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The mechanism of the inhibitory effect of polyamines on the induction of nitric oxide synthase: role of aldehyde metabolites

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1 We have recently found that in the presence, but not in the absence, of foetal calf serum, spermine inhibits the production of nitric oxide (NO) in cultured J774.2 macrophages stimulated with bacterial endotoxin (lipopolysaccharide; LPS) or with γ-interferon (IFN), showing that polyamines may act as suppressants of NO-mediated immune functions. Here, we have studied the mechanisms and the specificity of this inhibitory action.

2 Other polyamines, as well as spermine, inhibit the formation of NO in cultured J774.2 macrophages, with the order of potency being spermine > spermidine >> putrescine = cadaverine. This inhibition of NO formation is not due to any cytotoxic effect of these agents for they neither reduced mitochondrial respiration nor increased the release of lactate dehydrogenase into the supernatant.

3 Spermine is not a direct inhibitor of the activity of iNOS in induced J774.2 cells as measured by its lack of effect on the conversion of L-arginine to L-citrulline in homogenates. Neither spermine, nor its metabolites, interfere with the production of nitrite from NO or act as scavengers of NO. Thus, spermine is an inhibitor of the induction of iNOS.

4 Spermine inhibits nitrite formation in the presence of foetal, newborn or adult bovine serum, but not rat or human serum.

5 The effect of sper mine on nitrite production can be prevented by isoniazid, hydrazine or hydroxylamine, inhibitors of spermine oxidase, as well as by phenylhydrazine, an aldehyde inhibitor. We have, therefore, tested the effects of spermine dialdehyde or malon dialdehyde on the induction of iNOS. Spermine dialdehyde (SDA, 10^{-5} M) inhibits nitrite formation by IFN-activated J774.2 cells in the absence of serum when given as a pretreatment but not when given 6 h after stimulation. In contrast, malon dialdehyde was ineffective. Thus, aldehyde metabolites of spermine, such as SDA, account for the inhibitory effect of polyamines on the induction of NOS *in vitro*.

6 The inhibitory effect of polyamines on iNOS induction appears to be fairly specific to iNOS, for spermine does not inhibit LPS-induced production of prostaglandin $F_{2\alpha}$ or tumour necrosis factor.

Keywords: Nitric oxide; lipopolysaccharide; interferon; spermine; spermidine; putrescine; cadaverine; spermine dialdehyde; immunosuppression; cyclo-oxygenase; tumour necrosis factor; pregnancy, cancer

Introduction

Endotoxin (bacterial lipopolysaccharide, LPS) interleukin-1, tumour-necrosis factor and γ-interferon (IFN) induce nitric oxide (NO) synthase (iNOS) in a variety of cells including macrophages. L-Arginine-derived NO serves as a cytotoxic molecule that plays a key role in the antimicrobial activity of activated macrophages, and may play a role in the process of allograft rejection (Stuehr & Nathan, 1989; Nathan & Hibbs, 1991; Nathan, 1992; Stuehr & Griffith, 1992; Green & Nacy, 1993; Langrehr *et al.*, 1993). The formation of NO can be inhibited by competitive inhibitors of NOS such as the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA). The induction of iNOS in various cells *in vitro* can be inhibited by glucocorticoids, dihydropyridine calcium channel modulators and by certain cytokines, e.g. interleukin-4 (IL-4), IL-8, IL-10, transforming growth factor β (TGFβ) and platelet-derived growth factor (Stuehr & Nathan, 1989; Radomski *et al.*, 1990; Nathan & Hibbs, 1991; Nathan, 1992; Cunha *et al.*, 1993; Green & Nacy, 1993; Langrehr *et al.*, 1993; Szabó *et al.*, 1993a).

Polyamines are endogenous regulators of cell proliferation, differentation, functional activation and macromolecular biosynthesis although their mechanism of action is not clear (Gaugas, 1980; Selmeci *et al.*, 1985; Morgan, 1987). Certain polyamines inhibit the production of nitrite in immunostimulated macrophages (Szabó *et al.*, 1994; Pollack *et al.*, 1994). We have recently shown that spermine inhibits the production of NO in cultured J774.2 macrophages stimulated with bacterial endotoxin (lipopolysaccharide; LPS) or with γ-interferon (IFN) in the presence, but not in the absence, of foetal calf serum (FCS) (Szabó *et al.*, 1994). This study was designed to characterize this inhibitory action of spermine, by investigating in immuno-stimulated J774.2 macrophages in culture (i) whether polyamines other than spermine also inhibit nitrite accumulation, (ii) whether spermine has any direct effect on the activity of the iNOS, (iii) whether polyamines scavenge NO (and thereby e.g. form NO:spermine adducts) or alter the formation of nitrite and/or nitrate from NO, (iv) whether metabolism of polyamines plays a role in their inhibitory action, (v) the specificity of the serum needed for the inhibition and (vi) whether spermine also inhibits the induction of the cytokine-inducible isoform of cyclo-oxygenase (COX-2) or the production of tumour necrosis factor α (TNFα).

Methods

Cells and culture conditions

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) with 4×10^{-3} M L-glutamine and 10% foetal calf serum (FCS) as described (Szabó *et al.*, 1993b). Cells were cultured in 96-well plates with 200 μl culture medium until they reached confluence.

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Experimental protocols

To induce iNOS, fresh culture medium containing *E. coli* LPS (1 μg ml^{-1}) or IFN (50 U ml^{-1}) was added. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of the polyamines spermine, spermidine, putrescine, cadaverine and N^1-acetylspermine (all at 10^{-6}–10^{-4} M) or L-NMMA (3×10^{-3} M) on nitrite production, the above agents were added to the medium either simultaneously with or 6 h after stimulation of iNOS induction with LPS or IFN. To investigate whether spermine affects the activity or the induction of iNOS, spermine was also added 0.5 h before as well as 2, 4 or 6 h after LPS; nitrite production was measured 24 h after LPS treatment. The effect of spermine or N^1-acetylspermine (10^{-6}–10^{-4} M) on LPS- or IFN-induced nitrite production was also tested in DMEM without FCS. Moreover, the effect of spermine (10^{-4} M) on the production of nitrite by LPS (1 μg ml^{-1}) was also investigated at various concentrations of FCS (0.01, 0.1, 0.3, 1 and 3%). The effect of spermine (10^{-5} M) on nitrite production was also studied in DMEM in the presence of various concentrations (1 and 10%) of bovine (foetal calf, newborn calf or adult) sera as well as in the presence of human or rat serum. In addition, the effect of LPS-binding protein (LBP, 0.01–10 μg ml^{-1}) was tested on the induction of NOS both in the presence and absence of FCS.

Isonicotinic acid hydrazine (INH) and hydroxylamine (1 μM–1 mM), were used as inhibitors of spermine oxidase, an enzyme present in FCS (Morgan, 1987; Seiler, 1990). Phenylhydrazine (10^{-5} and 10^{-4} M), an aldehyde inhibitor, and genistein, a tyrosine kinase inhibitor (Levitzki, 1992), were also tested on the inhibition of the nitrite accumulation by spermine (10^{-4} M) in macrophages stimulated by LPS.

To assess whether spermine or spermine dialdehyde (see below) reacts directly with nitrite or nitric oxide, spermine (10^{-4} M) was added to either sodium nitrite (3×10^{-6}–10^{-4} M) or to the NO donor, 3-morpholinosydnonimine-*N*-ethylcarbamide (SIN-1, 10^{-5}–10^{-3} M) (Feelisch *et al.*, 1989) and nitrite was determined 3 h thereafter.

The effect of spermine dialdehyde (SDA) and malon dialdehyde (MDA, both at 10^{-7}–10^{-4} M) on nitrite production by IFN was studied in DMEM with or without FCS. As SDA has a short half-life in culture medium (see Discussion), it was added three times to the medium (together with LPS as well as 2 and 4 h thereafter).

Nitrite measurement

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of J774.2 macrophages as previously described (Szabó *et al.*, 1993a,b). Nitrite was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of cell culture medium. The optical density at 550 nm (OD_{550}) was measured by using an Anthos 1.2.1. microplate reader (Anthos Labtechnik, Salzburg, Austria). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium.

Cell viability and respiration

Cell viability was measured in 96-well plates using a colorimetric assay based on the standard kit obtained from Sigma (LDH Kit [LD]). Cells treated with 1% Triton X-100 for 24 h served as positive control.

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] to formazan (Szabó *et al.*, 1993a). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1} for 60 min). Culture medium was removed by aspiration and the cells solubilized in dimethylsulphoxide (DMSO, 100 μl). The extent of reduction of MTT to formazan within cells was quantitated by meausurement of OD_{550} using the Anthos microplate reader. The calibration curve for MTT-formazan was prepared in DMSO. MTT-formazan production by cells was calculated as μg MTT-formazan per well per min and expressed as a percentage of the values obtained from untreated cells.

Nitric oxide synthase assay

J774.2 cells were grown to confluence in 75 cm^2 culture flasks containing 40 ml DMEM. To induce iNOS, fresh culture medium containing *E. coli* LPS (1 μg ml^{-1}) was added. After 8 h, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and harvested with a Teflon cell-scraper into 5 ml volume of homogenization buffer composed of: 50 mM Tris HCl, 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-mercaptoethanol and 1 mM phenylmethylsulphonyl fluoride (pH 7.4). Cell suspensions were centrifuged at 800 *g* for 10 min and the pelleted cells were homogenized in buffer (composition as above) on ice using an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i. Br., Germany). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described (Szabó *et al.*, 1993b). Briefly, cell homogenate (100 μl) was incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM) and EGTA (5 mM) for 35 min at 37°C in HEPES buffer (pH 7.5). The reaction was stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.). Experiments performed in the absence of NADPH or in the presence of L-NMMA (3 mM) determined the extent of [^{3}H]-L-citrulline formation independent of a specific iNOS activity. By co-incubating the homogenates together with spermine (10^{-7}–10^{-4} M) in the presence of the above-mentioned cofactors for 35 min, we have investigated the direct effect of spermine on iNOS activity.

NO measurements using chemiluminescence

Appropriate volumes (150–300 μl) of samples of standard sodium nitrite and sodium nitrate solutions were injected through a septum into a reaction flask containing the reagent solution (see below), which was continuously flushed with nitrogen. The nitrogen and any NO produced was drawn through moisture and acid traps into a Dasibi 2107 chemiluminescence analyser (Dasibi Co, Japan), where NO was quantitated by its reaction with ozone (Cox & Frank, 1982; Walters *et al.*, 1987).

The reagent solutions used were: (a) 10 ml glacial acetic acid and 0.25 mg sodium iodide in 60 ml water at 85°C for conversion of nitrite and any potential spermine:NO adducts to NO; (b) 50 ml water containing 15.5 mg ferrous ammonium sulphate and 8 mg ammoniummolybdate for the conversion of both nitrate and nitrite to NO (Walters *et al.*, 1987). Moreover, NO was released from the authentic spermine: NO adduct (SperNO; a kind gift from Dr Larry Keefer at the National Cancer Institute, Frederick, U.S.A.) in the molar ratio of ~1.7 M NO/1 M adduct (i.e. ~85% of bound NO is released, $n = 3$). Under these conditions, N-nitroso compounds would also give a substantial response, and C-nitroso compounds a slight response.

All comparisons of NO detector responses were made using the same preparation of reagent solution, and dilution of the reagent solution did not exceed 10%. Under these conditions the coefficient of variation is <7%.

Addition of spermine to a solution of NO in water made no difference in the chemiluminescence response when it was injected into the reaction flask containing water at room

temperature. Repeated injections of NO, nitrite, nitrate or spermine:NO adducts over 30 min gave constant responses.

Measurements of tumour necrosis factor α

Murine TNFα was measured in 100 μl samples of cell culture supernatant using an ELISA kit (Genzyme, Kings Hill, Kent, UK) according to the manufacturer's instructions. Briefly, the kit is based on a solid-phase enzyme immunoassay employing the multiple antibody sandwich principle. A hamster monoclonal antibody specific for murine TNFα was coated on microtitre wells in a 96-well plate. TNFα present in standard, samples and tissue culture supernatants was captured by the solid-phase monoclonal antibody. Then a goat polyclonal anti-murine antibody, which binds to multiple epitopes on the TNFα contained on solid phase, was added. A third antibody, horseradish peroxidase-conjugated rabbit anti-goat IgG, was then added to bind anti-murine TNFα: murine TNFα immune complexes. The peroxidase enzyme was then reacted with peroxide substrate and OPD (chromagen) to produce colour which was proportional in intensity to the amount of TNFα present. Colour intenstiy was quantitated by measuring absorbance of 492 nm using the Anthos microplate reader. The presence of spermine (10^{-4} M) did not affect this assay ($n = 4$).

Measurement of prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ was measured in 100 μl samples of cell culture supernatant using a specific radioimmunoassay (Salmon, 1978). The presence of spermine (10^{-4} M) did not affect this assay ($n = 4$).

Materials

N^1-acetylspermine trihydrochloride, acrolein, bacterial lipopolysaccharide (*E. coli*, serotype No. 0127:B8), cadaverine hydrochloride, calmodulin, dexamethasone phosphate, DMEM, L-glutamine, hydroxylamine, isonicotinic acid, MTT, MTT-formazan, NADPH, phenylhydrazine hydrochloride, prostaglandin $F_{2\alpha}$ and anti-prostaglandin $F_{2\alpha}$ antibody, putrescine hydrochloride, sodium nitrite, spermidine hydrochloride, spermine hydrochloride and Dowex 50W anion exchange resin was obtained from Sigma Chemical Co. (Poole, Dorset, UK). N^G-methyl-L-arginine monoacetate (L-NMMA) and genistein were obtained from Calbiochem (Nottingham, UK). L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire, U.K.). Foetal and newborn calf serum and bovine serum were obtained from Advanced Protein Products (West Midlands, UK). Human serum and rat serum were prepared in our laboratory. Murine γ-interferon was obtained from Genzyme (West Malling, Kent, UK). Malon dialdehyde was purchased from Fluka (Gillinham, Dorset, UK). SIN-1 was a kind gift from Dr Rainer Henning at the Cassella AG (Frankfurt, Germany). Spermine dialdehyde was a kind gift from Drs Cathy Lau and Elizabeth Wang at the Marion Merrel Dow Laboratories (Don Mills, Ontario, Canada). Rabbit LPS-binding protein (LBP) was a kind gift of Dr Richard Ulevich at the Scripps Research Institute (La Jolla, CA, U.S.A.).

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean of n observations. One-way

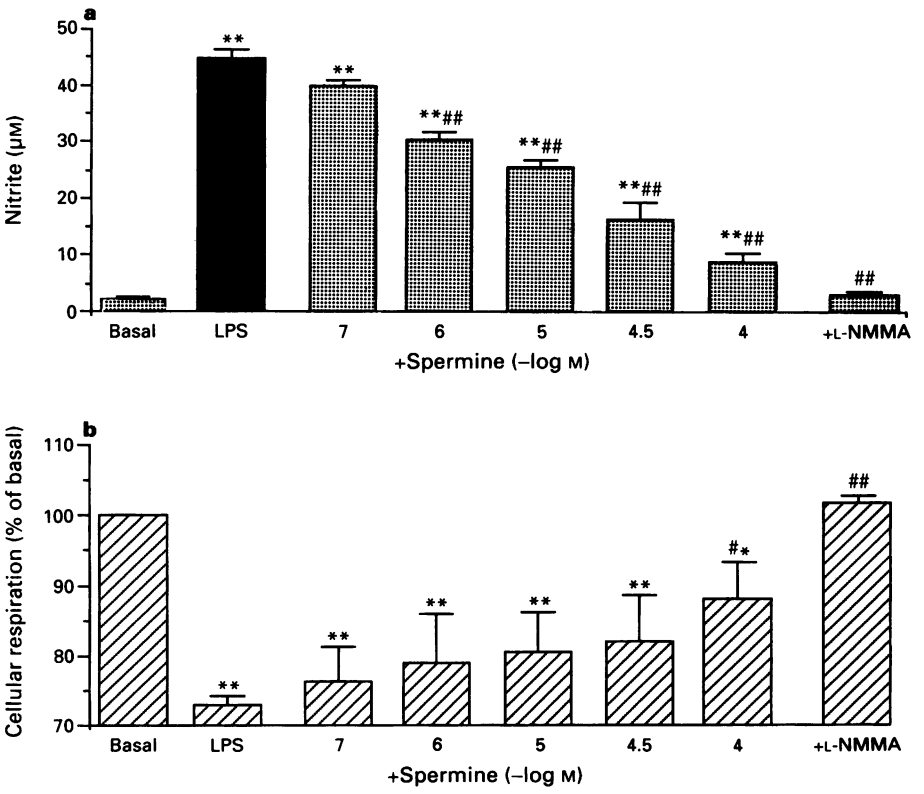

Figure 1 Spermine dose-dependently inhibits the formation of nitrite in J774.2 macrophages stimulated by LPS, without depressing cellular respiration. Depicted are the effects of spermine (10^{-7}–10^{-4} M) or L-NMMA (3×10^{-3} M) on nitrite accumulation (a), and on mitochondrial respiration (b) in J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h. Data are expressed as means ± s.e.mean of $n = 9$ wells from 3 experimental days. Formazan production in cells not treated with LPS amounted to 0.32 ± 0.07 μg formazan produced per well per min and was taken as 100%. $*P<0.05$ and $**P<0.01$ represent significant differences when compared to basal values. $\#P<0.05$ and $\#\#P<0.001$ represent significant differences when compared to the values seen with LPS. For abbreviations used in this and subsequent legends, see text.

analysis of variance was performed followed by an adjusted t test with P values corrected by the Bonferroni method. A P value less than 0.05 was considered to be statistically significant.

Results

Spermine inhibits the formation of NO

Nitrite concentration in the medium of J774.2 macrophages stimulated with LPS was 45 ± 2 μM after 24 h. Co-administration of spermine with LPS dose-dependently reduced the accumulation of nitrite in the medium (Figure 1a). The production of nitrite by these cells is due to NO, for the NOS inhibitor L-NMMA also inhibited nitrite accumulation.

The induction of iNOS in these cells was associated with an inhibition of mitochondrial respiration, which was also partially prevented by spermine (Figure 1b). Higher concentrations of spermine (at 10^{-3} M and above) caused a direct inhibition of mitochondrial respiration in J774.2 macrophages (data not shown). LPS caused a slight ($5 \pm 1\%$) increase in the LDH activity measured the culture medium of activated macrophages, which was unaffected by spermine or L-NMMA (not shown).

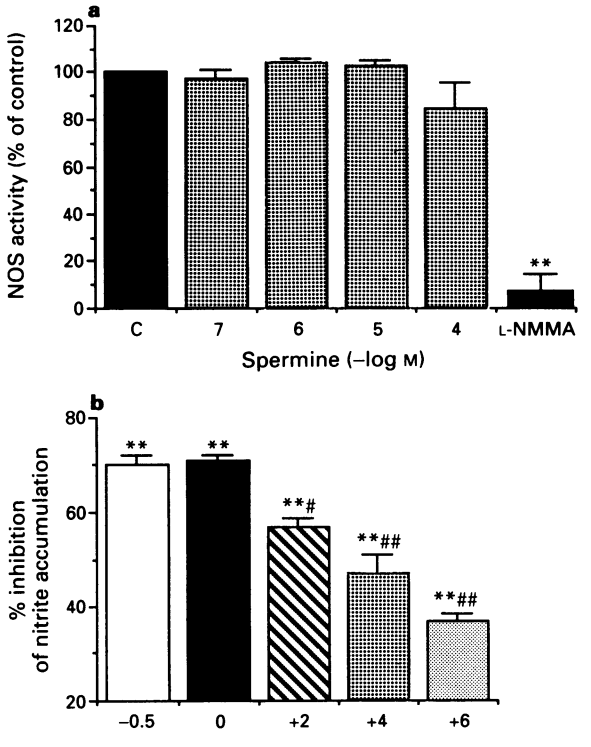

Figure 2 Spermine inhibits the induction of iNOS in response to LPS but it is not a direct inhibitor of iNOS activity. (a) The effect of spermine (10^{-6}–10^{-4} M) or L-NMMA (3×10^{-3} M) on the activity of iNOS in the homogenate of J774.2 macrophages. Data are expressed as means ± s.e.mean of triplicate determinations. iNOS activity in the homogenates was 2.07 ± 0.11 pmol mg^{-1} protein min^{-1} and was taken as 100%. $^{**}P<0.01$ represents significant inhibition. (b) The effect of spermine (3×10^{-5} M), when applied 0.5 h before LPS (– 0.5), together with LPS (0) or 2, 4 or 6 h after LPS (+ 2, + 4 and + 6, respectively) on nitrite accumulation in the medium of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) 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 inhibition, when compared to control; $\#P<0.05$ and $\#\#P<0.01$ represent significant decrease in the extent of inhibition, when spermine is applied 2, 4 or 6 h after LPS.

Spermine inhibits the induction, but not activity of iNOS

Spermine did not affect citrulline formation in the homogenates of J774.2 cells that had been pretreated for 8 h with LPS (Figure 2a). The inhibitory effect of spermine on nitrite accumulation in the culture medium of J774.2 macrophages was reduced in time-dependent fashion when spermine was given 2, 4 or 6 h after LPS to the tissue culture medium (Figure 2b). When given 6 h after LPS, the inhibitory effect of spermine (3×10^{-5} M) was reduced from $71 \pm 1\%$ to $37 \pm 2\%$ ($n = 6$, $P<0.01$).

Changes in the formation of nitrite, nitrate or NO: spermine adducts are not involved in the inhibitory action of spermine

Spermine does not interfere with the Griess reaction, for spermine given to sodium nitrite did not alter the levels of nitrite measured (Figure 3a), nor did it alter the accumulation of nitrite caused by the NO donor SIN-1 (Figure 3b).

Nitrite was also measured by chemiluminescence and the respective values obtained were similar to the NO production measured with the Griess reaction either under conditions that would convert only nitrite to NO (Figure 4a), or under conditions that would convert nitrite, nitrate as well as potential spermine:NO adducts or N-nitroso-compounds to NO (not shown).

The inhibitory effect of spermine on iNOS induction is dependent on bovine serum

Spermine did not cause an inhibition of nitrite accumulation when FCS was omitted from the medium (Figure 5a). Under

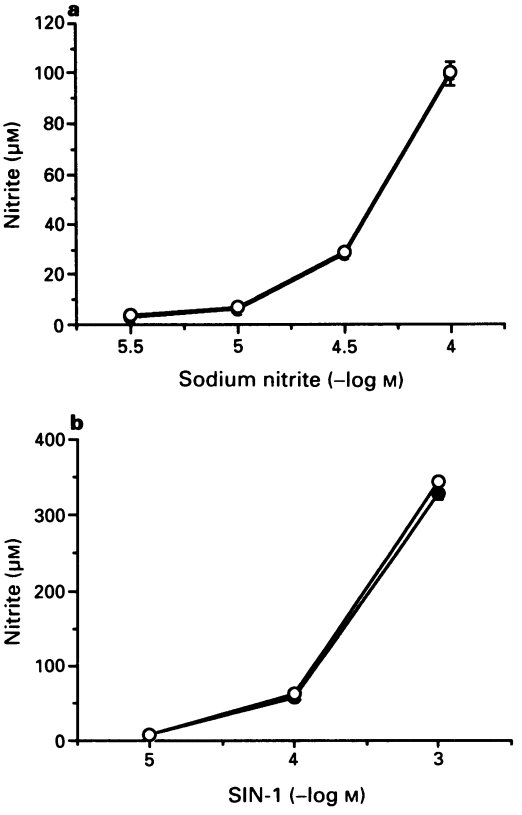

Figure 3 Spermine does not interfere with the Griess reaction and does not scavenge NO. Effect of spermine (10^{-4} M) on the levels of nitrite when sodium nitrite (3×10^{-6} M–10^{-4} M) is added to the culture medium (a) or on nitrite accumulation in the medium in response to the NO donor compound SIN-1 (10^{-5}–10^{-3} M). Data are expressed as means ± s.e.mean of quadriplicate wells: (●) nitrite levels in the absence of spermine; (○) nitrite levels in the presence of spermine.

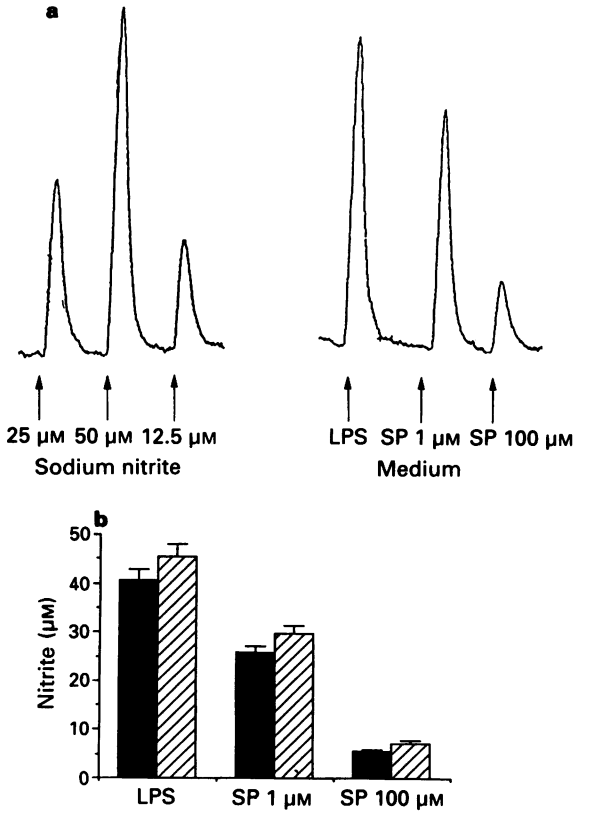

Figure 4 Spermine does not affect the conversion of NO to nitrite. (a) Chemiluminescence NO signals with various doses of sodium nitrite (left panel) or with tissue culture medium of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h without (LPS) or with spermine (SP 1 μM and SP 100 μM). (b) Nitrite levels in the medium of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h without (LPS) or with spermine (SP 1 μM and SP 100 μM), as determined by Griess reaction (hatched columns) or chemiluminescence (solid columns). Data are expressed as means ± s.e.mean of triplicate determinations.

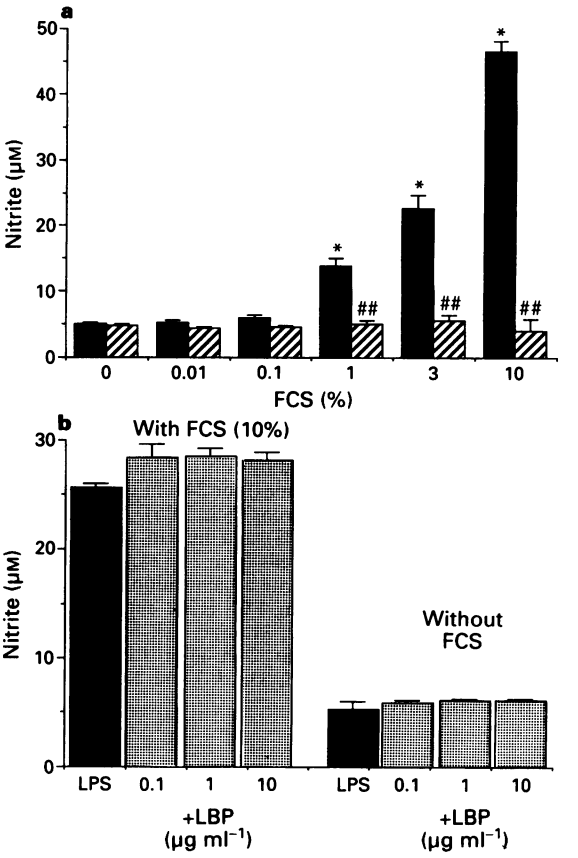

Figure 5 A serum factor, which is not LBP, is required for the induction of iNOS by LPS. Depicted are nitrite levels in the medium of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h. (a) Nitrite production at various concentrations (0–10%) of FCS in the absence (solid columns) and in the presence of spermine (10^{-4} M, hatched columns). (b) Effect of LBP (0.1–10 μg ml^{-1}) on nitrite production in the presence of 10% FCS (left panel) and without FCS (right panel). Data are expressed as means ± s.e.mean of $n = 9$ wells from three experimental days. Nitrite concentrations in wells not treated with LPS were <2.6 μM at all concentrations of FCS and were not affected by LBP. $^{**}P<0.01$ represent significant differences when compared to basal values. $\#\#P<0.01$ represent significant differences with spermine when compared to the values seen with LPS alone.

these conditions, the formation of nitrite by LPS was largely attenuated. Re-addition of FCS restored the ability of LPS to induce nitrite, and hence, iNOS (Figure 5a). When iNOS was induced by γ-interferon (50 U ml^{-1}), spermine caused a concentration-dependent inhibition of nitrite accumulation only in the presence of FCS. In contrast to LPS, the induction of iNOS by γ-interferon was enhanced when FCS was omitted from the culture medium (Figure 6).

We have tested the possibility that the lack of LPS-binding protein, normally present in all animal sera including FCS, is responsible for the lack of iNOS induction in the absence of FCS. Surprisingly, addition of LBP (1–30 μg ml^{-1}) did not enhance iNOS induction by LPS (Figure 5b) or by IFN (not shown) either in the presence or absence of FCS.

The inhibitory effect of spermine on iNOS induction by LPS or γ-interferon was also seen when FCS was replaced with bovine newborn serum (NS) or adult bovine serum (BS) (10% or 1%) in the culture medium. In fact, in the presence of NS or BS, the inhibition of nitrite accumulation by spermine (10^{-5} M) was slightly enhanced (Figure 7).

Role of oxidized metabolites of spermine in the inhibitory action

In the presence of FCS (10%), the inhibitory effect of spermine on nitrite production elicited by LPS was partially prevented by INH or hydroxylamine (0.1–1 mM, inhibitors of spermine oxidase) (Figure 8). These agents themselves did not significantly affect the production of nitrite by LPS in the

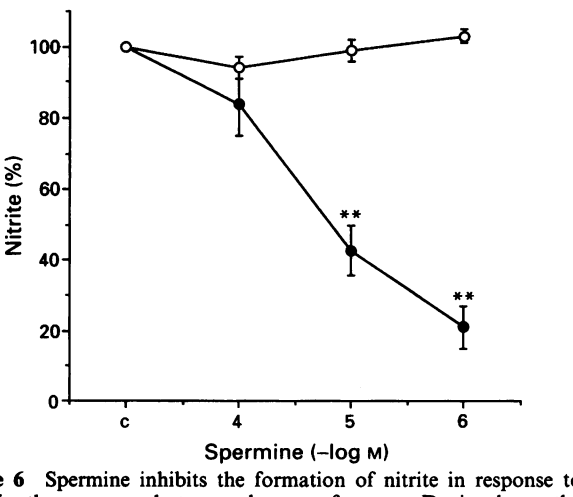

Figure 6 Spermine inhibits the formation of nitrite in response to IFN in the presence but not absence of serum. Depicted are the effect of spermine (10^{-7}–10^{-4} M) on nitrite accumulation in J774.2 macrophages stimulated by IFN (50 u ml^{-1}) for 24 h in the presence (●) or absence (○) of FCS (10%). Data are expressed as means ± s.e.mean of $n = 9$ wells from 3 experimental days. Nitrite in the medium at 24 h after IFN was 10 ± 1 μM in the absence and 28 ± 4 μM in the presence of serum; these values were considered as 100%. $^{*}P<0.05$ and $^{**}P<0.01$ represent significant inhibition of nitrite accumulation by spermine.

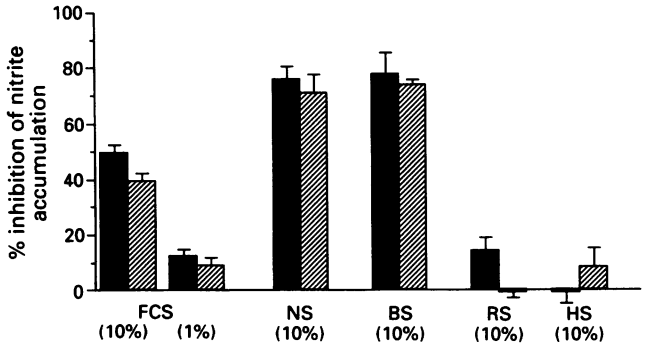

Figure 7 The inhibition of nitrite formation by spermine is only seen in the presence of bovine sera but not rat or human serum. Depicted are the effect of spermine (10^{-5} M) on nitrite accumulation in J774.2 macrophages stimulated by IFN (50 U ml^{-1}, solid columns) or LPS (1 μg ml^{-1}, hatched columns) for 24 h in the presence of FCS (FCS, 10 and 1%), newborn serum (NS, 10%), adult bovine serum (BS, 10%), rat serum (RS, 10%) and human serum (10%). Data (means ± s.e.mean of $n = 6-9$ wells from 2–3 experimental days) are expressed as % inhibition of nitrite accumulation. Nitrite production in the culture medium containing FCS (10% and 1%), NS, BS, RS and HS amounted to 71.6 ± 2.2; 31.6 ± 0.9; 35.5 ± 2.9; 39.1 ± 2 μM; 37.2 ± 1.0 and 37.6 ± 1.7 μM at 24 h after LPS and 48.1 ± 0.7; 66.1 ± 3.7; 42.7 ± 0.5, 45.5 ± 0.7; 33.5 ± 3 and 27.0 ± 1.4 μM at 24 h after IFN, respectively.

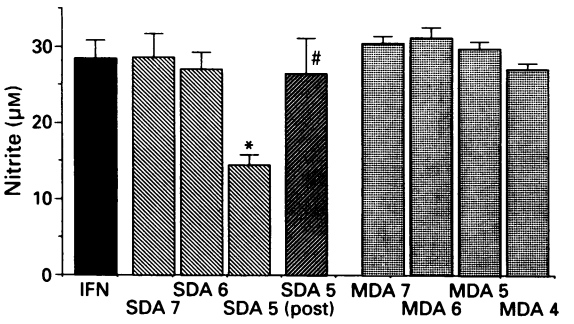

Figure 9 Spermine dialdehyde but not malon dialdehyde inhibits the induction of iNOS by IFN in serum-free medium. Depicted are the effects of spermine dialdehyde (SDA, $10^{-7}-10^{-5}$ M) and malon dialdehyde (MDA, $10^{-7}-10^{-5}$ M) applied together with IFN, or the effect of SDA applied 6 h after IFN (10^{-5}, post) on the nitrite accumulation in the medium of J774.2 macrophages stimulated by IFN (50 U ml^{-1}) for 24 h. Data are expressed as means ± s.e.mean of $n = 6-9$ wells from 2–3 experimental days. $^{*}P < 0.05$ represents significant inhibition of the effect on nitrite accumulation. $\#P < 0.05$ represents significant difference between the effect of SDA (10^{-5}) when applied together with LPS vs when given 6 h after LPS.

absence of spermine, nor did they affect mitochondrial respiration (data not shown). The aldehyde inhibitor, phenylhydrazine (10 or 100 μM), also attenuated the inhibitory effect of spermine (100 μM) on LPS-induced nitrite accumulation from 70 ± 9% (control) to 30 ± 2% and 8 ± 13%, respectively, ($n = 6$) (Figure 8).

Spermine can be metabolized by spermine oxidase to, *inter alia*, spermine dialdehyde (SDA) and malon dialdehyde (MDA). We found that SDA (10^{-5} M) inhibited the accumulation of nitrite in the culture medium of J774.2 cells stimulated with INF in the absence of serum (without being cytotoxic, not shown), but this effect was completely absent when it was added 6 h after IFN (Figure 9). In contrast, MDA (up to 10^{-4} M) did not inhibit nitrite formation (Figure 9). Like spermine (Figure 3), SDA did not directly affect the detection of nitrite by the Griess reaction, nor did it act as a scavenger of NO ($n = 6$, not shown).

The acetylated spermine metabolite N^1-acetylspermine caused an inhibition of nitrite accumulation that was similar to the one seen with spermine itself (Figure 10). Spermidine was less potent than spermine or its N^1-acetylated derivative,

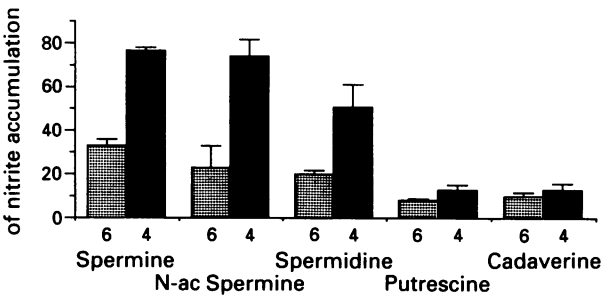

Figure 10 Polyamines inhibit LPS-induced nitrite production with variable potencies. Effect of spermine, N^1-acetyl-spermine (N-ac Spermine), spermidine, putrescine and cadaverine (10^{-6}, stippled columns and 10^{-4} M, solid columns) on nitrite accumulation in the medium of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h. Nitrite concentration at 24 h after LPS was 60.7 ± 1.4 μM. Data are expressed as means ± s.e.mean of $n = 9$ wells from 3 experimental days.

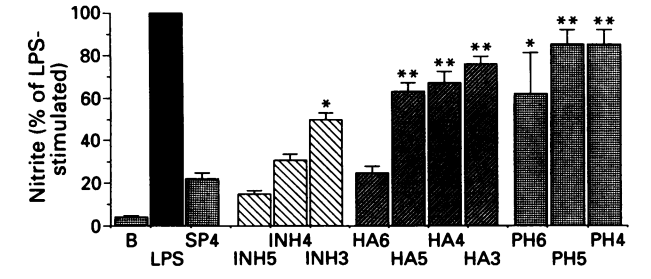

Figure 8 The inhibitory effect of spermine on nitrite accumulation is prevented by inhibitors of polyamine oxidase or by an aldehyde inhibitor. Depicted are the effects of isonicotinic acid (INH, $10^{-5}-10^{-3}$ M), hydroxylamine (HA, $10^{-6}-10^{-3}$ M) and phenhydrazine (PH $10^{-6}-10^{-4}$ M), applied together with spermine, on the inhibitory effect of spermine (SP, 10^{-4} M), on nitrite accumulation in the medium (with 10% FCS) of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h. Data are expressed as means ± s.e.mean of $n = 6-9$ wells from 2–3 experimental days. $^{*}P < 0.05$ and $^{**}P < 0.01$ represent significant inhibition of the effect of spermine by INH, HA or PH.

whereas putrescine and cadaverine were virtually ineffective (Figure 10). The presence of foetal calf serum was also required for the inhibitory effect of spermidine or N^1-acetylspermine on LPS-induced nitrite accumulation (not shown).

Acrolein may be a metabolite of spermine under certain conditions. It is however, unlikely that acrolein significantly contributes to the effects of spermine or SDA on NO formation for acrolein (up to 10^{-5} M), unlike spermine, did not inhibit nitrite formation in response to LPS, while it became cytotoxic at higher concentrations ($n = 3$).

Spermine does not inhibit LPS-induced production of TNFα or prostaglandin $F_{2\alpha}$

In addition to causing the expression of iNOS and, hence, NO formation, LPS also induces the expression of a novel isoform of cyclo-oxygenase (COX-2) resulting in the accumulation of large amounts of cyclo-oxygenase products such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Moreover, LPS causes the formation of murine TNFα in LPS-activated J774.2 cells (see: Discussion). Interestingly, this LPS-induced production of $PGF_{2\alpha}$ and murine TNFα was unaffected by spermine ($10^{-6}-10^{-4}$ M, Figure 11a and b).

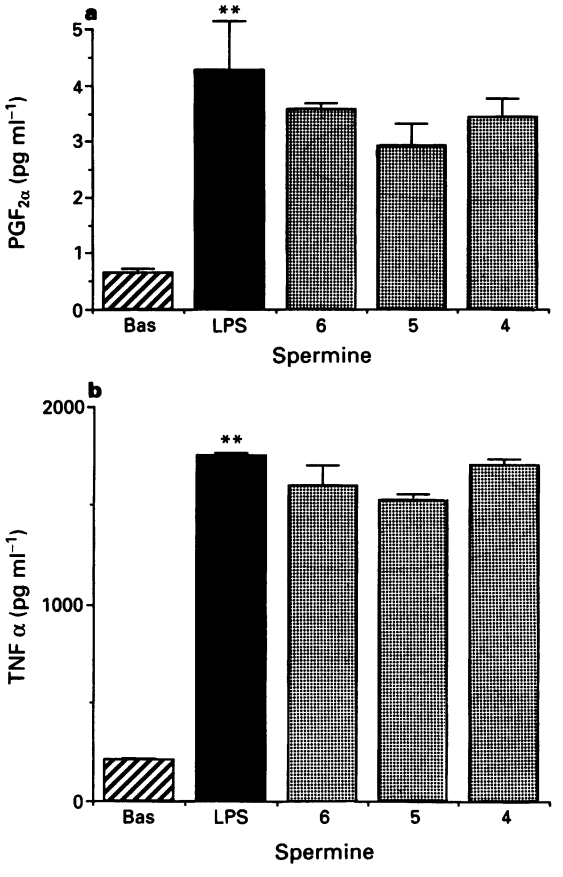

Figure 11 Spermine does not inhibit the LPS-induced induction of cyclo-oxygenase or the LPS-induced production of TNFα. Depicted are the effects of spermine (10^{-6}–10^{-4} M) on LPS-induced production of $PGF_{2\alpha}$ and (a) and TNFα (b) in the medium of J774.2 macrophages stimulated by LPS (1 μg ml^{-1}) for 24 h. Data are expressed as means ± s.e.mean of $n = 6$–9 wells from 2–3 experimental days. $^{**}P < 0.01$ represents significant increase in the production of $PGF_{2\alpha}$ or TNFα in response to LPS.

Discussion

The present study demonstrates that spermine, N^1 acetylspermine and spermidine, but not putrescine or cadaverine, inhibit the accumulation of nitrite in the medium of LPS-stimulated J774.2 macrophages. This inhibitory effect is dependent on the presence of bovine serum in the cell culture medium.

The hypothesis that spermine interferes with either induction or activity of iNOS, but not with NO directly (or with its detection), is supported by the findings that (i) neither spermine nor its metabolites scavenge NO or affect its conversion to nitrite or nitrate, (ii) spermine or its metabolites do not scavenge NO released by SIN-1 or bind to nitrite and (iii) similar levels of NO were determined by measuring nitrite with either the Griess reaction or chemiluminescence. Adducts of NO with spermine have recently been synthesized (Maragos *et al.*, 1991; Morley *et al.*, 1993). Formation of such adducts in the medium is not the mechanism by which spermine reduces the accumulation of nitrite in the medium, for any adducts of spermine with NO would also be converted to NO under the conditions used in our chemiluminescence experiments.

Spermine had no effect on the formation of citrulline by homogenates of J774.2 cells in which iNOS had previously been induced by LPS. Taken with the finding that spermine had a reduced potency when given to cells several hours after LPS, this strongly suggests that polyamines inhibit the induction of iNOS, rather than directly inhibiting iNOS activity.

The inhibitory effect of spermine was entirely dependent on the presence of serum. Moreover, the inhibition was observed either in the presence of foetal, newborn or adult bovine serum. Bovine serum contains polyamine oxidase (also known as spermine oxidase) (Tabor *et al.*, 1954; Yamada & Yasonobu, 1962) whereas spermine oxidase is not present in normal human or rodent sera (see Morgan, 1985; 1987). Our experiments with the spermine oxidase inhibitors, hydroxylamine and isonicotinic acid (Morgan, 1987; Seiler, 1990) demonstrate that the presence of this enzyme in the culture medium is a prerequisite to demonstrate an inhibitory effect of spermine on nitrite formation by LPS-activated J774.2 macrophages. Thus, we suggest that spermine oxidase converts spermine into active inhibitor(s) of iNOS induction by LPS or IFN. It is noteworthy that the levels of spermine oxidase in bovine serum increase after birth (Gahl & Pitot, 1982), which may explain our finding here (see Figure 7) that the degree of inhibition of iNOS induction afforded by spermine tended to increase when newborn or adult serum was used instead of FCS.

Spermine can be oxidized to N^1-acetylspermine by the enzymes polyamine oxidase and N-acetyltransferase (Gaugas, 1980; Selmeci *et al.*, 1985; Morgan, 1987). N^1-acetylspermine can then be further metabolized to e.g. carboxyethylputrescine, putrescine and/or aminopropionaldehyde (Gaugas, 1980; Selmeci *et al.*, 1985; Morgan, 1987) (see Figure 12). The finding that N^1-acetylspermine inhibits LPS-induced nitrite accumulation only in the presence, but not in the absence of serum, strongly suggests that the formation of N^1-acetylspermine and its subsequent further conversion may be involved in the inhibitory effect of spermine. Lower polyamine metabolites (such as putrescine), however, are much less potent inhibitors than spermine in J774.2 cells activated by LPS or IFN and, hence, are unlikely to play a major role in this inhibitory effect.

The inhibition of iNOS induction afforded by spermine in LPS-activated macrophages was prevented by phenylhydrazine, a compound that directly inactivates aldehydes. Thus, we suggest that aldehyde metabolites of spermine are involved in the observed inhibition. Such aldehyde metabolites could in principle directly inhibit the expression of iNOS or, alternatively, may be further metabolized to yield the active inhibitor. Potential aldehyde metabolites of spermine which may contribute to the inhibition of iNOS induction seen with spermine include spermine dialdehyde (SDA) or malon dialdehyde (MDA) which are generated from spermine by polyamine oxidases (Labib & Tomasi, 1981; Quash *et al.*, 1987; Lau *et al.*, 1990). MDA was ineffective in inhibiting nitrite production in LPS-activated macrophages, even when FCS was present, whereas SDA caused a significant inhibition of nitrite accumulation even in the absence of serum (Figure 9). However, the potency of SDA is less than the apparent potency of spermine. Thus, the formation of SDA by serum polyamine oxidases cannot fully account for the prevention of iNOS induction caused by spermine in J774.2 macrophages activated by LPS or IFN, especially for it is known that SDA has only a short half-life in the culture medium (Lau *et al.*, 1990). However, the formation of SDA may contribute to the inhibition of iNOS induction caused by spermine, and SDA may well synergize with other aldehyde metabolites. Thus, other, yet unidentified aldehyde metabolites of spermine must also contribute to the inhibition of iNOS induction seen with polyamines. Other aldehyde metabolites of spermine include small molecules such as propionaldehyde. Propionaldehyde has recently been proposed as an active aldehyde metabolite responsible for the inhibition of mast cell secretion by polyamines (Vliagoftis *et al.*, 1992).

The mechanism by which spermine or spermine-derived aldehydes inhibit the expression of iNOS warrants further investigation. Pharmacological agents that inhibit the induction of iNOS include glucocorticoids, growth factors, thrombin and dihydropyridine-type calcium channel modulators

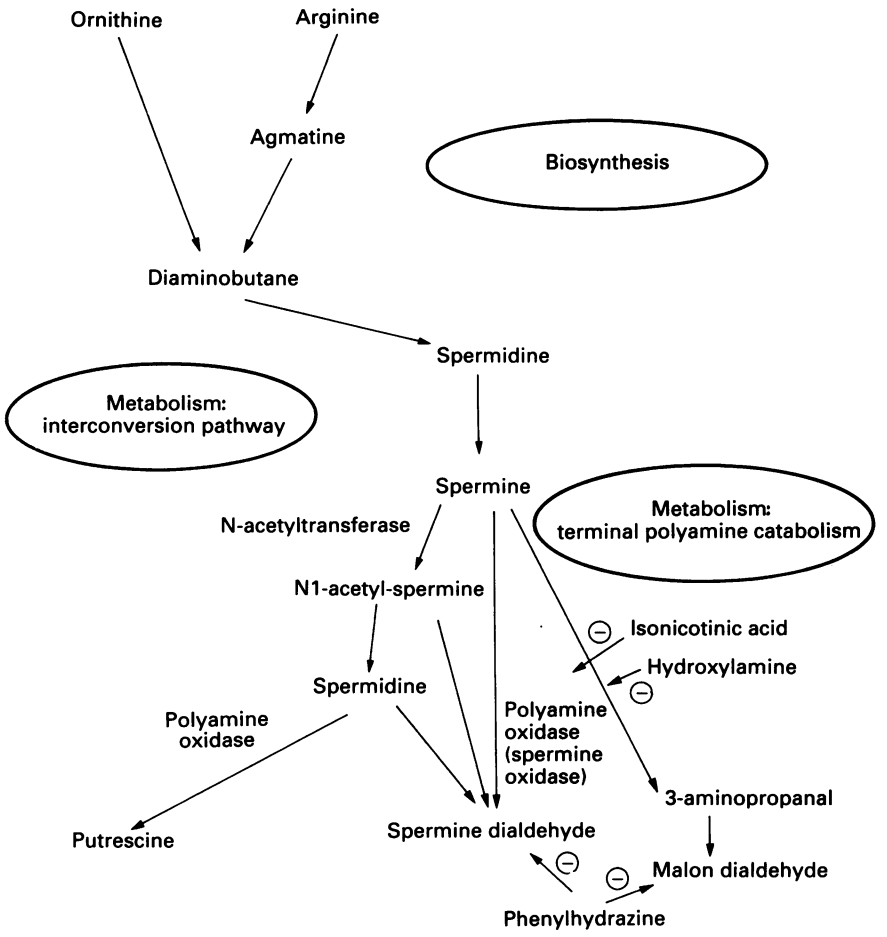

Figure 12 Metabolic pathways of polyamines in the presence of bovine serum.

(see Introduction), but their exact mode of action is not known. The induction of iNOS by LPS is, at least in part, dependent on the release of secondary cytokines such as TNFα and INF (*in vitro*) or TNFα, INF and interleukin-1 (*in vivo*) (Liew *et al.*, 1990; Evans *et al.*, 1992; Fortier *et al.*, 1992; Green *et al.*, 1993; Thiemermann *et al.*, 1992; Szabo *et al.*, 1993c). It is unlikely, however, that spermine or its metabolites inhibit the release of secondary cytokines for (i) spermine did not inhibit the release of TNFα in response to LPS; and (ii) spermine also inhibited nitrite production when iNOS was induced by IFN. Inhibition of tyrosine kinase has recently been shown to inhibit the induction of iNOS by LPS and IL-1 in cultured vascular smooth muscle cells (Marczin *et al.*, 1993) and in mouse peritoneal macrophages (Dong *et al.*, 1993). This mechanism is also involved in the induction of iNOS in J774.2 cells, for genistein, a tyrosine kinase inhibitor prevents nitrite production when given together with LPS, but its action is significantly reduced when given 6 h after the stimulus of induction (Szabo & Southan, unpublished data). There is also evidence for the involvement of the gene promoter region nuclear factor $NF_{\kappa}B$ (Sherman *et al.*, 1993) in the induction of iNOS. It is tempting to speculate that metabolites of spermine inhibit iNOS induction by interfering with the activity of tyrosine kinase and/or the activation of $NF\text{-}_{\kappa}B$. Although the mechanisms of the inhibitory effects of polyamines on iNOS induction reported here are unclear, it is likely that aldehyde metabolites of spermine interfere at a relatively early stage with the signal transduction pathway leading to the induction, i.e. prior to, or at the level of transcription.

Interestingly, in the absence of serum, the induction of iNOS by LPS, but not by IFN, was largely reduced, and the induction could be restored with re-addition of serum. Thus, the presence of a serum factor is necessary for the initiation of the induction process itself, but only with LPS and not IFN. A likely candidate might have been LPS-binding protein (LBP), a plasma protein that is required for many immunological actions of LPS. LBP is known to bind to LPS in order to activate the CD14 receptor located on the cell membrane (Raetz *et al.*, 1993; Hewerr & Roth, 1993). Here we show, however, that the lack of iNOS induction in the absence of serum is not simply due to the absence of LBP in the culture medium, and it must, therefore, be related to other factor(s) present in the serum (e.g. soluble CD14, growth factors, cytokines, hormones) or to other properties of serum (e.g. adherence properties). It is, however, noteworthy that the LBP-CD14 system is usually more functional at lower concentrations of LPS, rather than the relatively high ones used in the present study (Raetz *et al.*, 1991; Hewerr & Roth, 1993).

Polyamines are present in high concentrations in foetal and neoplastic tissues and in seminal fluid (Morgan & Illei, 1980). These tissues represent antigenic sites/challenges that often do not elicit appropriate immune response (Normann, 1985; Bulmer, 1992). In the presence of FCS, spermine and spermidine, but to a much lesser extent putrescine or cadaverine, inhibit the immune response in LPS-stimulated primary cell cultures of the spleen (Byrd *et al.*, 1977). Lau and co-workers (1984; 1990) described an immunosuppressant factor, originally termed suppression inhibitory factor, obtained from the supernatant of cultured mutant human lymphocytes, which they later identified as SDA (also known as dioxidized spermine). SDA may be useful in preventing graft-versus-host reactions in bone marrow transplantation (Lau *et al.*, 1992; Wang *et al.*, 1990).

The present study tentatively links these observations to

the induction of iNOS, a major cytotoxic macrophage effector molecule. Thus our data not only identify a novel inhibitor of iNOS induction (SDA) but also describe a possible mechanism for immunosuppression seen in tissues or biological fluids that contain high levels of polyamines. Our findings may have implications for the mechanism of host immunosuppression seen in pregnancy or in tumour-bearing organisms. For instance, it is well documented that pregnancy is associated with high levels of polyamines and polyamine oxidases in the plasma and in the amniotic fluid (Gaugas, 1980; Morgan & Illei, 1980; Gahl *et al.*, 1982). Moreover, many tumour cells also contain and secrete large amounts of polyamines. As polyamines are also produced by bacteria (Tait, 1985), they may serve as suppressants of the host immune response by inhibiting the induction of iNOS.

It is now evident that many of the actions of polyamines are dependent on their conversion by spermine oxidase. These effects of polyamines include the inhibition of lymphocyte proliferation and other immunosuppressive actions (Labib & Tomasi, 1981; Quan *et al.*, 1990; Lea *et al.*, 1991): inhibition of histamine release (Vligoftis *et al.*, 1992), and inhibition of neutrophil activation (Ferrante *et al.*, 1986). We, therefore, investigated the effect of spermine on other LPS-induced processes including the induction of COX-2 and the induction of the production of TNFα. LPS and several pro-inflammatory cytokines induce a distinct isoform of cyclooxygenase (COX-2) *in vitro* and *in vivo* that plays a key role e.g. in chronic inflammation (Masferrer *et al.*, 1992a,b; Vane, 1993). Our data show that spermine does not inhibit the induction of either COX-2 or the production of TNFα. Thus, we conclude that aldehyde metabolites of spermine are relatively selective inhibitors of the signal transduction pathway leading to the expression of iNOS in cultured macrophages.

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