significantly reduced when it was given 6 h after LPS. Spermine did not inhibit nitrite accumulation when foetal calf serum was omitted from the tissue culture medium. Thus, spermine is an inhibitor of the induction of iNOS, and its inhibitory activity requires the presence of a serum factor.

**Keywords:** Nitric oxide; lipopolysaccharide; spermine; polyamines

**Introduction** Endotoxin (bacterial lipopolysaccharide, LPS), interleukin-1, tumour-necrosis factor and γ-interferon (IFN) either alone or in combination induce nitric oxide (NO) synthase (iNOS) in macrophages resulting in the formation of large quantities of NO, which serves as a cytotoxic molecule with a key role in the antimicrobial activity of immune-stimulated macrophages (Green & Nacy, 1993).

The polyamines, spermine, spermidine and putrescine are endogenous regulators of proliferation, differentiation, functional activation and macromolecular biosynthesis in all mammalian cells (Selmeci *et al.*, 1985; Morgan, 1987). High concentrations of polyamines occur in foetal and neoplastic tissues and in seminal fluid. These tissues represent antigenic challenges that often do not elicit appropriate immune response of the host organism (see: Selmeci *et al.*, 1985; Normann, 1985; Morgan, 1987; Bulmer, 1992 for reviews). Here we show that spermine inhibits the induction of iNOS in activated macrophages.

**Methods** The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) with $4 \times 10^{-3}$ M L-glutamine and 10% foetal calf serum (FCS) (Szabó *et al.*, 1993). Cells were cultured in 96-well plates until confluence. To induce iNOS, fresh DMEM containing *E. coli* LPS (1 μg ml$^{-1}$) or γ-interferon (IFN, 50 u ml$^{-1}$) was added to the medium. Nitrite was measured after 24 h by the Griess reaction (Szabó *et al.*, 1993). Where appropriate, spermine ($10^{-6}$–$10^{-4}$ M), dexamethasone ($10^{-6}$ M) or $N^G$-monomethyl-L-arginine (L-NMMA, $3 \times 10^{-3}$ M) were added to the medium either together with LPS or 6 h after LPS. The effect of spermine ($10^{-6}$–$10^{-4}$ M) was also tested in DMEM without FCS. Mitochondrial respiration was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Szabó *et al.*, 1993). DMEM, L-glutamine, LPS (*E. coli*, serotype No. 0127:B8), dexamethasone phosphate, MTT and spermine hydrochloride were from Sigma (Poole, Dorset). $N^G$-monomethyl-L-arginine monoacetate (L-NMMA) was from Calbiochem (Nottingham). FCS was from AAP (West Midlands). Murine γ-interferon was from Genzyme (West Malling, Kent). Values are expressed as mean ± s.e.mean of $n$ observations, where $n$ represents the number of wells studies (9 wells from 3 independent experiments). Student's unpaired $t$ test was used to compare means between groups. A $P$ value $<0.05$ was considered significant.

**Results** LPS or IFN increased nitrite concentrations in the culture medium at 24 h (Figure 1). Co-administration of spermine with LPS dose-dependently reduced the accumulation of nitrite (Figure 1a). Nitrite production is due to induction of iNOS, for both L-NMMA (a NOS inhibitor, $3 \times 10^{-6}$ M) and dexamethasone (an inhibitor of iNOS induction, $10^{-6}$ M) inhibited nitrite accumulation (Figure 1). The induction of iNOS in the cells was associated with an inhibition of mitochondrial respiration, which was prevented by spermine or L-NMMA (Figure 1b).

The effects of spermine or dexamethasone were reduced when added to the medium 6 h after LPS (Figure 1a). This reduction was not due to accumulation of nitrite before addition of spermine or dexamethasone at 6 h, as there was no significant nitrite accumulation at this stage (nitrite concentration was 1.8 ± 0.5 μM 6 h after LPS vs. 1.4 ± 0.3 μM in control, $n = 12$).

Spermine did not inhibit nitrite accumulation when FCS was omitted from the medium (Figure 1a). Without FCS, the induction of nitrite production was attenuated by LPS, while it was slightly enhanced by IFN (Figure 1a).

**Discussion** We show that spermine inhibits the accumulation of nitrite in the medium of immune-stimulated J774.2 macrophages. This effect of spermine is less when added to the medium 6 h after stimulation, suggesting that spermine inhibits the induction, rather than activity of iNOS. Similarly, other inhibitors of iNOS induction, such as dexamethasone (Figure 1a), interleukin-10 (Cunha *et al.*, 1992) or dihydropyridine calcium channel modulators (Szabó *et al.*, 1993) also show less potency in inhibiting the formation of nitrite when given several hours after the stimulus of induction.

Cytotoxicity and/or a non-specific depression of cellular respiration does not account for the inhibition of nitrite accumulation seen with spermine, for spermine up to $10^{-4}$ M did not reduce viability. On the contrary, spermine partially reverses the LPS-induced inhibition of cellular respiration. This is likely to be due to inhibition of NO production by spermine, for NO mediates the inhibition of mitochondrial respiration in immune-stimulated macrophages (Green & Nacy, 1993).

The inhibitory effect of spermine on nitrite accumulation was only seen in the presence of FCS. This indicates that serum modulates the activity of spermine, possibly by providing an endogenous inhibitor of iNOS induction which synergizes with spermine or by activating spermine, e.g. through its metabolism.

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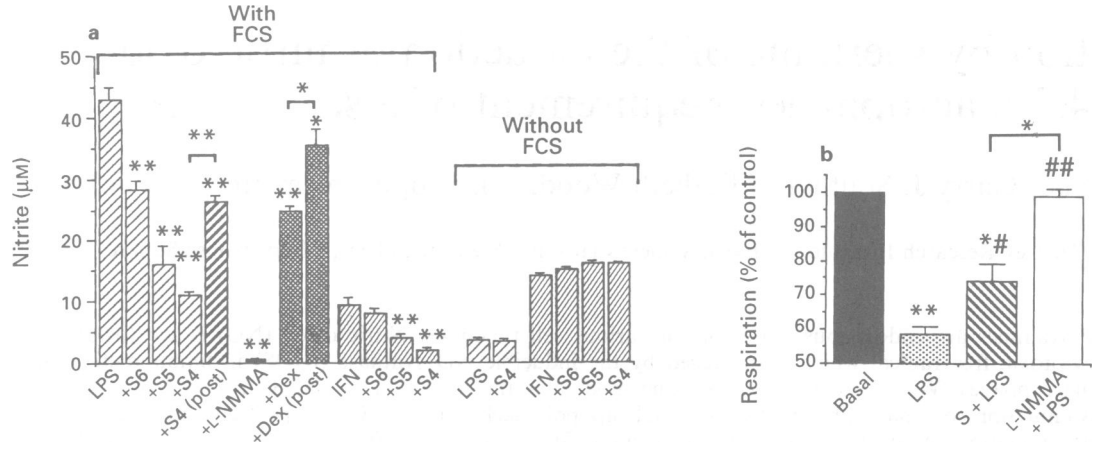


**Figure 1** Nitrite concentration in the supernatant of J774.2 macrophages 24 h after stimulation by bacterial lipopolysaccharide (LPS) or γ-interferon (IFN). Experiments were performed in DMEM either with 10% foetal calf serum (FCS) or without FCS. Basal nitrite (~2.5 μM) has been subtracted from the values. Depicted are the effects of spermine ($10^{-6}$, $10^{-5}$ and $10^{-4}$ M; S6, S5 and S4 respectively), $N^G$-monomethyl-L-arginine (L-NMMA, $3 \times 10^{-3}$ M) or dexamethasone (Dex, 1 μM) on nitrite accumulation. The effect of 100 μM spermine or Dex on nitrite accumulation was also studied when these agents were given 6 h after LPS (post). Data are expressed as means ± s.e.mean of $n = 9$ wells from 3 experimental days. $^*P < 0.05$ and $^{**}P < 0.01$ represent significant differences in the presence of various inhibitors, when compared to control, or between groups as indicated. (b) Spermine (S) or L-NMMA inhibit the depression of cellular respiration by LPS in J774.2 macrophages. Depicted are the effects of spermine ($10^{-4}$ M) and L-NMMA ($3 \times 10^{-3}$ M) on mitochondrial respiration in J774.2 macrophages stimulated by LPS for 24 h. Data are expressed as means ± s.e.mean of $n = 9$ wells from 3 experimental days. $^*P < 0.05$ and $^{**}P < 0.01$ represent significant differences when compared to basal values or between groups are compared as indicated; $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ represent significant restoration of the mitochondrial respiration in the presence of spermine or L-NMMA (together with LPS) when compared to LPS alone.

Interestingly, the induction of iNOS by LPS was markedly reduced in the absence of FCS. A likely explanation for this finding is that some serum factor is required for the induction of iNOS by LPS. A candidate is LPS-binding protein (LBP) which binds to LPS to form an LBD-LPS complex which then binds to the CD14 receptor on the cell membrane (Raetz *et al.*, 1991). Induction of NOS by IFN, however, was enhanced in the absence of FCS. This may be related to a possible non-specific binding of IFN to plasma proteins present in FCS.

Immunosuppression is frequently seen in tissues or biological fluids containing high levels of polyamines. We speculate that prevention of iNOS induction by polyamines may contribute to the well-documented host immunosuppression seen in pregnancy (see Bulmer, 1992) or in tumour-bearing organisms (see Normann, 1985). Polyamines are produced by various bacteria (see Morgan *et al.*, 1987), and so bacteria-derived polyamines may attenuate the host immune responses associated with the systemic inflammatory response syndrome.

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