MODULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION BY AN UREMIC CATABOLYTE, METHYLGUANIDINE, IN LPS-STIMULATED J774 MACROPHAGES.

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Running Title: Inhibition of iNOS expression by methylguanidine

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Abstract

1. We have investigated whether methylguanidine (MG), an uremic toxin, modulates the expression of the inducible nitric oxide synthase (iNOS) and nitric oxide (NO) release *in vitro* in LPS-induced J774 macrophages.

2. Addition of MG (0.01-1mM) or L-NAME (0.01-1mM), 30 min before LPS challenge, inhibited significantly (37.77 % inhibition, $P<0.001$; n=12) but only at the highest concentration tested LPS-induced nitrite production.

3.When J774 macrophages were pretreated with MG or L-NAME (0.01-1mM) 18h prior stimulation with LPS, MG significantly ($P<0.001$) inhibited, in a concentration related manner, LPS-stimulated nitrite production reducing nitrite levels by 40.26 % (n=12), 31.85 % (n=9) and 26.68 % (n=6) for MG 1, 0.1 and 0.01 mM respectively.

4. MG, added to the culture medium of J774 macrophages 24 h after LPS challenge, also reduced significantly and in a concentration-related manner nitrite production inhibiting NO release by 50.0 % ($P<0.001$, n=32), 26.0 % ($P<0.05$, n=23) and 3.18 % for MG 1, 0.1 and 0.01 mM respectively. In this condition when the culture medium was supplemented with L-arginine (10mM; L-ARG) the inhibitory effect of MG (1mM) on nitrite production was reversed to 10.86 % significantly different ($P<0.05$) from MG-inhibitory effect observed in normal medium.

5. Preincubation of cells with MG (1mM; 24 h) 30 min and 18 h prior to activation with LPS resulted in inhibited iNOS protein expression by 44.16±5.7 % ($P<0.01$; n=3) and 30.47±3.9 % ($P<0.01$; n=3). In the same conditions L-NAME (1mM) did not modify significantly iNOS expression.

7. MG (1mM) or L-NAME (1mM) added to the culture medium 24 h after LPS challenge did not modify iNOS protein expression.

8. Our results show that MG inhibited LPS-induced nitrite production in the murine macrophages cell line J774. This inhibitory effect could be partly due to the antagonistic

activity on iNOS protein that is reversed by L-Arg supplementation and partly related to the inhibitory effect on iNOS expression .

9. Parallel experiments employing L-NAME (0.01-1 mM) have been carried out for comparison in all the three different experimental settings. Similar results on LPS-induced nitrite production but different results on iNOS expression have been obtained for L-NAME.

Key words: macrophages; bacterial lipopolysaccharide; nitric oxide, inducible nitric oxide synthase, methylguanidine; L-arginine; L-NAME

Introduction

It has been well established that an immunodeficient state often paradoxically coexists with signs of activation of immune system in patients with chronic renal failure (CRF) (Deschamps–Lastscha, 1993). While chronic activation of monocytes has been recognized as a key factor responsible for the immunological disorders, the existing immunodeficiency may reflect inhibition of nitric oxide (NO) synthesis normally induced in macrophages as in part responsible of the host defense mechanism. More recently Le Meur and coworkers (1996) suggested the possibility that CRF-associated-immunodeficiency could be linked to multiple and complexes alterations of cytokine network and that target cells such as monocytes, T or B lymphocytes, fibroblasts and endothelial cells may be involved. In this disease chronic activation of monocyte functions has been recognized as a key factor responsible for immunological disorders associated to CRF that is accentuated rather than corrected by replacement dialysis therapy (Kawano et al., 1993; Kelly, 1994; Deenitchina et al., 1995). In 1975 Jones and Burnett hypothesized that high blood concentration of various endogenous nitrogen compounds may be responsible of uraemic symptoms and immunodisfunctions. In 1990 Yokozawa and coworkers and more recently Mendes-Ribeiro (1996) demonstrated that uraemia is often accompained by accumulation of L-arginine analogue N^G-monomethyl-L-arginine, methylguanidine (MG) and its precursor creatinine. These results have been recently clarified by Aucella and coworkers (1998) who indicated that there are two key factors responsible for the different cytokine release: one is the different absorption of inflammatory mediators on the dialysis membrane surface and another one could be related to the different sterilization method used for haemodialysis membranes that may be responsible for the higher serum levels of monokines, including TNFα, in patients with CRF in haemodialysis (Kawano et al., 1993). In this regard we have previously demonstrated that methylguanidine (MG), an uremic toxin, inhibits NO production of both the constitutive and inducible isoforms of NO synthase (Sorrentino et al., 1997), that *in vivo* pretreatments in rats with MG, simulating a

pattern similar to the uremic conditions, significantly inhibited serum levels of TNF-α measured *in vivo* 2h after LPS challenge and also *in vitro* MG (1mM) reduced significantly TNF-α released by LPS-induced J774 (Autore et al., 1999)

In order to examine whether the uraemic toxin MG could modulate iNOS activity and/or iNOS expression and whether these effects could be related to the time or concentration of MG added to J774 medium, in this study we have examined *in vitro* the ability of MG to influence iNOS expression and/or NO release in the murine macrophage cell line J774 stimulated with LPS. Parallel experiments employing L-NAME were also carried out for comparison.

METHODS

Cell culture

Murine macrophage cell line, J774, was maintained in continous culture in DMEM supplemented with $NaHCO_3$ (42 mM), penicillin (100 units ml^{-1}), streptomycin (100 units ml^{-1}), glutamine (2 mM) and foetal calf serum (10 %). 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 95 % air and 5 % CO_2 atmosphere.

Determination of the cytotoxic activity

J774 (3.5×10^4) were plated on 96-well microtiter plates and allowed to adhere at 37°C in 5 % CO_2, 95 % air for 2 h. Thereafter the medium was replaced with 50 μl of fresh medium and 75 μl aliquot of 1:2 v v^{-1} serial dilution of MG (0.01-1 mM, dissolved in culture medium) was added and the cells incubated for 24, 48, 72 h. The cells viability was assessed accordingly to the method of Mosman, 1983. Briefly, 25μl of 3-(4,5-dimethyltiazol-2yl)-2,5-phenyl-2H-tetrazolium bromide] (MTT, 5 mg ml^{-1}) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 100 μl

of a solution containing 50 % (v:v) N,N-dimethilformamide, 20 % (w:v), SDS (sodium dodecyl sulphate) with an adjusted pH of 4.5 (Opipari et al., 1992). The optical density (OD) of each well was measured with a microplate spectrophotometer equipped with a 620 nm filter. The viability of J774 cell line in response to treatment with compounds was calculated as: % dead cell = 100-(OD treated/ OD control) ×100.

Analysis of nitrite production

Prior to each experiment, harvested cells were plated at a seeding density of $2.5x10^5$ cells ml^{-1} ·Cells were activated with LPS ($6x10^3$units ml^{-1}) alone or in combination with MG (0.01-1 mM) or L-NAME (0.01-1 mM), used as reference drug. In our studies MG and L-NAME were also added to the culture medium of J774 30 min or 18 h before and simultaneously with LPS.

The culture medium was replaced with fresh DMEM plus MG or L-NAME after each different incubation time. NO release was determined 24 h after LPS-activated J774 by measuring accumulated nitrite levels in the culture medium by the Griess reaction (Green et al., 1982) and expressed as μM NO_2^- released in medium from $2.5x10^5$ cells ml^{-1} released in medium 24 h after LPS challenge or as percentage inhibition calculated versus LPS alone.

Western Blot analysis for iNOS expression

Confluent monolayers of J774 in well culture plates ($1.5x10^6$ cells/well) were preincubated with MG (1mM) or L-NAME (1mM) for 30 min or 18 h respectively and then incubated with either DMEM alone or DMEM containing LPS ($6x10^3$ u ml^{-1}) alone or in combination with MG (1mM) or L-NAME (1mM). After 24 h incubation with LPS, cells were scraped off, washed with ice-cold phosphate buffered saline (PBS) [(mM): 140 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 K_2HPO_4 (pH 7.4)], and harvested by centrifugation (5000 g, 10 min at 4°C) and resuspended in a buffer containing 20 mM TRIS HCl (pH 7.5), 1 mM Na^+ orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} leupeptin, 10 mM NaF, 150 mM NaCl, 10 mg ml^{-1} tripsin inhibitor and 1% Nonidet P 40. Protein concentration was estimated by the Bio-

Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (70 µg) of cell lysates were dissolved in Laemmli's sample buffer, boiled, and run on a SDS-PAGE minigel (8 % polyacrylamide) and transferred for 40 minutes at 5 mA cm^2 into 0.45 µm hybond polyvinyldeme difluoride membrane. Membranes were blocked for 40 minutes in PBS 1× containing 5 % (w v^{-1}) non fat milk and subsequently probed overnight at 4 °C with mouse monoclonal anti-iNOS antibody (1:10.000 dilution in PBS 1× containing 5 % w v^{-1} non fat milk and 0.1 % Tween-20). Blots were then incubated, after four washes in PBS 1× containing 5% w/v non fat milk and 1% Tween 20, with horseradish peroxidase conjugated goat anti-mouse IgG (1:5.000) for 1 h at room temperature. Immunoreactive bands were visualized using ECL detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS on X ray film were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD U.S.A.) and the data are expressed as a percentage of the values obtained from samples containing cells with LPS alone.

Materials

Escherichia coli lipopolysaccharide (activity $6x10^6$ u mg^{-1}) was obtained from Fluka (Italia). Methylguanidine, N^G-nitro-L-arginine methylester, [3-(4,5-dimethyl-thiazolyl-2yl) 2.5 diphenyl tetrazolium bromide], PBS, NaCl, KCl, Na_2HPO_4, K_2HPO_4 ,Tris HCl, Na+ orthovanadate, phenylmethylsulfonylfluoride, bovine serum albumin were obtained from Sigma Chemical Co., Italy. Kodak X-Omat film**,** Hybond polyvinyldeme difluoride membrane, ECL detection system, Hybond polyvinyldeme difluoride membrane were from Amersham Life Science Ltd Buckinghamshire (U.K.). Lleupeptin, tripsin, Nonidet, Biorad reactive, Laemmli's sample buffer, polyacrylamide, non fat milk, Tween 20, horseradish peroxidase conjugated goat anti-mouse

Mouse monoclonal antibody for inducible nitric oxide synthase was from Jackson (U.K.).

Dulbecco's Modified Eagle's Medium, penicillin/streptomycin, HEPES, glutamine, Foetal calf serum and horse serum were from Hy Clone (Euroclone, U.K.).

Statistics

Data are expressed forn nitrite production as μM NO_2^- released in medium from $2.5x10^5$ cells ml^{-1}. For Western Blot anlysis data are expressed as percentage of inhibition calculated versus LPS alone. All values are means±s.e.mean of measurements in at least three different experiments with five replicates in each. Prism software version 2.01, GraphPad Inc.was used to perform statistical analysis. Comparisons were made using the one way ANOVA or using unpaired Student's t-test. The level of statistical difference was defined as $P<0.05$.

RESULTS

Inhibition of nitrite production by MG or L-NAME preincubated with cells 30 min or 18 h before LPS challenge

NO production in J774 cells incubated for 24 hours with LPS ($6x10^3$ u ml^{-1}; 24 h) was significantly ($P<0.0001$) increased from a basal value of untreated cells of 0.05±0.03 μM to 15.98± 0.8 μM 24 h^{-1} (n=36).

MG added to the culture medium of incubation in a range from 0.01-1 mM inhibited NO formation. As shown in figure 1a LPS-stimulated J774 released 15.33±1.02 μmol l^{-1} NO_2^- (n=18) in the medium. MG, added to the culture medium 30 min before and simultaneously to LPS, only at the highest concentration (1 mM) significantly inhibited NO release ($P<0.001$; 37.77 %; Fig.1a). When MG (0.01-1 mM) was added to the culture medium 18 h before and simultaneously to LPS reduced significantly ($P<0.001$) and in a concentration-dependent manner LPS-induced NO production giving rise to 40.26 % inhibition , 1mM; 31.85 % inhibition, 0.1 mM; 26.68 % inhibition, 0.01 mM respectively with $P<0.001$; Figure 2a). Parallel experiments were carried out with a well known NO synthase inhibitor, L-NAME. Results obtained with MG were comparable to those obtained with L-NAME (0.01-1 mmol l^{-1}) both when incubated for 30 min than for 18 h before LPS challenge (Figure 1b and 2b).

Effect of MG and L-NAME on NO release from already expressed iNOS

In previous experiments MG was added before any expression of iNOS; in another set of experiments J774 macrophages were stimulated with LPS ($6x10^3$ u ml^{-1}) and 24 h later the medium was replaced with MG- or L-NAME-medicated medium (0.01-1 mmol l^{-1}) and the incubation was continued for further 24 h. After the first incubation NO released in the medium was 14.69±0.61 μmol l^{-1}, while NO production in the second incubation (24 h) was 5.34±0.37 (n=52). MG (0.01-1 mM), added 24 h after LPS challenge, markedly and significantly ($P<0.001$) reduced NO release both at the highest concentration (50 % inhibition,

P<0.001) than at lower concentration 0.1 mM (26 % inhibition , P<0.05, Figure 3a). Similar results have been obtained with L-NAME (0.01-0.1 mM), added 24 h after LPS addition. In this condition L-NAME decreased significantly and in a concentration related manner NO release by LPS-stimulated J774 (Figure 3b.).

In this experimental condition L-ARG (10mM) supplementation significantly (P<0.05) reversed both MG than L-NAME inhibitory effect on NO release. In fact MG (1mM) added to the culture medium of LPS-stimulated-J774 24 h after LPS challenge gives rise to 50.0±9.0 % (P<0.001) inhibition in normal medium This inhibitory effect is significantly reversed in medium containing L-ARG (10mM) to 10.86±1.8 % inhibition) that is significantly different (P<0.05) from MG inhibitory effect (Figure 3a). Similar results have been obtained for L-NAME (1mM). L-NAME in normal medium gives rise to a significant inhibition (64.79±2.8 % with P<0.001 versus LPS) of NO release. This inhibitory effect is significantly reversed with L-ARG (10 mM) to 24.11±5.3 % that is significantly (P<0.001) different from L-NAME inhibitory effect (Figure 3b.)

Western Blot analysis for iNOS expression

To determine whether the inhibitory effect caused by MG on NO released by LPS-stimulated J774 could also be due to the inhibition of iNOS expression, Western Blot analysis was carried out on whole cell lysates using a monoclonal antibody for murine iNOS. In lysates from LPS-stimulated ($6x10^3$ u ml^{-1} 24 h^{-1}) but not from unstimulated cells the iNOS antibody recognised as a single 130 kDa protein band which migrated at a molecular weght of 130 kDa (Figure 4a. and Figure 5a)

The level of iNOS protein was markedly attenuated in lysates from J774 cells preincubated with MG (1mM) or L-NAME (1mM) 30 min before and then activated with LPS plus MG (1mM) or L-NAME (1mM). MG, but not L-NAME, added to the culture medium only 30

min before LPS challenge, significantly reduced iNOS expression (44.16± 7.9 % inhibition ; n=3; P<0.05,Figure 4b.). A representative blot from these experiments is shown in Figure 4a.

Similar results have been obtained when MG (1mM) or L-NAME (1mM) was preincubated with J774 18 h before LPS challenge. The level of iNOS protein was markedly and significantly attenuated also in lysates from J774 cells preincubated with MG (1mM) or L-NAME (1mM) 18 h before and then activated with LPS plus MG (1mM) or L-NAME (1mM). In this experimental setting MG, but not L-NAME, significantly reduced iNOS expression (30.47±3.9 % inhibition; n=3; P<0.01, Figure 5b). A representative blot from these experiments is shown in Figure 5a.

The effect of MG on iNOS expression has also been observed incubating LPS-induced J774 with MG or L-NAME (1mM) added 24 hours after LPS challenge. In this experimental setting both MG and L-NAME did not influence significantly iNOS.

In all the different experimental settings MG (0.01-1 mM) or L-NAME (0.01-1 mM) did not affect significantly mitochondrial reduction of MTT to formazan (data not shown) both when incubated for 30 min, 18 h before LPS challenge and or 24 after LPS suggesting that these compounds at the concentration tested are not cytotoxic to J774 cells even at the highest concentration used (1 mM).

DISCUSSION

The present study demonstrates that preincubation of J774 macrophages with MG significantly affected LPS-induced nitrite production. Its inhibitory effect is both time- and concentration-dependent.. The maximal response was obtained following 18 h pretreatment of cells with 1 mM MG prior activation with LPS. Although addition of MG only for 30 min before LPS challenge also inhibited accumulated nitrite levels, the inhibition was relatively less compared to responses produced after 18 h preincubation because MG, preincubated for 30 min before LPS activation, was effective only at the highest concentration used (1 mM). On the other hand its inhibitory effect was effective still at 0.01-0.1 mM, when MG was added to J774 medium 18 h before LPS challenge, this effect is concentration dependent and appears to involve also regulation of iNOS expression at the molecular levels resulting in inhibited enzyme protein expression and thus NO production. These findings suggest that MG could act with a dual mechanism of action on NO release. One mechanism may be directly linked to the inhibition iNOS expression, another one may be related to capability of MG to act as iNOS antagonist.

Induction of iNOS is a time-dependent process requiring *de novo* protein synthesis. Enzyme expression is detected after about 6 h from LPS activation of cells, reaching a maximum at 12 h and is maintained over aperiod of 24-48 h (Assreuy et al., 1993). Thus the fact that the addition of MG just 30 min before LPS challenge significantly inhibited iNOS expression and NO release strongly suggest that all these effects may be linked to the inhibitory effect at certain upstream events probably associated with induction of the enzyme such as TNF-α release. In fact, Western blot analysis of lysates obtained from cells activated in the presence of LPS alone or in combination with MG showed that LPS-induced iNOS expression was significantly inhibited in the presence of MG, probably indicating decreased iNOS gene transcription and/or mRNA stability.

A growing body of evidence suggests that NO may be involved in the inflammatory processes, such as host defence mechanisms, induced by TNF-α (Moncada et al., 1991). NO is generated in macrophages by an inducible, calcium independent isoform of iNOS that is expressed to a significant level following stimulation with phlogogenic agents, as LPS (Moncada et al., 1991) and/or cytokines, including TNF-α (Drapier et al.,1988; Chester et al., 1998).

TNF-α is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammation and as an important factor in host defence functions (Berczi et al., 1996). It is not generally thought to be produced costitutively by normal cells, but rather to be induced by invasive stimuli in the setting of infectious diseases. In this role, macrophages and monocytes are thought to be the cells which contribute most to the local and systemic TNF-α release in response to bacterial, viral and parasitic organisms and products. The consequences of endogenous TNF-α can be either beneficial or life threatening depending on the amount, time course and distribution of released cytokine.

In this study we have simulated *in vitro* a pattern similar to the uremic conditions by incubating J774-macrophages for 30 min or 18 h in medium containing high concentrations of MG (1mM) and then we have stimulated J774 macrophages with LPS in presence of MG or L-NAME. In the experimental setting in which MG was added to J774 medium 18 h before LPS challenge, we have previously demonstrated (Autore et al., 1999) that, in this condition, MG inhibits *in vitro* TNF-α release, measured 3 h after LPS activation. In this paper we also demonstrated that MG is able to inhibit not only NO release but also iNOS expression. In the same experimental conditions L-NAME showed almost the same inhibitory effect, compared to MG effect, on TNF-α and NO release while did not show any significant inhibitory effect on iNOS expression. Similar results have been obtained by Deakin (1995) and Ruetten & Thiemmermann (1996) with aminoguanidine (AMG). In their papers they have demostrated

that the immunological stimulated TNF-α release is markedly attenuated by nitric oxide inhibitors (Deakin et al., 1995) and by AMG (Ruetten & Thiemmermann, 1996), an endogenous compound which is structurally-tightly-linked to MG.

Previously we have demonstrated (Sorrentino et al., 1997) the ability of MG to link and to inhibit a semicrude preparation of both constitutive (neuronal and endothelial) and inducible isoforms (macrophagic) of NOS but the relevance of the present paper regarded the ability of MG to regulate not only iNOS activity but although iNOS expression and on the basis of our previous results obtained both *in vitro* in LPS-treated-J774 macrophages than *in vivo* in LPS-treated rats we could hypothesize that the effects observed could be due or tightly linked to the inhibition of TNF-α release (Autore et al.,1999).

The ability of MG to inhibit iNOS expression and/or NO production may have significant implications in the pathogenesis of CRF where it has been reported that these patients often present an immunodeficient state that paradoxically coexists with signs of activation of immune system cells. Moreover, this is accentuated rather than corrected by replacement dialysis therapy (Deschamps-Latscha, 1993; Kelly, 1994; Deenitchina et al., 1995). It is obvious that MG concentration in CRF patients could be quite different from the concentration used in our experimental model. Indeed, plasma concentration of MG in CRF patients have been calculated at around 27 μM (Orita et al., 1981). It is worth noting, however that MG can accumulate intracellularly, reaching concentrations which are 7 fold higher than those found in serum (Orita et al., 1981).

In summary our results indicate that high concentration of MG could interfere not only in inhibiting iNOS activity but also in inhibiting iNOS expression. The ability of MG to inhibit NO release and iNOS expression may have significant implications in the pathogenesis of CRF where it has been reported that patients often present an immunodeficient state paradoxically coexisting with signs of activation of immune system cells. In conclusion our results strongly demonstrate that MG is a potent modulator of NO release and that its

inhibitory effect is not only due to a competitive antagonism of iNOS but also to an inhibitory activity on iNOS expression. These effects together with the ability of MG to inhibit TNFα release, as demonstrated in our previous study (Autore et al., 1999), could, at least in part, account for the immunodisfunction associated to CRF

The relevance of these results is that in CRF patients the presence of high serum concentration of MG could interfere with NOS inhibitory activity (Sorrentino et al., 1997) and /or in cytokine network not only trough reduction of TNFα release (Autore et al., 1999) but also in inhibiting iNOS expression. This study points out a possible pathophysiological role for iNOS activity and expression in immunodisfuntions of haemodialitic patients where elevation of iNOS expression could be considered a physiological response to invasive stimuli that is drastically reduced when high concentrations of the uremic catabolyte, MG, are present in the blood.

REFERENCES

1. Assreuy, J., Cunha, F.J., Liew, F.Y. & Moncada, S. (1993) Feed back inhibition of nitric oxide synthse activity by nitric oxide. *Br. J. Pharmacol*., **108**, 833-837
2. Aucella, F., Vigilante, M., Grandone, E., Colaizzo, D., Margaglione, M**.,** Modoni, S., Orlandini, G., Stallone, C. (1998) Reduction of mononuclear cytokine production in hemodialysis patients treated with steam-sterilized low-flux polysulphone membranes. *Int. J.Artif.Organs*, **21** (4), 210-215
3. Autore, G., Marzocco, S., Sorrentino, R., Mirone, V., Baydoun, A. & Pinto, A. (1999) In vitro and in vivo TNFα synthesis by methylguanidine, an uremic catabolyte. *Life Science*, in press
4. Berczi, I, Chalmers, I.M., Nagy, E., Warrington, R.J. (1996), The immune effects of neuropeptides. *Bailliers Clin Rheumatol*.,**10** (2), 227-257

5. Chester, A.H., Borland, J.A., Buttery, L.D., Mitchell, J.A., Cunningham, D.A., Hafizi, S., Hoare, G.S., Springall, D.R., Polak, J.M., Yacoub, M.H. (1998) Induction of nitric oxide synthase in human vascular smooth muscle: interactions between proinflammatory cytokines. *Cardiovascul. Res*.,**38** (3), 814-821
6. Deakin, A.M., Payne, A.N., Whittle, B.J. & Moncada, S. (1995), The modulation of IL-6 and TNF alpha release by nitric oxide following stimulation of J774 cells with LPS and IFN-gamma. *Cytokine*, **7** (5), 408-416
7. Deenitchina, S.S., Ando, T., Okuda, S., Kinukawa, N., Hirakata, H., Nagoshima, A., Fujishima, M. (1995) Cellular immunity in hemodialysis patients: a quantitative analysis of immune cell subsets by flow cytometry. *Am. J. Nephrol*., **15** (1), 57-65
8. Deschamps-Lastscha, B. (1993) *Curr. Opin. Nephrol. Hypertens*.,**2** (6), 883-891
9. Drapier, J.C., Wietzerbin, J. , Hibbs, J.B. Jr (1988), Interferon.γ and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. *Eur. J. Immunol*. **18** (10), 1587-1592
10. Green, L.C., Wagner, D.A., Glogowski, J.J., Skipper, P.L., Wishnok, J.S 6 Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite and ^{15}N-nitrite in biological fluids. *Anal.Biochem.*,**126**, 131-138
11. Jones, J.D. & Burnett, P.C. (1975) Creatinine metabolism and toxicity. *Kidney Int.* Suppl.,**3**, 294-298
12. Kawano, Y., Takaue, Y., Motoyoshi, K., Minakuchi, J., Kawashima, S., Saito, S., Hirao, A., Sato, J., Shimizu, T, Kuroda, Y.(1993) Measurements of serum levels of macrophage colony stimulating factor (m-CSF) in patients with uraemia. *Exp. Hematol*., **21**(2), 220-223
13. Kelly, C.J. (1994) T cell function in chronic renal failure and dialysis. *Blood Purif*.,**12** (1), 36-41

14. Le Meur, Y., Fixe, P., Aldigier, J.C., Leroux-Robert, C., Praloran, V. (1996), Macrophage colony stimulating factor involvement in uremic patients. *Kidney Int*, **50**(3), 1007-1012
15. Mendes-Ribeiro, A.C., Roberts, N.B., Lane, C., Yaqoob, M., Ellory,J.C. (1996), Accumulation of the endogenous L-arginine analogue NG-monomethylL-arginine in human end-stage-renal failure patients on regular emodialysis. *Exp. Physiol.*, **81** (3), 475-481
16. Moncada, S., Palmer, R.M.J., Higgs, E.A.(1991) Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol Rev*., **43**, 109-142
17. Opipari, A.W. Jr., Hu, H.M., Yabkowitz, R., Dixit, V.M. (1992), The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity. *J Biol Chem*., **267** (18), 12424-12427
18. Ruetten, H., Thiemmermann, C. (1996) Prevention of the expression of inducible nitric oxide sinthase by aminoguanidine or aminoethyl-isothiourea in macrophages and in the rat. *Bioch. Biophys. Res. Commun.*,**225** (2), 525-530
19. Sorrentino, R., Pinto, A. (1995) Effect of methylguanidine on rat blood pressure: role of endothelial nitric oxide synthase. *Br. J. Pharmacol*., **115** (3), 510-514
20. Sorrentino, R., Sautebin, L., Pinto, A. (1997) Effect of methylguanidine, guanidine and structurally-related compounds on constitutive and inducible nitric oxide synthase activity. *Life Science*,**61** (13), 1283-1291
21. Yokozawa, T., Fujitsuka, N., Oura, H. (1990) Production of methylguanidine from creatinine in normal rats with renal failure.**56** (3), 249-254

Fig 1 (a, b). Concentration-dependent effect of LPS-induced nitrite production in J774 macrophages by MG (1a) and L-NAME (1b). Cells were incubated with LPS ($6x10^3$ u ml^{-1}) alone (control) or in combination with MG (0.01-1mM) or L-NAME (0.01-1 mM) added simoultaneously and **30 min before** LPS challenge. Accumulated nitrite in the culture medium was determined 24 h after LPS addition by the Griess reaction. Results are expressed as μmol l^{-1} NO_2^- (basal LPS levels were 15.33±1.02 μM NO_2^- 24 h^{-1}) and values are the mean±s.e.m. of at least 3-6 independent experiments with 5 replicates in each. *** Denotes values significantly different from control at $P<0.001$.

Fig 2 (a,b). Concentration-dependent inhibition of LPS-induced nitrite production in J774 macrophages by MG and L-NAME. Cells were incubated with LPS ($6x10^3$ u ml^{-1}) alone (control) or in combination with MG (0.01-1mM) or L-NAME (0.01-1 mM) added simoultaneously and **18 h before** LPS challenge. Accumulated nitrite in the culture medium was determined 24 h after LPS addition by the Griess reaction. Results are expressed as μmol l^{-1} NO_2^- (basal LPS levels were 16.64±0.58 μM NO_2^- 24 h^{-1}) and values are the mean±s.e.m. of at least 3-6 independent experiments with 5 replicates in each. *** Denotes values significantly different from control at $P<0.001$, * $P<0.05$.

Fig 3 (a,b). Concentration-dependent inhibition of LPS-induced nitrite production in J774 macrophages by MG or L-NAME, added 24 h after LPS challenge, and reversal inhibition of LPS-induced nitrite production in J774 macrophages by MG or L-NAME with L-ARG supplementation (10mM). Cells were incubated with LPS ($6x10^3$ u ml^{-1}) alone (control) or in combination with MG (0.01-1 mM) or L-NAME (0.01-1 mM) with or without L-ARG (10mM) added **24 h after** LPS challenge. The effect of MG or L-NAME on accumulated nitrite in the culture medium was determined 48 h after LPS addition by the Griess reaction. Results are expressed as μmol l^{-1} NO_2^- and values are the mean±s.e.m. of at least 3-6

independent experiments with 3 replicates in each *** Denotes values significantly different from control at P<0.001, * P<0.05

FIGURE 4. (a,b) Western blot analysis of inducible NO synthase in J774 cells. Lysates (70 µg protein) from untreated cells (c) and from cells preincubated with MG or L-NAME (1mM) for 30 min (a,b) before LPS and reincubated with LPS ($6x10^3$ u ml^{-1}; 24 h) alone or in combination with either MG (1mM) or L-NAME (1mM) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with a mouse monoclonal anti-iNOS antibody. The blot is representative of 3 independent experiments. (a) A representative blot from these experiments (b) Relative intensity of iNOS protein bands quantitated by scanning densitometry. Data are expressed as percentage of inhibition calculated versus LPS alone.

FIGURE 5. (a,b) Western blot analysis of inducible NO synthase in J774 cells. Lysates (70 µg protein) from untreated cells (c) and from cells preincubated with MG or L-NAME (1mM) for 18 h before LPS and reincubated with LPS ($6x10^3$ u ml^{-1}; 24 $^{h-1}$)alone or in combination with either MG (1mM) or L-NAME (1mM) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with a mouse monoclonal anti-iNOS antibody. These blots are representative of 3 independent experiments. (a) A representative blot from these experiments (b) Relative intensity of iNOS protein bands quantitated by scanning densitometry. Data are expressed as percentage of inhibition calculated versus LPS alone.

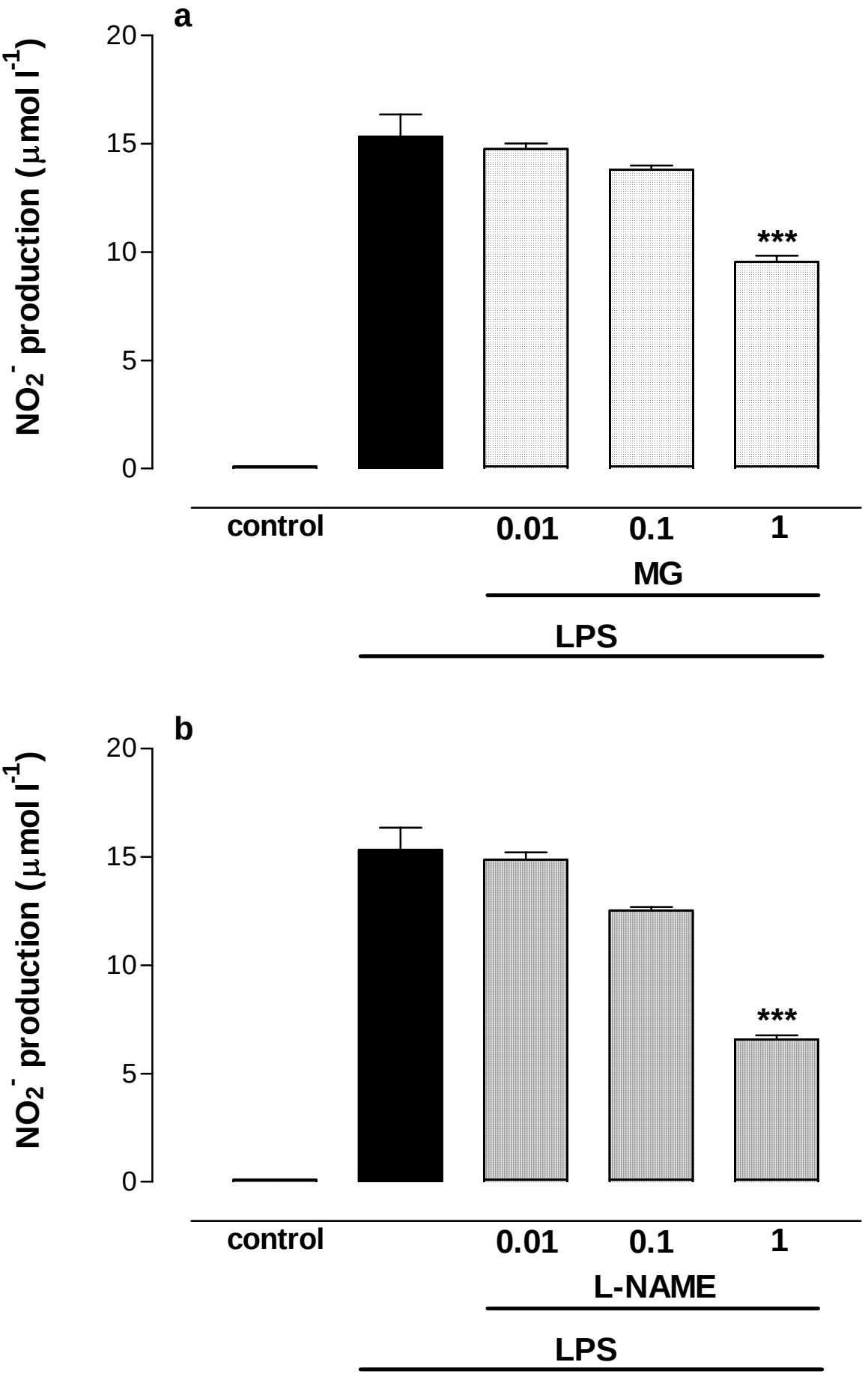
a
20
15
10
5
0
NO_2^- production (μmol l^{-1})
***
control
0.01
0.1
1
MG
LPS
b
20
15
10
5
0
NO_2^- production (μmol l^{-1})
***
control
0.01
0.1
1
L-NAME
LPS

Figure 1

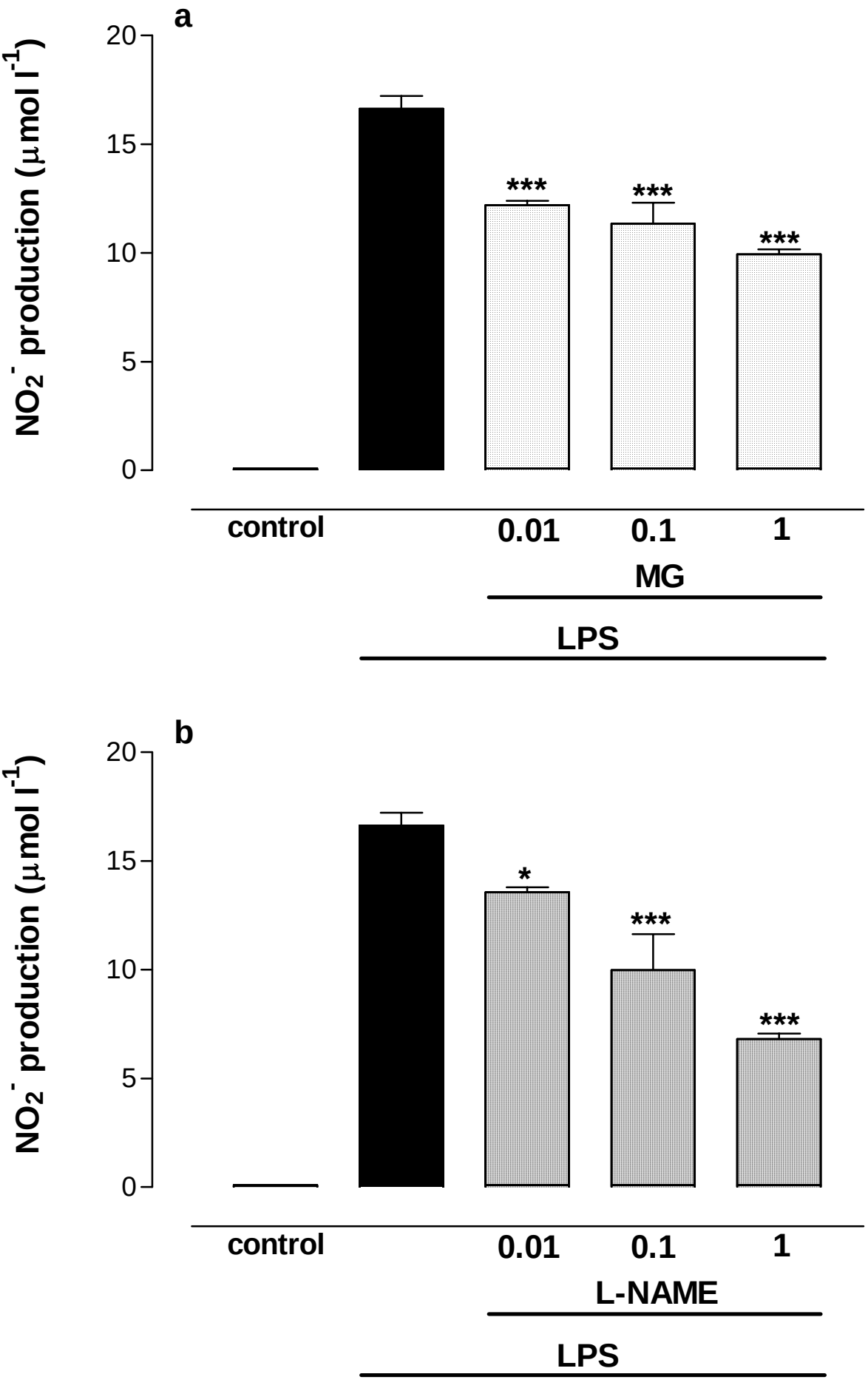
a
20
15
10
5
0
NO_2^- production (μmol l^{-1})
***
***
***
control
0.01
0.1
1
MG
LPS
b
20
15
10
5
0
NO_2^- production (μmol l^{-1})
*
***
***
control
0.01
0.1
1
L-NAME
LPS

Figure 2

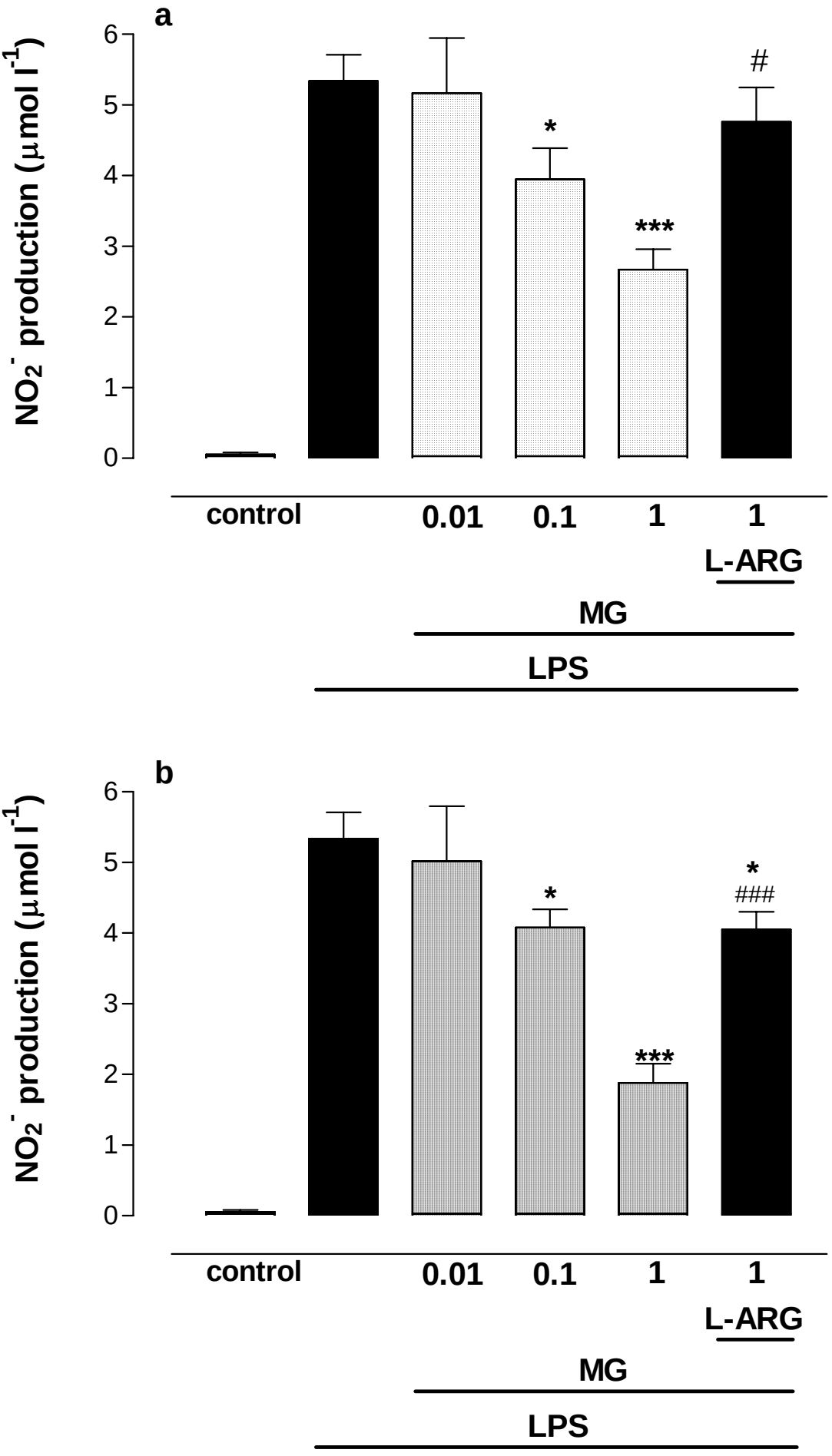
a
NO_2^- production (μmol l^{-1})
0
1
2
3
4
5
6
*
***
#
control
0.01
0.1
1
1
L-ARG
MG
LPS
b
NO_2^- production (μmol l^{-1})
0
1
2
3
4
5
6
*
***
*
###
control
0.01
0.1
1
1
L-ARG
MG
LPS

Figure 3

a: a photo of representative blot

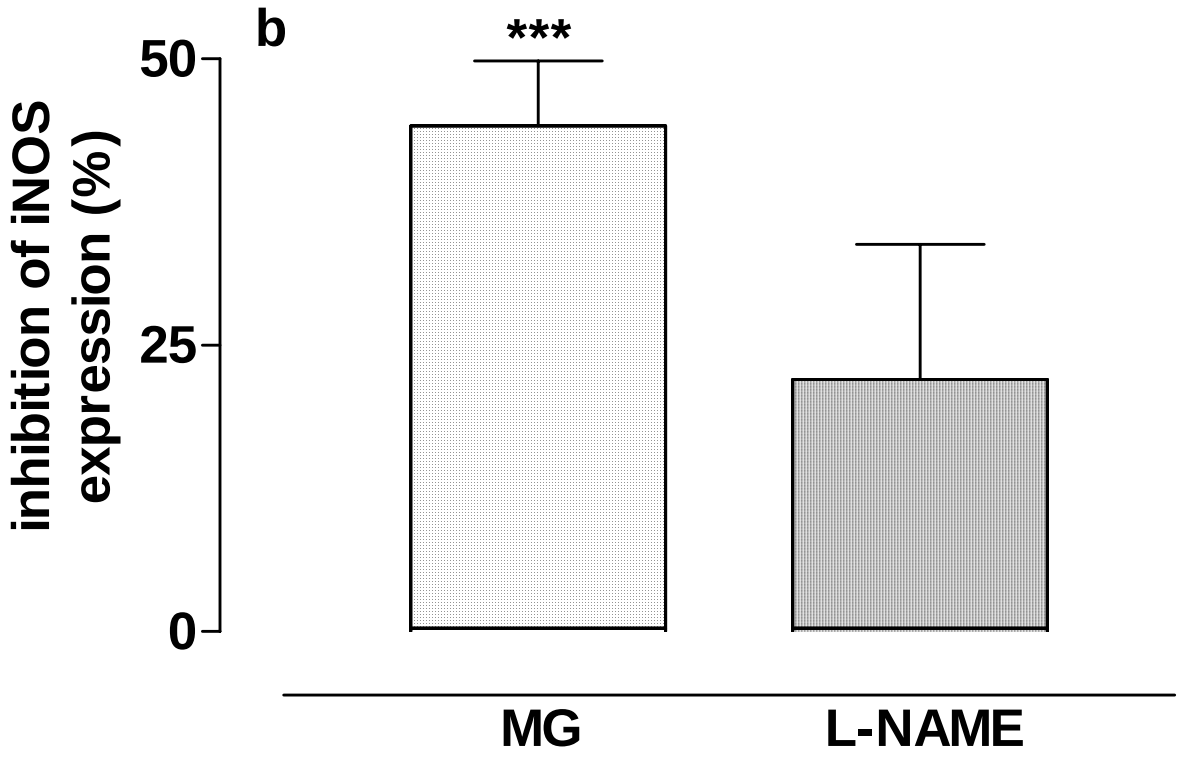

Figure 4

a: a photo of representative blot

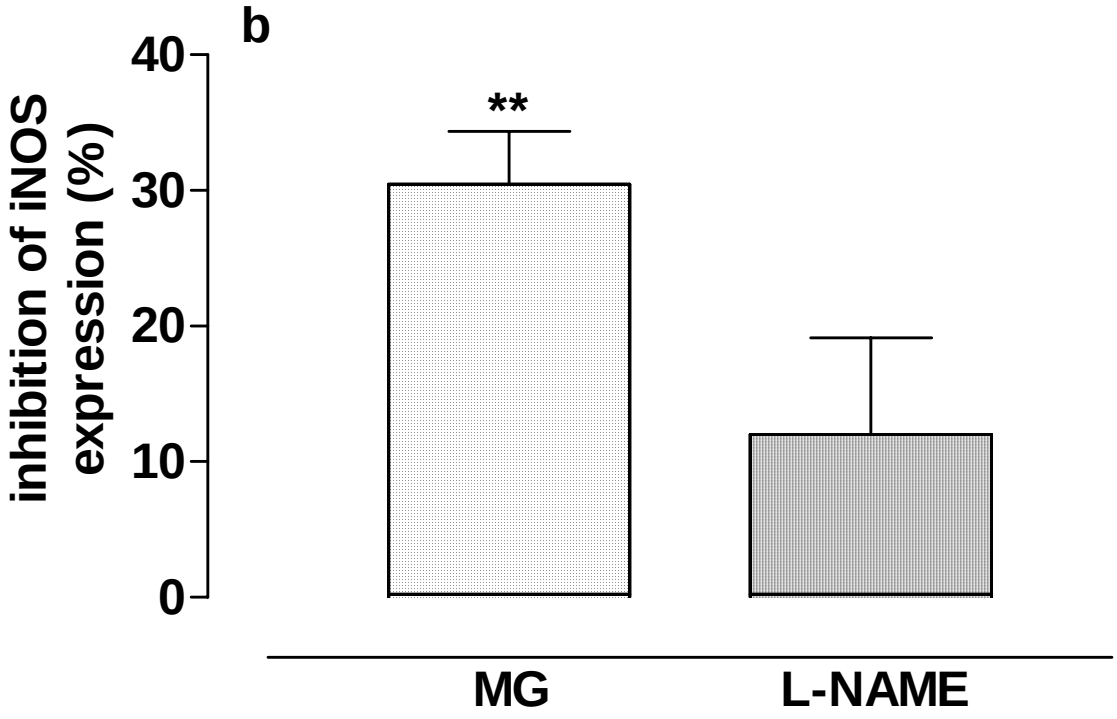

Figure 5