Polyamine regulation of nitric oxide production in LPS-activated macrophages

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Introduction

Polyamines are physiological cellular constituents essential for cell growth and differentiation, and regulate a multitude of cellular functions (1-4). Nitric oxide (NO) is an effector molecule in both the cardiovascular and nervous systems (5,6). Intracellularly, NO and polyamines are derived from arginine, the latter via the rate-limiting enzyme ornithine decarboxylase (ODC; 7). This enzyme, like the inducible nitric oxide synthase (iNOS), is induced by proinflammatory cytokines and bacterial lipopolysaccharide (LPS), resulting in enhanced enzyme activity and increased polyamine biosynthesis (8,9). While the increase in polyamine synthesis would have important implications for cell growth and proliferation, it is not clear how this might affect iNOS pathway.

Inhibition of polyamine biosynthesis impairs the phagocytic capacity of macrophages (10) and can block macrophage activation by tumour necrosis factor (11). Recently, exogenous polyamines have been shown to inhibit NO production in LPS-activated J774 cells (12) and by isolated neuronal NO synthase (13). However, these effects required relatively high concentrations of polyamines compared to those found in plasma and in intact cells (14), and appear to be due to aldehyde metabolites resulting from polyamine oxidation by the amine oxidase present in calf serum (15-17). In this study we have explored the effects of both endogenous and exogenous polyamines on the inducible L-arginine-NO pathway by examining whether inhibition of ornithine decarboxylase (ODC) and thus of polyamine biosynthesis (7), regulates NO production in lipopolysaccharide-activated J774 cells, a murine macrophage cell line.

Methods

Materials

Tissue culture reagents, LPS, MTT, N^G-monomethyl-L-arginine, EGTA, Dowex (AG 50W-8), 2-methylornithine (2-MeO) and polyamines were obtained from Sigma (Poole, UK). Monoclonal iNOS antibody was from Affiniti Research Products Ltd (Nottingham, UK); nitrocellulose membrane from Anderman and Co. (Kingston-upon-Thames, UK); ECL detection system from Amersham Life Sciences (Little Chalfont, UK); scanning densitometry equipment (UVP E.A.S.Y. Plus Enhanced Analysis System) was from Ultra-Violet Products Ltd. (Cambridge, UK). Other chemicals were from either Sigma or Merck and of the highest grade obtainable. Difluoromethylornithine (DFMO)was a gift from Marrion Merrell Dow, Cincinnati, Ohio, USA. N^1,N^{12}-Diacetylspermine was prepared as described elsewhere (18).

Cell culture
J774 cells were maintained in continuous culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 42 mM $NaHCO_3$, penicillin (100 units/ml) and streptomycin (100 units/ml), 2 mM glutamine and 10% foetal calf serum. Monolayers of cells were routinely harvested by gentle scraping with a Teflon cell-scraper, diluted 1:10 in fresh medium and cultured to confluency at 37°C. For use, cells were resuspended in fresh medium containing either foetal calf or horse serum at 5 x 10^5 cells/ml. Aliquots (200 μl) were dispensed into the inner 60 wells of 96-well tissue culture plates, the outer wells of which each contained 200 μl phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM $KHPO_4$, pH 7.4).

Measurement of iNOS activity
Confluent monolayers of cells were activated with *Eschericia coli* lipopolysaccharide (LPS, serotype 0111:B4; 0.1 μg/ml) alone or in combination with inhibitor (1-10 mM) and, or, polyamines (25-100 μM). When applied, inhibitor was either added simultaneously with LPS or pre-incubated with cells for 24 or 48 h prior to stimulation with LPS. Two protocols for cell treatment were used. In the first (Protocol 1) culture medium was replaced with fresh medium plus treatment (inhibitor, polyamine) every 24 h; in the second (Protocol 2), all treatments were added directly into the existing culture medium without replacing the latter. Nitrite production was assayed after 24 h by measuring the accumulated levels in the culture medium using the Griess reaction (19), as described previously (20).

Cell Viability
Cell viability was monitored by measuring the mitochondrial-dependent reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT) to formazan (21). Briefly, after each experimental protocol, cells in 96-well plates were incubated with MTT (0.5 mg ml^{-1}) for 4 h at 37°C. An equal volume of 10% SDS in 0.01 M HCl was then added to all wells and incubated for a further 3 h to dissolve the accumulated crystals of formazan. Absorbance was read at 560 nm using a Multiskan II plate reader (Titertek). In some experiments the formazan was solubilised using dimethylsulphoxide (22).

Polyamine transport
Uptake of radiolabelled polyamines (0.25-10 μM) was measured in cells cultured in 96-well plates for 24 h in the presence or absence of LPS (1 μg/ml) essentially as described elsewhere (23,24). Briefly, cell monolayers were rinsed with HEPES-buffered Hanks balanced salt solution, then incubated with 0.25-10 μM radiolabelled polyamine (approximately 100 μCi/mMol) in HEPES-Hanks. After 15 min incubation the plates were placed in melting ice and ice-cold PBS added to each well. After three rinses with PBS, the protein content of the cell monolayers was measured using Coomassie Blue (25). Cell material was then dissolved in formic acid (25 M) and the amount of radioactivity taken up determined by liquid scintillation counting. Apparent kinetic constants were derived by computer fitting the uptake data (calculated as pmol/μg protein/h) to Michaelis-Menten plots.

Assay of isolated iNOS activity
Cells (approximately 9.5 x 10^6) in 75 cm^2 culture flasks were activated with LPS (1 mg/ml) for 18 h then harvested by scraping into 1 ml of homogenization buffer (50 mM Tris-HCl, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 2 mg/ml aprotinin). The cells were lysed by three cycles of freezing (-70°C) and thawing (37°C) then the lysate was centrifuged for 60 min at 10,000 *g*, 4°C.

Activity of iNOS in the cell-free supernatant was determined by monitoring the conversion of L-(^{3}H)arginine to L-(^{3}H)citrulline (26), in the presence or absence of N^G-monomethyl-L-arginine (L-NMMA; 100 μM), EGTD (1 mM), DFMO (1-10 mM), or polyamines (0.001-1 mM). Briefly, 20 μl of 10,000 *g* supernatant, plus compound of choice, was added to 100 μl of assay buffer (50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine, 1 mM dithiothreitol, 100 mM NADPH, 1 mM L-citrulline, 20 mM L-arginine and 0.5 mCi/ml L-(^{3}H)arginine) and incubated for 20 min at 37°C. Unreacted (^{3}H)arginine was removed by adding 1 ml of a 1:1 suspension of Dowex (AG 50W-8) in water to each sample. The supernatant, containing the (^{3}H)citrulline, was transferred into β-vials and radioactivity measured by liquid scintillation counting.

Western blot analysis of iNOS expression

Confluent monolayers of J774 cells in 24-well culture plates (5 x 10^5 cells/well) were pre-incubated with either DMEM alone or with DMEM containing DFMO (10 mM). The medium was removed after 24 h and replaced with fresh DMEM or with DMEM containing LPS (1 μg ml^{-1}) and/or DFMO (10 mM). Incubations were terminated after 18 h by rapid aspiration of the cell supernatant followed by washing with ice-cold PBS. The cell monolayers were lysed in buffer (63.5 mM Tris-HCl, pH 6.8; 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 1 mM 4-(2-aminoethyl)-benzenesufonylfluoride and 50 mg/ml leupeptin). Samples were then probed for iNOS protein levels as described previously (27). Briefly, lysates (20 μg protein/lane) were separated by SDS-PAGE on 8% polyacrylamide gel, then transferred onto nitrocellulose membrane. Membranes were blocked in STT (100 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween-20, pH 7.4 containing 1% (w/v) BSA), then probed overnight with mouse monoclonal anti-iNOS antibody. Blots were washed with STT and incubated with horseradish peroxidase-conjugated rabbit anti-mouse I_gG. After further washing in STT, immunoreactive bands were visualised using the ECL detection System. Protein bands were quantified by scanning densitometry and the data expressed as a percentage of the value obtained for samples from cells treated with LPS alone.

Statistics

All values are means ± SEM of measurements from at least three different experiments with 5-10 replicates in each. Statistical analyses were performed using a multiple means comparison test ((28); validated using the Longley set of 'stiff' data (29,30) with the overall confidence levels set at 99% ($P<0.01$).

Results

Effects of DFMO on LPS-stimulated nitrite production

Incubation of J774 cells with LPS (0.1 μg/ml) according to Protocol 1 stimulated NO production from a basal value of 0.02 ± 0.003 to 0.76 ± 0.01 nmole/μg protein/24 h ($n=13$). This increase in nitrite production was further potentiated, dose-dependently, by DFMO, which at 10 mM (added 24 h before LPS) increased nitrite levels by a further 52 ± 5.9% (Figure 1). Similar results were obtained when 2-MeO was substituted for DFMO. This response is not dependent on sera type since DFMO potentiation occurred in the presence of either foetal calf or horse serum (Table 1).

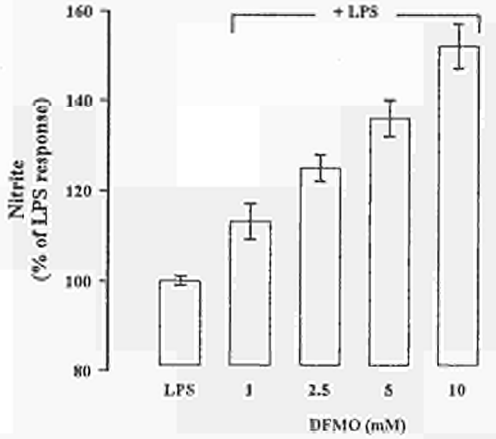

Figure 1. Concentration-dependent potentiation of LPS-induced nitrite production in J774 macrophages by 2-difluoromethylornithine (DFMO). Cells cultured in calf serum-containing medium were incubated with DFMO for 24 h prior to activation with LPS (0.1 μg/ml). Nitrite was determined after a further 24 h. Results are expressed as % of the LPS response. Values are means ± SEM of 5 independent experiments with 5 replicates in each. All values in the DFMO-treated group are significantly different from LPS alone ($P<0.01$).

Table 1. Effects of foetal calf or horse serum on potentiation of LPS-induced nitrite production by DFMO.

	Nitrite (nmole/μg protein/24 h)	
	Foetal calf serum (10%)	Horse serum (10%)
Control	0.004 ± 0.001	0.001 ± 0.005
DFMO (10 mM)	0.006 ± 0.002	0.004 ± 0.006
LPS (0.1 μg/ml)	0.60 ± 0.04	0.56 ± 0.005
LPS + DFMO	0.91 ± 0.06*	0.83 ± 0.02*

*$P<0.01$ compared to LPS alone

Although addition of DFMO simultaneously with, or for 48 h prior to LPS, enhanced nitrite levels, the increases observed were relatively less compared to those seen after 24 h pre-treatment (Figure 2). Addition of DFMO for 48 h without replacement of medium, followed by activation of cells with LPS, again without replacement of medium (Protocol 2) resulted in a marked decrease in nitrite levels of approximately 50%, compared to LPS alone (31). Supplementation of the medium with 1 mM exogenous arginine did not reverse the inhibition caused by DFMO. Replacement of DFMO by 2-MeO gave similar results.

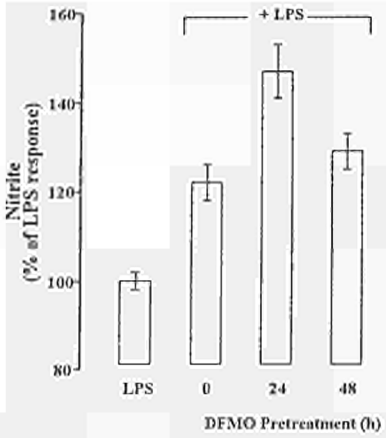

Figure 2. Time-dependent potentiation of LPS-induced nitrite production in J774 macrophages by DFMO. Cells cultured in calf serum-containing medium were incubated with DFMO (10 mM) for 0, 24 or 48 h prior to activation by adding LPS (0.1 μg/ml). Accumulated nitrite was determined after a further 24 h. Results are expressed as % of the LPS response. Values are means $\pm$ SEM of 5 independent experiments with 5 replicates in each. All values in the DFMO-treated group are significantly different from LPS alone ($P<0.01$).

The potentiation of LPS-stimulated nitrite production by DFMO (Protocol 1) was critically time dependent in that the effect was greater when cells were pre-incubated with DFMO for 24 h prior to activation with LPS. Addition of DFMO simultaneously with, or up to 12 h after, LPS resulted in a time-dependent decrease in the ability of DFMO to enhance nitrite levels, independent of serum type (Table 2). Incubation of cells for up to 48 h with DFMO in the absence of LPS, using either protocol, failed to enhance nitrite accumulation above basal values. Furthermore, mitochondrial reduction of MTT to formazan was not significantly altered under any of the conditions examined, suggesting that inhibition of ODC by either DFMO (irreversible) or 2-MeO (reversible) does not cause any adverse effects to J774 cells even when applied at high concentrations and for prolonged periods.

Table 2. Time-dependent regulation of LPS-induced nitrite production by DFMO.

	Nitrite (nmoles/μg protein/24 h)
Control	0.004 $\pm$ 0.001
LPS (0.1 μg/ml)	0.60 $\pm$ 0.04
LPS (0.1 μg/ml) + DFMO (10 mM) 3 h post LPS	0.77 $\pm$ 0.06*
LPS (0.1 μg/ml) + DFMO (10 mM) 6 h post LPS	0.70 $\pm$ 0.03**
LPS (0.1 μg/ml) + DFMO (10 mM) 12 h post LPS	0.63 $\pm$ 0.04

*$P<0.01$, **$P<0.05$ compared to LPS alone

Table 3. Effects of exogenous polyamines, added concomitantly with agonist, on nitrite release from LPS-activated J774 cells cultured in horse serum-containing medium.

Polyamine	Concentration (μM)				
	0	10	20	50	100
Putrescine	100 ± 1	104 ± 2	103 ± 1	97 ± 2	93 ± 3*
N^1-acetylputrescine	100 ± 1	101 ± 3	102 ± 1	98 ± 3	93 ± 1*
Spermidine	100 ± 1	103 ± 1	99 ± 3	97 ± 3	95 ± 2
N^1-acetylspermidine	100 ± 3	105 ± 3	105 ± 1	99 ± 5	94 ± 5
N^8-acetylspermidine	100 ± 3	107 ± 1	107 ± 2	102 ± 4	100 ± 2
Spermine	100 ± 1	105 ± 1	103 ± 3	91 ± 7*	87 ± 4*
N^1-acetylspermine	100 ± 1	107 ± 2	107 ± 2	93 ± 9*	90 ± 7*
N^1,N^{12}-diacetylspermine	100 ± 1	105 ± 3	105 ± 2	95 ± 7	98 ± 5

Values are percentages of controls (LPS only); *$P<0.01$ compared to controls

Effects of polyamines on LPS-stimulated nitrite accumulation

Addition of exogenous polyamines did not increase nitrite release from LPS-stimulated J774 cells cultured in medium containing horse serum (Table 3). Slight inhibition (5-6%) was observed at 100 μM. In contrast, and as shown by others (12,13,17), 50 μM spermine reduced LPS-stimulated nitrite release by 57% in the presence of calf serum- (Table 4), but by only 8% in horse serum-containing medium, which has lower levels of amine oxidase (32). This was increased to 68% and 13%, respectively, at 100 μM spermine. In the absence of LPS polyamines, at concentrations up to 1 mM, did not alter basal levels of nitrite release.

Table 4. Effects of exogenous spermine, added concomitantly with agonist, on nitrite release. from LPS-activated J774 cells cultured in foetal calf serum-containing medium.

	Nitrite (nmol/μg protein/24 h)
Control	0.08 ± 0.03
LPS (0.1 μg/ml)	1.64 ± 0.31
Spermine (μM) 10	1.78 ± 0.10
20	1.75 ± 0.13
50	0.68 ± 0.08*
100	0.53 ± 0.09*

*Significantly less than LPS values ($P<0.01$)

Effects of LPS-stimulation on polyamine transport

Uptake was linear with time up to 30 min and was temperature-dependent, being reduced approximately 3-fold at 0°C compared to 37°C. Putrescine uptake was non-saturable, being linear with concentration up to 10 μM, and was significantly enhanced ($P<0.01$) by

approximately 30% in the presence of LPS. Uptake of spermidine and spermine could be modelled by Michaelis-Menten kinetics (Table 5), LPS treatment reduced the rate of uptake but did not affect binding of polyamine to transporter (33).

Table 5. Kinetic constants for spermidine and spermine transport by J774 cells.

	Spermidine		Spermine	
	K_m[1]	V_{max}[2]	K_m	V_{max}
− LPS	7.3 ± 0.5	70 ± 6	2.3 ± 0.2	89 ± 5
+ LPS[3]	7.5 ± 0.4	20 ± 1	2.5 ± 0.2	56 ± 2

[1] μM; [2] pmol/μg protein/h; [3] 1 μg/ml

Effects of polyamines or ornithine on DFMO modulation of LPS-stimulated nitrite production
Addition of putrescine (25-100 μM) concomitantly with DFMO did not enhance the potentiating effect (Protocol 1) but, in experiments in which DFMO or 2-MeO inhibited nitrite release (Protocol 2), this was reversible by exogenous putrescine in both foetal calf and horse serum-containing medium (Figure 3). Supplementation of the culture medium with ornithine (5 mM) did not alter basal or LPS-stimulated nitrite release in the presence or absence of DFMO, although ornithine can inhibit arginine uptake in these cells (Baydoun, unpublished data). Again, replacement of DFMO by 2-MeO gave similar results.

Effects of DFMO or polyamines on iNOS activity
In order to ascertain whether DFMO altered the activity of iNOS once induced, experiments were carried out to explore the direct effects of DFMO or polyamines on enzyme activity by monitoring the conversion of L-(^{3}H)arginine to citrulline using lysates from LPS-activated J774 cells. Unstimulated J774 cells had no detectable levels of NOS activity. Following activation with LPS (1 mg/ml) total NOS activity was significantly elevated (159 ± 11 pmoles L-(^{3}H)citrulline mg protein^{-1} 20 min^{-1}; n=3) and was unaffected by removal of calcium from the reaction buffer using 1 mM EGTA. Conversion of L-(^{3}H)arginine to citrulline was, however, markedly attenuated by L-NMMA, which at 100 mM inhibited L-(^{3}H)citrulline production by 79%. In contrast neither DFMO (1-10 mM) nor putrescine, spermidine, or spermine (0.001-1 mM) inhibited conversion of (^{3}H)arginine to (^{3}H)citrulline (Table 6).

Western blot analysis
To determine whether the potentiation caused by DFMO was due to the enhanced iNOS expression, Western blot analysis was carried out on whole cell lysates using a monoclonal antibody for murine iNOS. In lysates from LPS-activated (1 mg/ml; 24 h), but not from untreated cells, the iNOS antibody recognised a protein band which migrated at a molecular weight of ~130 kDa. The levels of iNOS protein, determined by scanning densitometry, were significantly enhanced in lysates from J774 cells activated with LPS in the presence of DFMO (10 mM; 24 h pre-LPS; Figure 4).

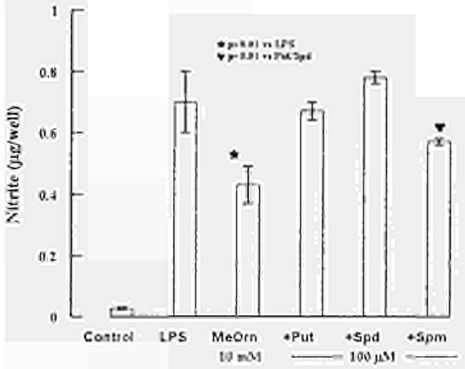

Figure 3. Inhibition of LPS-induced nitrite accumulation by 2-MeO and its reversal by polyamines.

Table 6. Effects of DFMO, putrescine, spermidine and spermine on cytosolic iNOS activity.

		L-(H^3)citrulline production*
Control		100
+ EGTA	1mM	103
+ L-NMMA	100 μM	30
+ DFMO	1.0 mM	103
	2.5 mM	101
	5.0 mM	104
	10.0 mM	106
+ Putrescine	1 μM	113
	10 μM	90
	100 μM	112
	1000 μM	111
+ Spermidine	1 μM	98
	10 μM	101
	100 μM	108
	1000 μM	111
+ Spermine	1μM	102
	10 μM	96
	100 μM	101
	1000 μM	91

*Values, as % activity of enzyme alone, are from 2-3 experiments with 3 replicates in each

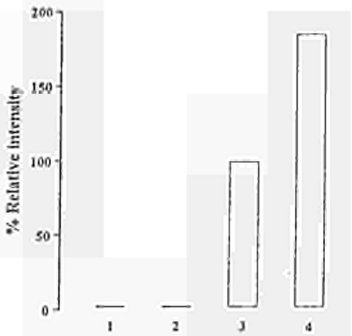

Figure 4. Western blot analysis of iNOS expression in J774 macrophages. Expression of iNOS protein in whole cell lysates was detected using a selective monoclonal antibody followed by scanning densitometry: 1, unstimulated cells (control); 2, cells + DFMO (10 mM; 48 h); 3, cells + LPS (0.1 μg/ml, 24 h); 4, cells + DFMO (10 mM, 48 h), + LPS (0.1 μg/ml, 24 h).

Discussion

The present study demonstrates that pre-incubation of J774 macrophages with DFMO, an irreversible inhibitor of ODC, or 2-MeO, a reversible inhibitor, potentiates LPS-induced nitrite production when the medium is changed every 24 h. This effect, which is associated with an increase in iNOS protein, is not mediated by a direct action of DFMO or polyamines on iNOS since addition of DFMO or putrescine, spermidine or spermine to cell lysates did not inhibit enzyme activity, measured by the conversion of L-(^{3}H)arginine to L-(^{3}H)citrulline. Thus the effects observed may involve regulation of iNOS expression at the molecular level. Incubation with DFMO reduces intracellular putrescine and spermidine levels by 60-70% at 24 h and to below detectable levels at 48 h, although spermine concentrations are unaffected (34). Depletion of intracellular putrescine and spermidine by 24 h of DFMO treatment, leads to a marked decrease in the rate of cell replication. Exposure of macrophages to LPS results in enhanced activity and expression of ODC, which reaches a maximum at 4 h, and which precedes the rise in iNOS activity (maximal at 12 h) and subsequent nitrite release, which begins to plateau at 24 h (35-37). Since the only known action of DFMO to date involves inhibition of ODC (7), it is therefore reasonable to conclude that this novel effect of DFMO results from depletion of intracellular putrescine and, or, spermidine pool(s). More importantly, our data suggest that expression of iNOS, at least *in vitro*, may be critically regulated by endogenous polyamines. Thus, by preventing this increase in ODC activity, DFMO may have a downstream effect on the induction of NO synthase. However, addition of exogenous polyamines did not alter the potentiating effect of DFMO on nitrite release. Although pre-incubation of cells with DFMO prior to activation potentiated nitrite production, addition of the drug to cells several hours after LPS caused only marginal changes in accumulated nitrite levels, suggesting that the actions of the two agents are temporally related.

Pre-incubation of J774 macrophages with DFMO, or 2-MeO, without change of medium, results in inhibition of LPS stimulated nitrite release and this is reversible by exogenous putrescine, the product of ornithine decarboxylation and precursor of spermidine and spermine (3,4). The inhibitory effects of both DFMO and 2-MeO on nitrite production were

not reversed by excess L-arginine, suggesting that substrate availability was not a limiting factor. Not surprisingly, exogenous ornithine also did not alter the effects of DFMO.

Adequate intracellular levels of polyamines are necessary for optimal cell growth and replication of all cell types examined so far. Intracellular concentrations of the polyamines are in the high micromolar range and at physiological pH all their amino groups will be positively charged. Hence the majority of the polyamines will be sequestered in some way and it is probable that only the 'free' polyamine pool is physiologically active. Polyamines can influence the transcriptional and translational stages of protein synthesis (38), stabilise membranes (39), alter intracellular free calcium levels (40-42), and have important neurophysiological functions (43).

In contrast to the findings reported by Sazbò *et al.* (12,17), it is unlikely that the effects observed in our study involve aldehydes. The acetylation of primary amino groups is the first step in the intracellular metabolism of polyamines (44). This is followed by oxidation by an intracellular polyamine oxidase (for review see reference 15), resulting in the production of acetylated aldehydes which are relatively inactive and non-cytotoxic (18). Modulation of LPS-induced nitrite production by DFMO does not appear to be dependent on extracellular amine oxidases, in that the effects observed were similar in both foetal calf and horse serum, although the latter has much lower levels of amine oxidases (32).

The apparent conflict between the these results and those published earlier (31) could be a consequence of the response of the cells to the growth stimulus resulting from the application of fresh serum-containing medium, which would also supply small quantities of polyamines. Treatment with DFMO, although reducing intracellular putrescine and spermidine to trace levels, does not completely abolish ODC activity and hence small amounts of putrescine may be formed and will transiently alter the intracellular pool of 'free' polyamines. Addition of fresh serum-containing medium to cultured cells will induce both ODC and *S*-adenosylmethionine decarboxylase, which regulates the synthesis of spermidine and spermine from putrescine (45,46). Furthermore, growth factors are known to increase polyamine transport (47) in addition to their inductive effects on ODC (48,49). Also, as shown here, LPS increases putrescine transport in macrophages. Hence, under these circumstances, despite the presence of DFMO, sufficient putrescine and/or spermidine may be available for the synthesis of iNOS protein. Without the inductive effect of fresh serum on ODC synthesis and polyamine uptake, the free putrescine/spermidine pools could be below the levels required for efficient iNOS synthesis.

We conclude that alteration of intracellular polyamine levels, in particular the pool of 'free' putrescine or spermidine, activates a signalling pathway regulating the activity and/or stability of iNOS under both physiological and pathophysiological conditions. Our findings could also account, at least in part, for the cytostatic, and indeed antitumour, properties of DFMO.

Acknowledgements

This work was supported by the British Heart Foundation. We thank Marrion Merrell-Dow for the gift of DFMO.

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