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Inhibition of ornithine decarboxylase potentiates nitric oxide production in LPS-activated J774 cells

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1 We have examined whether modulation of the polyamine biosynthetic pathway, through inhibition by α-difluoromethylornithine (DFMO) of the rate limiting enzyme, ornithine decarboxylase (ODC), modulates NO synthesis in J774 macrophages.

2 DFMO potentiated LPS-stimulated nitrite production in both a concentration- and time-dependent manner, increasing nitrite levels by 48±5% at 10 mM. This effect was observed in cells pre-treated with DFMO for 24 h prior to stimulation with LPS. Addition of DFMO 12 h after LPS failed to potentiate LPS-induced nitrite production.

3 Supplementation of the culture medium with horse serum (10%) in place of foetal calf serum (10%) caused no significant change in either LPS-induced nitrite production or in the ability of DFMO (10 mM) to potentiate LPS-induced NO synthesis.

4 Metabolism of L-[^{3}H]arginine to L-[^{3}H]citrulline by partially purified inducible nitric oxide synthase (iNOS) was not significantly altered by either DFMO (1–10 mM) or by putrescine (0.001–1 mM), spermidine (0.001–1 mM) or spermine (0.001–1 mM). iNOS activity was also unaffected by 1 mM EGTA but was markedly attenuated (70±0.07%) by L-NMMA (100 μM).

5 Pre-incubation of cells with DFMO (10 mM; 24 h) prior to activation with LPS resulted in enhanced (~2 fold) iNOS protein expression.

6 These results show that DFMO potentiates LPS-induced nitrite production in the murine macrophage cell line J774. Since the only known mechanism of action of DFMO is inhibition of ODC, and thus polyamine biosynthesis, we conclude that expression of iNOS can be critically regulated by endogenous polyamines.

Keywords: Macrophages; bacterial lipopolysaccharide; nitric oxide; inducible nitric oxide synthase; polyamines; α-difluoromethylornithine; ornithine decarboxylase

Introduction

Polyamines are physiological cellular constituents essential for cell growth and differentiation, and regulate a multitude of cellular functions (Morgan, 1987a; Jänne *et al*., 1991). In mammalian cells, the naturally occurring polyamines putrescine, spermidine and spermine are synthesized from the cationic amino acid L-arginine which is metabolized by arginase to yield ornithine and subsequently polyamines *via* the rate-limiting enzyme ornithine decarboxylase (ODC; Morgan, 1987a; McCann & Pegg, 1992). This enzyme, like the inducible nitric oxide synthase (iNOS), is induced by proinflammatory cytokines and bacterial lipopolysaccharide (LPS) resulting in enhanced enzyme activity and thus polyamine synthesis (Tjandrawinata *et al*., 1994). While this would have important implications in terms of cell growth and proliferation it is not clear what effects this might have on the inducible nitric oxide synthase pathway, which also utilizes L-arginine for the synthesis of nitric oxide (NO).

Recently, exogenous polyamines have been shown to inhibit NO production in LPS-activated J774 cells (Szabó *et al*., 1994a,b) and by isolated neuronal NO synthase (Hu *et al*., 1994). These effects however required relatively high concentrations of polyamines compared to those found in plasma and in intact cells (Morgan, 1990), and appear to be due to accumulation of aldehyde metabolites resulting from polyamine oxidation by an amine oxidase present in calf serum (Szabó *et al*., 1994a). The question of whether there is any interaction between the polyamine and iNOS pathways or whether endogenous polyamines regulate iNOS expression and/or activity still remains to be established.

In this study we have examined the effects of endogenous polyamines on the inducible L-arginine-NO pathway by examining whether α-difluoromethylornithine (DFMO), a potent and selective inhibitor of ODC (McCann & Pegg, 1992), regulates LPS-induced NOS expression or NO production in the murine macrophage cell line J774. The dependency of the actions of DFMO on serum amine oxidases was investigated by examining its effects in cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing either foetal calf serum or horse serum, the latter having low levels of amine oxidases (Blaschko, 1962).

Methods

Cell culture

The murine macrophage cell line J774 was maintained in continuous culture in DMEM supplemented with $NaHCO_3$ (42 mM), penicillin (100 units ml^{-1}) and streptomycin (100 units ml^{-1}), 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.

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Analysis of nitrite production

Prior to each experiment, cells were harvested and plated at a seeding density of 10^5 cells per well in 96-well microtiter plates. Confluent monolayers of cells were activated with 0.1 μg ml^{-1} LPS (*E. coli*, serotype 0111:B4) alone or in combination with DFMO (1–20 mM). In these studies DFMO was either added simultaneously with LPS and/or polyamines, or pre-incubated with cells for 24 or 48 h prior to stimulation, replacing the culture medium with fresh DMEM plus DFMO every 24 h. In another series of experiments, DFMO (10 mM) was added to the culture medium at 3, 6 and 12 h after LPS. NO production was determined 24 h after LPS-activation of cells by measuring accumulated nitrite levels in the culture medium as previously described (Cirino *et al*., 1996). Briefly, 50 μl of sample was incubated with 0.1 U nitrate reductase for 15 min at 37°C in the presence of FAD (5 nmoles) and NADPH (50 nmoles). This was followed by a further 5 min incubation of samples with lactate dehydrogenase (10 U) and sodium pyruvate (10 μmoles). Total nitrite was subsequently determined by the Griess reaction (Green *et al*., 1982) and expressed as nmoles μg protein^{-1} 24 h^{-1}. Cell protein was measured for each well using Brilliant Blue G (Bradford, 1976). Briefly, culture medium was removed from well, cells washed three times with 200 μl phosphate buffered saline and incubated at room temperature for 30 min with 200 μl of Brilliant Blue G reagent (0.01% w/v Coomassie Brilliant Blue G-250 , 5% w/v ethanol and 10% orthophosphoric acid. Absorbance was measured at 620 nm using a Multiskan plate reader (Flow, U.K.) and the amount of protein per well determined by reference to a standard curve constructed using bovine serum albumin as standards.

Western blot analysis of iNOS expression

Confluent monolayers of J774 cells in 24-well culture plates (5×10^5 cells per 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 (0.1 μg ml^{-1}) and/or DFMO (10 mM). Incubations were terminated after 24 h by rapid aspiration of the cell supernatant followed by washing with ice-cold phosphate-buffered saline (mM): 140 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4 (pH 7.4). Cells were lysed in buffer containing 63.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 1 mM 4-(2-aminoethyl)-benzenesufonylfluoride (AEBSF) and 50 mg ml^{-1} leupeptin. Samples were then probed for iNOS protein levels as described previously (Cirino *et al*., 1996). Briefly, lysates (20 g protein per lane) were separated by SDS–PAGE electrophoresis on 8% polyacrylamide gel and transferred for 3 h at 0.8 mA cm^2 onto 0.2 mm nitrocellulose membrane (Anderman and Co., Kingston-upon-Thames, Surrey, U.K.). Membranes were blocked for 2 h at room temperature in 100 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween-20, pH 7.4 (STT) containing 3% (w/v) BSA and subsequently probed overnight with mouse monoclonal anti-iNOS antibody (1 : 2500 dilution in STT containing 0.2% (w/v) BSA). Blots were washed with STT (6 × 5 min) and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1 : 10,000) for 1 h at room temperature. Following further washing (6 × 5 min) in STT, immunoreactive bands were visualized using the ECL detection System (Amersham Life Sciences, Little Chalfont, U.K.). Protein bands were quantified by scanning densitometry (UVP E.A.S.Y. Plus Enhanced Analysis System; Ultra-Violet Products Ltd, Cambridge, U.K.) and the data expressed as a percentage of the value obtained for samples from cells treated with LPS alone.

Analysis of isolated iNOS activity

J774 macrophages (9.5×10^6 cells) in 75 cm^2 culture flasks were activated with LPS (1 μg ml^{-1}) for 18 h and harvested with a cell scraper into 1 ml of homogenization buffer containing 50 mM Tris-HCl, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor and 2 μg ml^{-1} aprotinin. Cells were lysed by freezing the suspension at −70°C and thawing at 37°C three times. The lysate was then centrifuged at $10{,}000 \times g$ for 60 min at 4°C. Activity of the partially purified enzyme was determined by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the absence and presence of N^G-monomethyl-L-arginine (L-NMMA; 100 μM), ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; 1 mM), DFMO (1–10 μM), putrescine (0.001–1 mM), spermidine (0.001–1 mM) or spermine (0.001–1 mM) as described by Brown *et al*. (1992). Briefly, 20 μl of $10{,}000 \times g$ supernatant plus compound of choice was added to 100 μl of assay buffer consisting of 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine, 1 mM dithiothreitol, 100 μM NADPH, 1 mM L-citrulline, 20 μM (0.5 μCi ml^{-1}) L-[^{3}H]arginine. The reactions were incubated for 20 min at 37°C and L-[^{3}H]citrulline separated from L-[^{3}H]arginine by adding 1 ml of a 1 : 1 suspension of Dowex (AG 50W-8) in water to each sample. The supernatant was transferred into β-vials and radioactivity measured by liquid scintillation counting.

Assessment of cell viability

Cell viability was determined under different experimental conditions by monitoring mitochondrial-dependent reduction of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) to formazan as described by Mossmann (1983). 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. Absorbance was read at 560 nm using a Multiskan II plate reader (Titertek). Readings obtained from treated cells were compared to controls.

Materials

Tissue culture reagents, bacterial lipopolysaccharide from *E. coli* (serotype 0111:B4), polyamines, nitrate reductase, NADPH, FAD, lactate dehydrogenase, sodium pyruvate, MTT, EGTA and Dowex (AG 50W-8) were obtained from Sigma (Poole, U.K.). L-NMMA was purchased from Alexis Corporation (Nottingham, U.K.). Monoclonal antibody for inducible nitric oxide synthase was from Affiniti Research Products Ltd (Nottingham, U.K.). Other chemicals were from Sigma or BDH and of the highest analytical grade obtainable. Difluoromethylornithine was a gift from Marion Merrell Dow (Cincinnati, OH, U.S.A.).

Statistics

All values are means ± s.e.mean of measurements in at least three different experiments with five replicates in each. Statistical analyses were performed using a parametric multiple means comparison Peritz *F* test (Harper, 1984) which was validated using the Longley set of 'stiff' data (Lachenbruch, 1983; Wilson, 1983). The overall confidence level was set at

99% ($P<0.01$). Statistical analyses were performed on raw data. Normalization of the data (as percentage of controls) did not alter the significance levels (P values) of differences found.

Results

Effects of DFMO on LPS-stimulated nitrite production

Incubation of J774 cells with LPS (0.1 μg ml^{-1}) stimulated nitric oxide production, elevating nitrite levels from a basal value of 0.19 ± 0.01 to 1.35 ± 0.14 nmole μg protein^{-1} 24 h^{-1}. This was further potentiated in a concentration-dependent manner by DFMO which produced maximal effects at 10 mM, increasing LPS-stimulated nitrite levels by a further $48\pm5\%$ (Figure 1). This response is not dependent on serum type since at 10 mM DFMO enhanced LPS-induced nitrite production equally in the presence of either foetal calf (10%) or horse serum (10%) (Table 1).

Potentiation of nitrite release by DFMO was not affected by exogenous putrescine (10–100 μM) or spermidine (10–100 μM) in the presence of calf serum but was markedly attenuated by non-physiological concentrations of spermine which at 100 μM reduced responses to both LPS and LPS plus DFMO by $76\pm12\%$ and $64\pm9\%$ respectively. Physiological concentrations of spermine (10 μM) were without effect (data not shown).

Although addition of DFMO simultaneously with or for 48 h prior to LPS enhanced nitrite levels, the increases observed were relatively less compared to that after 24 h pre-treatment (Figure 2). Similarly, DFMO was less effective in potentiating nitrite production when applied to the culture medium upto 12 h after LPS (Table 2). Incubation of cells for up to 48 h with DFMO (10 mM) alone failed to enhance nitrite accumulation above basal values. Over this prolonged period of incubation, mitochondrial reduction of MTT to formazan was not significantly altered, suggesting that DFMO does not cause any adverse effects to J774 cells even when applied at high concentration and for relatively long periods (data not shown).

Table 1 Effects of foetal calf or horse serum on potentiation of LPS-induced nitrite production by α-difluoromethylornithine (DFMO)

Conditions	*Nitrite levels* (nmoles μg protein^{-1} 24 h^{-1})
Cells cultured in 10% foetal calf serum:	
Control	0.22 ± 0.017
DFMO (10 mM)	0.25 ± 0.021
LPS (0.1 μg ml^{-1})	1.07 ± 0.11
LPS (0.1 μg ml^{-1}) + DFMO (10 mM)	1.59 ± 0.13*
Cells cultured in 10% horse serum:	
Control	0.18 ± 0.02
DFMO (10 mM)	0.15 ± 0.02
LPS (0.1 μg ml^{-1})	0.98 ± 0.06
LPS (0.1 μg ml^{-1}) + DFMO (10 mM)	1.47 ± 0.11*

Cells cultured in DMEM containing either foetal calf or horse serum were pre-treated with DFMO for 24 h and then incubated with fresh medium containing LPS and/or DFMO. Accumulated nitrite in the culture medium was determined after a further 24 h incubation period by the Griess reaction. Results are expressed as the means ± s.e.-mean of three separate experiments with five replicates in each. *Denotes $P<0.01$ compared to responses obtained with LPS alone.

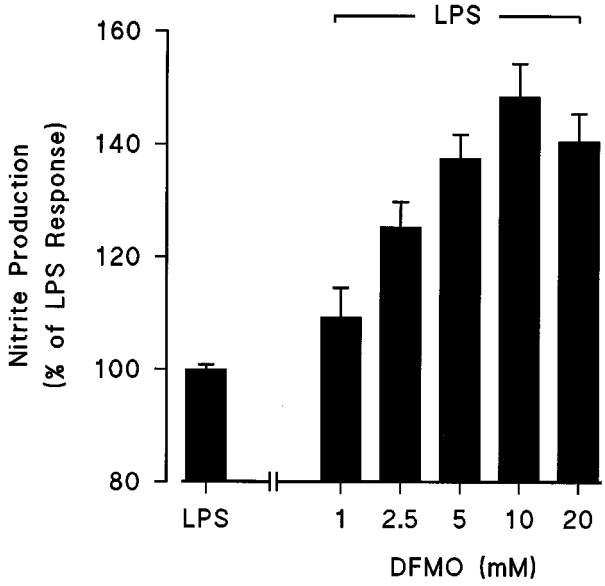

Figure 1 Concentration-dependent potentiation of LPS-induced nitrite production in J774 macrophages by α-difluoromethylornithine (DFMO). Cells cultured in DMEM containing 10% foetal calf serum were incubated with DFMO for 24 h prior to activation with LPS (0.1 μg ml^{-1}). Accumulated nitrite in the culture medium was determined after a further 24 h by the Griess reaction as described in the Methods. Results are expressed as percentages of the LPS response and values are the means ± s.e.mean of three independent experiments with five replicates in each. All values in the DFMO treated group are significantly different at $P<0.01$ from the LPS control.

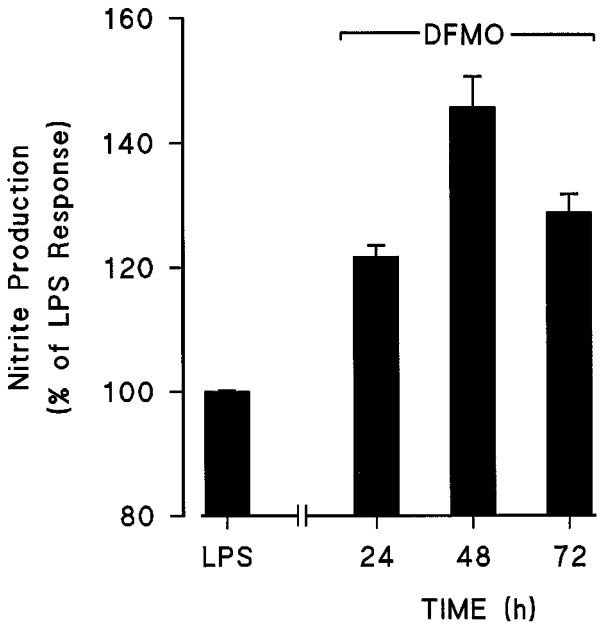

Figure 2 Time-dependent potentiation of LPS-induced nitrite production in J774 macrophages by α-difluoromethylornithine (DFMO). Cells cultured in DMEM containing 10% foetal calf serum were preincubated with DFMO (10 mM) for 0, 24 or 48 h prior to activation with LPS (0.1 μg ml^{-1}). Accumulated nitrite in the culture medium was determined by the Griess reaction after a further 24 h incubation period. Results are expressed as percentages of the LPS response and values are the means ± s.e. mean of five independent experiments with five replicates in each. All values in the DFMO treated group are significantly different at $P<0.01$ from the LPS control.

Table 2 Time-dependent regulation of LPS-induced nitrite production by of α-difluoromethyl-ornithine (DFMO)

Conditions	*Nitrite levels* (nmoles μg protein^{-1} 24 h^{-1})
Control	0.23±0.02
LPS (0.1 μg ml^{-1})	1.04±0.11
LPS (0.1 μg ml^{-1}) +DFMO (10 mM), 3 h post LPS	1.35±0.08*
LPS (0.1 μg ml^{-1}) +DFMO (10 mM), 6 h post LPS	1.16±0.11
LPS (0.1 μg ml^{-1}) +DFMO (10 mM), 12 h post LPS	0.96±0.09

Cells cultured in DMEM containing 10% foetal calf serum were activated with LPS in the absence (LPS) and presence of DFMO added at 3, 6 and 12 h after LPS. Accumulated nitrite in the culture medium was determined by the Griess reaction 24 h after addition of LPS. Results are expressed as the means±s.e.mean of at least three separate experiments with five replicates in each. *Denotes $P<0.01$ compared to responses obtained with LPS alone.

Effects of DFMO on isolated iNOS activity

In order to ascertain whether DFMO or polyamines altered the activity of iNOS once induced, experiments were carried out exploring the direct effects of DFMO (1–10 mM) 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 NOS activity. Following activation with LPS (1 μg ml^{-1}) total NOS activity was significantly elevated (159±11.4 pmoles L-[^{3}H]citrulline mg protein^{-1} 20 min^{-1}) and was unaffected by removal of calcium from the reaction buffer using 1 mM EGTA. L-NMMA, (100 μM) inhibited L-[^{3}H] citrulline production by 70%. In contrast neither DFMO (1–10 mM) nor putrescine (0.001–1 mM), spermidine (0.001–1 mM) or spermine (0.001–1 mM) caused any significant change in the production of L-[^{3}H]citrulline (Table 3).

Table 3 Effects of α-difluoromethylornithine (DFMO), putrescine, spermidine and spermine on isolated inducible nitric oxide synthase (iNOS) activity

Conditions		*L-[H^3]citrulline production* (% of control)
Control		100±3.3
+EGTA,	1 mM	103±2.3
+L-NMMA,	100 μM	30±2.7
+DFMO	1 mM	103±3.5
	2.5 mM	101±6.9
	5.0 mM	104±5.5
	10.0 mM	106±3.4
+putrescin	1 μM	113±6.8
	10 μM	90±7.1
	100 μM	112±5.7
	1000 μM	111±6.9
+spermidine	1 μM	98±7.4
	10 μM	101±3.9
	100 μM	108±5.2
	1000 μM	111±6.7
+spermine	1 μM	102±5.6
	10 μM	96±6.6
	100 μM	101±6.3
	1000 μM	91±7.2

Production of L-[H^3]citrulline from L-[H^3]arginine was determined as described in Methods and expressed as a percentage of that produced by enzyme in the absence of other compounds. Values obtained for polyamines are the mean of six replicates in two independent experiments with three replicates in each. All other values are the mean of three independent experiments with three replicates in each.

Western blot analysis

To determine whether the potentiation caused by DFMO was due to enhanced iNOS expression, Western blot analysis was carried out on whole cell lysates using a monoclonal antibody for murine iNOS. In these studies, the antibody recognized a single 130 kDa protein band in lysates from LPS-activated (0.1 μg ml^{-}; 18 h) cells but not from cells incubated either with DMEM alone or DMEM plus DFMO (10 mM). Moreover, iNOS expression was increased by 92±11% ($n=4$) in cells pre-incubated with DFMO (10 mM) for 24 h prior to activation with LPS. A representative blot from these experiments is shown in Figure 3.

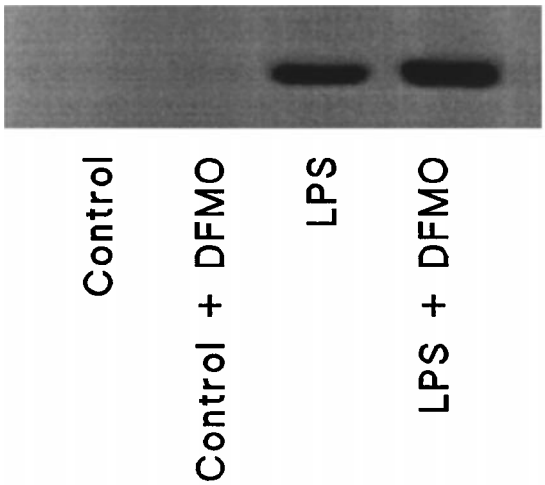

Figure 3 Western blot analysis of inducible nitric oxide synthase (iNOS) expression in J774 macrophages. Expression of iNOS protein in whole cell lysates were detected as described in the Methods using a selective monoclonal anti-iNOS antibody. Lysates were obtained from control cells, cells incubated with α-difluoromethylornithine (DFMO, 10 mM) alone for 24 h following 24 h pre-treatment, cells stimulated with LPS (0.1 μg ml^{-1}, 24 h) alone, and cells incubated with LPS (0.1 μg ml^{-1}, 24 h) and DFMO (10 mM) following 24 h pre-incubation with DFMO (10 mM).

Discussion

The present study demonstrates that pre-incubation of J774 macrophages with DFMO potentates LPS-induced nitrite production. This effect is both time- and concentration-dependent and appears to involve regulation of iNOS expression at the molecular level resulting in increased enzyme protein expression and thus NO production. The maximal response was obtained following 24 pretreatment of cells with 10 mM DFMO prior to activation with LPS. Although addition of DFMO simultaneously with LPS also enhanced accumulated nitrite levels, the potentation was relatively less compared to responses produced after 24 h pre-incubation. Furthermore, when added 12 h after LPS, DFMO failed to potentate LPS-induced nitrite production. These findings suggest that the effects of DFMO are not mediated directly on iNOS but rather at certain upstream events associated with induction of the enzyme.

Induction of iNOS is a time dependent process requiring *de novo* protein synthesis. Enzyme expression is detected ~6 h after activation of cells, reaching a maximum at 12 h and is maintained over a period of 24–48 h (Assreuy *et al*., 1993). Thus the fact that addition of DFMO 12 h post LPS failed to alter LPS-induced nitrite production strongly suggests that DFMO has no effect on iNOS activity once induced. This was confirmed in experiments carried out to examine the direct effect of DFMO on the partially purified enzyme isolated from LPS activated J774 cells. In these studies DFMO did not alter the metabolism of [^{3}H]arginine to citrulline by iNOS. In contrast, Western blot analysis of lysates obtained from cells activated in the presence of LPS alone and in combination with DFMO showed LPS-induced iNOS protein expression was significantly augmented in the presence of DFMO, demonstrating increased iNOS gene transcription and/or mRNA stability.

The only known action of DFMO is irreversible inhibition of ODC by covalently binding to the enzyme (Poulin *et al*., 1992; McCann & Pegg, 1992). It is therefore reasonable to suggest that the novel actions observed in our study results from inhibition by DFMO of ODC with a consequential depletion of intracellular polyamines, especially putrescine and spermidine which are sequentially metabolized to spermine. These polyamines can be depleted by 60–70% at 24 h and to below detectable levels 48 h after exposure to DFMO (Mamont *et al*., 1978). Under these conditions, the constraint on iNOS expression exerted by endogenous polyamines is overcome resulting in over expression of iNOS protein. In this regard it is interesting that exposure of RAW 264 macrophages to LPS and interferon-gamma results in enhanced ODC activity and polyamine synthesis. Induction of ODC occurs relatively rapidly with maximal enzyme activity peaking at 4 h. Moreover this response precedes the induction of iNOS (Tjandrawinata *et al*., 1994). Our current findings are therefore consistent with the suggestion that the early upregulation in ODC activity and subsequent increase in endogenous polyamines modulate the induction of iNOS by down regulating iNOS protein expression. This coercion is presumably overcome following inhibition of ODC by DFMO.

In an attempt to further validate this hypothesis additional experiments were carried out examining the effects of exogenous polyamines on DFMO-potentiated nitrite release. The results obtained suggest that spermine may be the critical regulatory polyamine since accumulated nitrite levels were markedly attenuated by spermine and not by spermidine or putrescine. However, inhibitory responses were only observed at relatively high non-physiological concentrations, thus questioning the selectivity of these effects. At lower concentrations spermine failed to alter either LPS-induced or DFMO potentiated nitrite production. This may reflect the highly positively charged nature of spermine which, once inside the cell, may become predominantly bound to intracellular components. The free intracellular concentration may thus be very low, especially under conditions in which synthesis is inhibited and supply is dependent on influx from low extracellular pools.

The ability of spermine to inhibit nitrite synthesis is supportive of other reports which have shown that exogenous polyamines, taken up by specific transport mechanisms (Seiler & Dezeure, 1990; Seiler *et al*., 1996), inhibit NO synthesis by endothelial constitutive NOS (Galea *et al*., 1996), neuronal constitutive NOS (Hu *et al*., 1994; Galea *et al*., 1996) or macrophage inducible NOS (Sazbò *et al*., 1994a,b). However, the relatively high concentrations required questions the selectivity and relevance of such effects since the inhibitions observed may simply reflect non-specific cytotoxic action of spermine metabolites generated by serum amine oxidases. This suggestion is supported by a recent report by Sazbò *et al*. (1994a) in which inhibition of iNOS protein expression was found to be critically dependent on the metabolism of spermine to toxic aldehydes by amine oxidases present in foetal calf serum.

In our studies, modulation of LPS-induced nitrite production by DFMO was not 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 (Blaschko, 1962). Moreover, unlike exogenous metabolism, the acetylation of primary amino groups is the first step in the intracellular metabolism of polyamines (Casero & Pegg, 1993). This is followed by oxidation by an intracellular polyamine oxidase (Morgan, 1989), resulting in the production of acetylated aldehydes which are relatively non-cytotoxic (Morgan, 1987b). Thus the effects of endogenous polyamines, acting intracellularly, are likely to be more specific.

We are aware that the data presented here differ from our previous preliminary findings that DFMO could apparently inhibit LPS-induced NO synthesis (Morgan, 1994). This discrepancy appears to be related to the protocol of induction. In the present study culture medium was replaced every 24 h with fresh medium containing DFMO and/or LPS as appropriate. In contrast, in our previous study cells were incubated with DFMO for periods of up to 48 h followed by activation with LPS without replenishing the incubation medium. This procedure is likely to have resulted in the progressive depletion of DFMO, thus overcoming its inhibitory actions on ODC. This hypothesis is supported by the fact that (i) expression of ODC is repressed by polyamines (Hayashi, 1989), (ii) depletion of endogenous polyamine pools by DFMO enhances ODC gene transcription (Alhonen-Hongisto *et al*., 1985, 1987; Leinonen *et al*., 1987) and (iii) ODC has a short half-life (under 30 min) which is further reduced (to ~3 min) in the presence of DFMO (McCann & Pegg, 1992). Thus the rapid turnover together with the derepression and over expression of ODC may inevitably lead to the depletion of DFMO. Frequent replenishment of culture medium with DFMO may therefore be required to overcome its depletion and maintain its inhibitory actions (Pegg, 1986).

We conclude that DFMO depletion of endogenous polyamine pools regulates iNOS expression, modulating cellular signalling mechanisms associated with the induction of iNOS protein. These findings may have significant implications for the potential application of DFMO and could account, at least in part, for the cytostatic and indeed antitumour properties of this compound.

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References

ALHONEN-HONGISTO, L., HIRVONEN, A., SINERVIRTA, R. & JANNE, J. (1987). Cadaverine supplementation during a chronic exposure to difluoromethylornithine allows an overexpression, but prevents gene amplification of ornithine decarboxylase in L1210 mouse leukaemia cells. *Biochem. J.,* **247,** 651 – 655.

ALHONEN-HONGISTO, L., KALLIO, A., SINERVIRAT, R., SEPPANEN, P., KONTULA, K.K., JANNE, O.A. & JANNE, J. (1985). Difluoromethylornithine-induced amplification of ornithine decarboxylase gene in Ehrlich ascites carcinoma cells. *Biochem. Biophys. Res. Commun.,* **126,** 734 – 740.

ASSREUY, J., CUNHA, F.Y., LIEW, F.Y. & MONCADA, S. (1993). Feedback inhibition of nitric oxide synthase activity by nitric oxide. *Br. J. Pharmacol.,* **108,** 833 – 837.

BLASCHKO, H. (1962). The amine oxidases of mammalian blood plasma. *Adv. Comp. Physiol. Biochem.,* **1,** 67 – 116.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 – 254.

BROWN, J.L., TEPPERMAN, B.L., HANSON, P.J., WHITTLE, B.J.R. & MONCADA, S. (1992). Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. *Biochem. Biophys. Res. Commun.,* **184,** 680 – 685.

CASERO, R.A. & PEGG, A.E. (1993). Spermidine/spermine N^{-1}-acetyltransferase: the turning point in polyamine metabolism. *FASEB J.,* **7,** 653 – 661.

CIRINO, G., WHEELER-JONES, C.P.D., WALLACE, J.L., DEL SOLDATO, P. & BAYDOUN, A.R. (1996). Inhibition of inducible nitric oxide synthase expression by novel nonsteroidal anti-inflammatory derivatives with gastrointestinal-sparing properties. *Br. J. Pharmacol.,* **117,** 1421 – 1426.

GALEA, E., REGUNATHAN, S., ELIOPOULOS, V., FEINSTEIN, D.L. & REIS, D.J. (1996). Inhibition of mammalian nitric oxide synthases by agmatin, an endogenous polyamine formed by decarboxylation of arginine. *Biochem. J.,* **316,** 247 249.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal. Biochem.,* **126,** 131 – 138.

HARPER, J.F. (1984). Peritz *F* test: BASIC program of a robust multiple comparison test for statistical analysis of all differences among group means. *Comput. Biol. Med.,* **14,** 437 – 445.

HAYASHI, S. (1989). Multiple mechanisms for the regulation of mammalian ornithine decarboxylase. In *Ornithine Decarboxylase: Biology, Enzymology, and Molecular Genetics*, ed. Hayashi, S. pp.35 – 43. Oxford: Pergamon Press.

HU, J., MAHMOUD, M.I. & EL-FAKAHANY, E.E. (1994). Polyamines inhibit nitric oxide synthase in rat cerebellum. *Neurosci. Lett.,* **175,** 41 – 45.

JÄNNE, J., ALHONEN, L. & LEIONEN,, P. (1991). Polyamines: from molecular biology to clinical applications. *Ann. Med.,* **23,** 241 – 259.

LACHENBRUCH, P.A. (1983). Statistical programs for microcomputers. *Byte,* **8,** 560 – 570.

LEINONEN, P., ALHONEN-HONGISTO, L., LAINE, R.A.U., JANNE, O.A. & JANNE, J. (1987). Human myeloma cells acquire resistance to diflurormethylornithine by amplification of ornithine decarboxylase gene. *Biochem. J.,* **242,** 199 – 203.

MAMONT, P.S., DUCHESNE, M.-C., GROVE, J. & BEY, P. (1978). Antiproliferative properties of DL-α-difluoromethylornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. *Biochem. Biophys. Res. Commun.,* **81,** 58 – 66.

MCCANN, P.P. & PEGG, A.E. (1992). Ornithine decarboxylase as an enzyme target for therapy. *Pharmacol. Ther.,* **54,** 195 – 215.

MORGAN, D.M.L. (1987a). Polyamines. *Essays Biochem.,* **23,** 82 – 115.

MORGAN, D.M.L. (1987b). Oxidised polyamines and the growth of vascular endothelial cells: prevention of cytotoxic effects by selective acetylation. *Biochem. J.,* **242,** 347 – 352.

MORGAN, D.M.L. (1989). Polyamine oxidase and oxidised polyamines. In *Physiology of the Polyamines*. eds. Bachrach, U. & Heimer, Y. **vol. 1,** pp. 203 – 229. Boca Raton FL: CRC Press.

MORGAN, D.M.L. (1990). Polyamines and cellular regulation: perspectives. *Biochem. Soc. Trans.,* **18,** 1080 – 1084.

MORGAN, D.M.L. (1994) Difluoromethylornithine (DFMO), an inhibitor of nitrite production by macrophages? *Biochem. Soc. Trans.,* **22,** 389S.

MOSSMAN, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* **65,** 55 – 63.

PEGG, A.E. (1986). Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.,* **234,** 249 – 262.

POULIN, R., LU., L., ACKERMANN, B., BYE, P. & PEGG, A.E. (1992). Mechanism of the irreversible inactivation of mouse ornithine decarboxylase by α-difluoromethylornithine. *J. Biol. Chem.,* **267,** 150 – 158.

SEILER, N., DELCROS J.G. & MOULINOUX J.P. (1996). Polyamine transport in mammalian cells – an update. *Int. J. Biochem. Cell Biol.,* **28,** 843 – 861.

SEILER, N. & DEZEURE, F. (1990). Polyamine transport in mammalian cells. *Int. J. Biochem.,* **22,** 211 – 211.

SZABO, C., SOUTHAN, G.J., THIEMERMANN, C. & VANE, J.R. (1994a). The mechanism of the inhibitory effect of polyamines on the induction of nitric oxide synthase: role of aldehyde metabolites. *Br. J. Pharmacol.,* **113,** 757 – 766.

SZABO, C., SOUTHAN, G.J., WOOD, E., THIEMERMANN, C. & VANE, J.R. (1994b). Inhibition by spermine of the induction of nitric oxide synthase in J774.2 macrophages: requirement of a serum factor. *Br. J. Pharmacol.,* **112,** 355 – 356.

TJANDRAWINATA, R.R., HAWEL III, L. & BYUS, C.V. (1994). Regulation of putrescine export in lipopolysaccharide or IFN-γ activated murine monocytic-leukemic RAW 264 cells. *J. Immunol.,* **152,** 3039 – 3052.

WILSON, S.R. (1983). Benchmark data sets for the flexible evaluation of statistical software. *Comp. Statist. Data Anal.,* **1,** 29 – 39.

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