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ScienceDirect
Life Sciences 80 (2007) 1282–1291
Life Sciences
www.elsevier.com/locate/lifescie

Oxalomalate affects the inducible nitric oxide synthase expression and activity

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Received 6 March 2006; accepted 15 December 2006

Abstract

Inducible nitric oxide synthase (iNOS) is an homodimeric enzyme which produces large amounts of nitric oxide (NO) in response to inflammatory stimuli. Several factors affect the synthesis and catalytic activity of iNOS. Particularly, dimerization of NOS monomers is promoted by heme, whereas an intracellular depletion of heme and/or L-arginine considerably decreases NOS resistance to proteolysis. In this study, we found that oxalomalate (OMA, oxalomalic acid, α-hydroxy-β-oxalosuccinic acid), an inhibitor of both aconitase and NADP-dependent isocitrate dehydrogenase, inhibited nitrite production and iNOS protein expression in lipopolysaccharide (LPS)-activated J774 macrophages, without affecting iNOS mRNA content. Furthermore, injection of OMA precursors to LPS-stimulated rats also decreased nitrite production and iNOS expression in isolated peritoneal macrophages. Interestingly, α-ketoglutarate or succinyl-CoA administration reversed OMA effect on NO production, thus correlating NO biosynthesis with the anabolic capacity of Krebs cycle. When protein synthesis was blocked by cycloheximide in LPS-activated J774 cells treated with OMA, iNOS protein levels, evaluated by Western blot analysis and ^{35}S-metabolic labelling, were decreased, suggesting that OMA reduces iNOS biosynthesis and induces an increase in the degradation rate of iNOS protein. Moreover, we showed that OMA inhibits the activity of the iNOS from lung of LPS-treated rats by enzymatic assay. Our results, demonstrating that OMA acts regulating synthesis, catalytic activity and degradation of iNOS, suggest that this compound might have a potential role in reducing the NO overproduction occurring in some pathological conditions.

Keywords: Nitric oxide; iNOS; Oxalomalate; Krebs cycle; Protein degradation

Introduction

Nitric oxide is an endogenous short-lived free radical that freely diffuses within the cells, from formation to action site. NO is synthesized by a family of homodimeric enzymes named NO synthase, having L-arginine and molecular oxygen as substrates and NADPH as electron donor (Moncada et al., 1991). The activity of NOS is provided by heme with pentacoordinated Fe^{2+}, tetrahydrobiopterin (H_4B), FMN, FAD, Zn^{2+} and the Ca^{2+}-binding protein calmodulin as cofactors and prosthetic groups (Alderton et al., 2001). The enzymatic reaction consumes five electrons necessary for the sequential oxidation of L-arginine terminal guanidine nitrogen (Aktan, 2004). Three NOS isoforms have so far been described; among these two NOS isoforms (i.e. neuronal or type-1 NOS and endothelial or type-3 NOS) are constitutively expressed in cells (cNOS), synthesizing picomoles of NO in response to increased Ca^{2+} ions, while a third isoform, namely inducible NOS (iNOS or type-2 NOS) synthesizing nanomoles of NO, is typically expressed in response to inflammatory stimuli in a variety of immune cells. The expression of iNOS may be beneficial in host defence or in modulating the immune response (Hobbs et al., 1999); indeed, the massive NO production by iNOS from macrophages during infections inhibits the growth of many pathogens (Bogdan, 2001).

NOS isoforms are only active as homodimers (Hemmens and Mayer, 1998) and their activity is dependent on a number of cofactors. The dimerization of NOS monomers is promoted by heme,

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0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.12.008

resulting in rapid conformational changes that, by cooperative action of BH_4 and L-arginine, lead to a stable and active enzyme (Gorren and Mayer, 2002). Moreover, an intracellular depletion of heme, BH_4 and/or L-arginine considerably contributes to decrease the resistance of NOS enzymes to proteolysis (Dunbar et al., 2004).

Oxalomalate, a tricarboxylic acid structurally related to citrate, has long been known to be a powerful competitive inhibitor of both aconitase (Ruffo et al., 1962; Adinolfi et al., 1969) and NADP-dependent isocitrate dehydrogenase (Ingebretsen, 1976). OMA can be produced *in vitro* by a non-enzymatic aldolic condensation between oxaloacetate and glyoxylate (Adinolfi et al., 1971; Johanson and Reeves, 1977). Moreover, the reaction occurs also *in vivo* in animals injected with equimolar concentration of both compounds (Festa et al., 2000) or could occur in mammalian cells under physiological conditions when both oxaloacetate and glyoxylate are present, the latter being synthesized and catabolized in cells of vertebrates (Davis et al., 1989; Bais et al., 1991; Fujiwara and Noguchi, 1995). Aconitase, which is required for the first step of citric acid cycle, is structurally identical to the cytoplasmic aconitase/IRP-1, a bi-functional protein that controls the expression of the most important proteins involved in iron metabolism at post-transcriptional level (Eisenstein, 2000; Cairo and Pietrangelo, 2000). In detail, c-Acon/IRP-1 through [4Fe–4S] cluster assembly/disassembly, switches from the aconitase form, to the IRP-1 form (Haile et al., 1992), in response to the iron level in the cells (Guo et al., 1994; Pantopoulos, 2004).

OMA is able to interfere with iron-containing proteins and iron metabolism (Festa et al., 2000), since it acts as an inhibitor of IRP-1 RNA-binding capacity (Santamaria et al., 2004). The activity of c-Acon/IRP-1 is also regulated by NO signalling (Pantopoulos et al., 1996) and nitration of this protein occurs in macrophages during inflammation with a consequent change in its activity (Gonzalez et al., 2004). Moreover, NO modulates also the activity of several other proteins (Kim and Ponka, 2002, 2003) including mitochondrial aconitase, an iron-dependent enzyme (Drapier et al., 1993) and isocitrate dehydrogenase (Yang et al., 2002).

In the present study we evaluated the effect of OMA on nitrite production and iNOS protein expression in J774 murine macrophages activated with bacterial lipopolysaccharide (LPS). In addition, we evaluated the ex vivo effect of OMA precursors on nitrite production and iNOS protein expression in peritoneal macrophages from LPS-stimulated rats. Moreover, considering that OMA interacts with key enzymes of the tricarboxylic acid cycle, we examined the effect of Krebs cycle intermediates on NO production in OMA-treated LPS-stimulated J774 cells. Furthermore, we evaluated the possible mechanism of OMA action on iNOS protein biosynthesis, stability and/or degradation. Finally, we investigated the OMA effect on the activity of iNOS from lung of LPS-treated rats.

Materials and methods

Animals

Male Wistar (Charles River) rats, weighing 250–300 g, were used after one week of adaptation to the housing conditions. Rats were housed in a controlled environment and provided with standard rodent chow and water *ad libitum*. Animal care was in compliance with Italian regulations (D.M. 116192) on protection of animals used for experimental and other scientific purpose, as well as with the EEC regulations (O.J. of E.C. L 358/1 18/12/1986).

Macrophage cell cultures and treatments

The monocyte/macrophage cell-line J774 (from American Tissue Cell Collection) was grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in 5% CO_2 and 95% air. The cells were plated in 24-well culture plates at density of 2.5×10^5 cells/ml/well and allowed to adhere for 2 h at 37 °C. The medium was replaced with fresh medium and cells were treated with OMA (2.5 and 5 mM) for 2 h before being stimulated with lipopolysaccharide (LPS) from *Salmonella typhosa* (1 μg/ml) for a further 24 h. In some experiments, cells were incubated with succinyl-CoA (5 mM), α-ketoglutarate (5 mM) and sodium pyruvate (5 mM) 1 h after LPS stimulation, in the presence or absence of OMA (5 mM). In other experiments, OMA (5 mM) and dexamethasone (DXM) (10 μM) were added to the cells 2 h before or 12 h after LPS (1 μg/ml). In experiments with cycloheximide (CHX), J774 cells were firstly treated with OMA (5 mM) for 2 h, then stimulated with LPS for 12 h and next CHX (10 μg/ml) was added for 2 h, to stop protein synthesis in the cells. In addition, experiments were performed in LPS-stimulated J774 cells exposed to OMA (5 mM) for 2 h after CHX treatment. To inhibit proteasome-mediated protein degradation, J774 cells were incubated with carbobenzoxyl–L-leucinyl–L-leucinyl–L-leucinal (MG132) (2.5, 5, 10 and 20 μM) for the last 2 h of LPS stimulation.

Cell cultures of primary macrophages were obtained from male Wistar rats (250–300 g) treated as described below. Briefly, rats were injected subcutaneously with aliquots of a solution that yielded 92.6 mg of neutralized oxaloacetic acid and 50.3 mg of sodium glyoxylate per 100 g of body weight 2 h before intraperitoneal LPS injection (6 mg/kg). The control animals were injected with physiological solution. Five hours after LPS or saline administration rats were sacrificed and macrophages were harvested from the peritoneal lavage fluid as described by Carnuccio et al. (1989). The peritoneal macrophages were grown in DMEM supplemented with 2 mM glutamine, 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% foetal bovine serum and 1.2% sodium pyruvate. Macrophages were separated from red blood cells by adhesion on plastic Petri dishes for 1 h, then non-adherent cells were removed by washing and fresh DMEM was added to adherent macrophages. Macrophages were then incubated for further 3 h before nitrite detection in the cell medium.

Cell viability assay

Cell viability was assessed by measuring the level of mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) as substrate (Hansen et al., 1989). The assay was based on the redox