Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation

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The activation of cultured Raw 264.7 murine macrophages with interferon γ and lipopolysaccharide results in the expression of inducible nitric oxide synthase (i-NOS) and the subsequent production of nitric oxide. In the present study, the i-NOS expressed in these activated cells was characterized for possible post-translational protein modification by endogenous tyrosine protein kinases. Western-blot analysis using phosphotyrosine antibodies revealed that i-NOS was phosphorylated on tyrosine residues and that this was an early event coinciding with the appearance of newly synthesized i-NOS. A brief exposure of

activated cells to vanadate, a tyrosine phosphatase inhibitor, significantly increased the level of i-NOS tyrosine phosphorylation, suggesting that tyrosine phosphatases are dynamically involved in the regulation of this process. Vanadate treatment of activated cells also resulted in a rapid increase in enzyme activity, occurring within 5 min of exposure. Taken together, these results demonstrate that tyrosine kinases and phosphatases are involved in the post-translational modification of i-NOS and may potentially play a role in modulating the functional activity of the enzyme in macrophages.

INTRODUCTION

Nitric oxide (NO), the product of a catalytic transformation of L-arginine to citrulline by nitric oxide synthases (NOSs), is an important messenger molecule in biological systems (for reviews, see [1,2]). The three principle isoforms of NOS identified and cloned to date are: the constitutively expressed brain NOS [3], the endothelial NOS [4], and the macrophage inducible (i-) NOS [5,6]. Both the brain NOS and endothelial NOS enzymes are regulated by Ca^{2+} through the regulatory Ca^{2+} -binding protein calmodulin and require the addition of both components when assaying enzyme activity [7,8]. Although the i-NOS isoform is also a calmodulin-dependent enzyme, it does not require the addition of Ca^{2+} or calmodulin when assaying activity, since the enzyme's high affinity for these two cofactors keeps the i-NOS in a predominantly Ca^{2+} -and calmodulin-bound state during cell-extract preparation and purification [9].

Post-translational modification and regulation of NOS activity by protein kinases has been demonstrated for both the endothelial and brain isoforms. The phosphorylation of endothelial NOS on serine residues by unidentified protein kinases has been reported and shown to be associated with the translocation of the enzyme from the cell membrane to the cytosol [10]. Several specific protein kinases have been shown to phosphorylate the brain NOS, including cAMP-dependent protein kinase [11,12], calcium calmodulin-dependent protein kinase II [13], and protein kinase C [12,13]. Phosphorylation by cyclic AMP-dependent protein kinase was shown to have no effect on brain NOS activity [11,12], whereas phosphorylation by Ca^{2+} /calmodulin-dependent protein kinase II decreased activity [13]. Both positive and negative effects on brain NOS activity as a result of protein kinase C activation have been reported [12,13].

Studies on protein kinase regulation of i-NOS activity have focused primarily on the regulation of gene expression. Protein kinase C, for example, has been shown to mediate interferon γ (IFN- γ)-stimulated i-NOS expression in macrophages [14]. Tyrosine kinases have been implicated in regulating gene expression in macrophages that have been treated with lipopolysaccharide (LPS) [15]. Similarly, tyrosine kinases have been shown to mediate interleukin 1 β -induced i-NOS activity in the rat insulin secretory cell line, RINm-5F [16], and to mediate LPS and IFN- γ co-induced i-NOS activity in glial cells [17].

Little is known, however, about protein kinase phosphorylation of the i-NOS isoform or the post-translational effects that protein kinases may have on i-NOS cellular localization or function. Using the RAW 264.7 murine macrophage cell line, we studied the phosphorylation of i-NOS by cellular tyrosine kinases. We also investigated whether inhibiting tyrosine phosphatases could lead to elevations in the level of i-NOS phosphorylation or to alterations in enzyme activity.

MATERIALS AND METHODS

Materials

Reagents were purchased as follows: murine IFN- γ , BioSource International; tetrahydrobiopterin (THB), Research Biochemicals International; L-[2,3- ^3H]arginine (36.1 Ci/mmol), NEN Research Products; Dowex 50WX-8 resin (Na^+ form), sodium orthovanadate, Aldrich; horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-phosphotyrosine antibody, blocking reagent, Zymed Laboratories, Inc.; Protein A-Sepharose, Pharmacia Biotech Inc.; Hybond-ECL nitrocellulose, Hyperfilm-ECL X-ray film, ECL Western blotting detection kit,

Abbreviations used: * NOS, nitric oxide synthase; i-NOS, inducible nitric oxide synthase; THB, tetrahydrobiopterin; L-NMMA, N^G -monomethyl-L-arginine; IFN- γ , interferon γ ; LPS, lipopolysaccharide; DTT, dithiothreitol; AEBSEF, 4-(2-aminoethyl)benzenesulphonyl fluoride; HRP, horseradish peroxidase; PMA, phorbol-12-myristate 13-acetate; FBS, foetal bovine serum.

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Amersham International; Sea Kem LE agarose, FMC Bio-Products; Bio-Rad Protein Assay kit, Tris-buffered saline [TBS; 10 mM Tris/HCl (pH 8.2)/50 mM NaCl], Bio-Rad Laboratories; HRP-conjugated donkey anti-rabbit IgG, Axell laboratories; Ecoscint A, National Diagnostics; 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), Boehringer-Mannheim; RPMI 1640, all other cell culture products, TRIzol reagent, Superscript reverse transcriptase, GIBCO-BRL; Prime RNase inhibitor was from 5 Prime-3 Prime Inc.; β -actin PCR primers, Clontech; i-NOS PCR primers, synthesized in-house; AmpliTaq polymerase, Perkin-Elmer; phorbol 12-myristate 13-acetate (PMA), LPS (*Escherichia coli* strain 055:B5), Triton X-100, aprotinin, dithiothreitol (DTT), NADPH, fetal bovine serum (FBS), (L-*N*^G-monomethyl-L-arginine (L-NMMA), all other chemicals, Sigma.

Cell cultures and treatments

RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in culture medium (RPMI 1640 containing 10% heat-inactivated FBS, 1 mM sodium pyruvate, 2 mM glutamine, 50 units/ml each of penicillin and streptomycin) at 37 °C in a 5%-CO₂ humidified incubator. For measuring nitrite release, cells were grown for 24 h in 96-well plates at 2.5×10^4 cells/well in 100 μ l of culture medium. The next day, the culture medium was replaced with 100 μ l of fresh medium containing the indicated concentrations of IFN- γ and/or LPS and grown for various times out to 72 h. For measuring citrulline formation or for Western-blot analysis, 10^7 cells in 10 ml of culture medium were seeded in 75 cm² flasks and grown for 24 h. The specified components were then added to the medium for the times indicated followed by extract preparation as described below.

Whole-cell-extract preparation

For preparation of crude soluble extracts, culture flasks were chilled on ice without removing the culture medium and the cells harvested by gentle scraping into a centrifuge tube. Cells were centrifuged at $1000 \times g$ for 5 min at 4 °C followed by resuspension in 1 ml of ice-cold PBS and transferred to a 1.5 ml microcentrifuge tube. Cells were then centrifuged at $1000 \times g$ for 5 min at 4 °C in a microcentrifuge, followed by resuspension in 1 ml of ice-cold PBS. Cell lysis was accomplished by first centrifuging the cells as in the previous step and resuspending the cell pellet in 500 μ l of ice-cold lysis buffer [1% Triton X-100/20 mM Tris/HCl (pH 8.0)/137 mM NaCl/10% glycerol/0.2 mM AEBSF/10 mg/ml aprotinin/1 mM sodium orthovanadate]. The suspension was gently vortex-mixed for 10 s and allowed to incubate on ice for 30 min to complete cell lysis. The mixture was centrifuged at $14000 \times g$ for 30 min at 4 °C in a microcentrifuge and the supernatant transferred to a new microcentrifuge tube. Protein concentrations were determined using the Bio-Rad DC protein assay kit according to the manufacturer's instructions.

Cytosol and particulate fractionation of cell extracts

Cytosol and particulate fractions were separated by high-speed ultracentrifugation. Briefly, cells were treated with a combination of 100 ng/ml LPS and 10 unit/ml IFN- γ for 16 h, harvested by gentle scraping, and washed in PBS as described above. Cell lysis was achieved by homogenizing the cell suspension with a Polytron homogenizer in 2 ml of ice-cold hypotonic buffer [50 mM Hepes (pH 7.4)/1 mM EGTA/1 mM DTT/10 μ g/ml aprotinin/0.2 mM AEBSF] until the cells were completely disrupted as assessed by viewing the cell lysate under a microscope. The

homogenate was then centrifuged at $105000 \times g$ for 2 h at 4 °C, followed by removal and storage of the supernatant on ice. The particulate fraction was resuspended in 4 ml of ice-cold hypotonic buffer and the ultracentrifugation step repeated. The washed particulate fraction was detergent-solubilized by resuspending the pellet in 1 ml of hypotonic buffer containing 1% Triton X-100 and gently vortex-mixing for 10 s followed by incubating on ice for 30 min. The insoluble debris was then sedimented at $14000 \times g$ for 30 min in a microcentrifuge at 4 °C and the resulting supernatant transferred to a new tube for use in the enzyme assays or Western-blot experiments.

Measurement of NOS activity

The activity of NOS was quantified either by measuring the appearance of nitrite in 50 μ l of medium sample [18] or by measuring the amount of citrulline formed in 30 min from 50 μ g of cell extract protein as previously described [19].

Generation of polyclonal antibodies to murine i-NOS

Rabbit polyclonal antibodies specific for murine i-NOS were obtained through a contractual agreement with Research Genetics (Huntsville, AL, U.S.A.). Briefly, an amino acid multiple antigen peptide (MAP) consisting of the first 17 amino acids of the murine i-NOS protein sequence (MACPWKFLFRVKS-YQGD) [5,6] was synthesized on an Advanced Chemtech model 350 multiple peptide synthesizer using small scale 'Fmoc' (fluoren-9-ylmethoxycarbonyl) synthesis. The peptides were emulsified with Freund's adjuvant and injected into four subcutaneous dorsal sites for a total volume of 1.0 ml (0.5 mg of peptide) per immunization. Rabbits received two boosters similar to the initial immunization at weeks 2 and 6, and serum was collected on weeks 4, 8 and 10. Only the third-bleed serum antibodies purified by affinity chromatography using the immunizing peptide were used in these studies.

Immunoprecipitation

Soluble extracts from 10^7 cells were prepared from cells co-stimulated with IFN- γ and LPS as described above. Samples (300 μ g of protein) were incubated in 1 ml of lysis buffer overnight at 4 °C with 1 μ g of affinity purified i-NOS antibody in a silicone-treated microcentrifuge tube. The antigen-antibody complexes were immunoprecipitated by adding 100 μ l of Protein A-Sepharose beads and gently rotating the tubes at 4 °C for 1 h. The mixture was then microcentrifuged for 20 s at $14000 \times g$ at 4 °C and the pellet washed three times in the cell lysis buffer. Samples were then resuspended in 30 μ l of 2 $\times$ SDS/PAGE loading buffer [100 mM Tris/HCl (pH 6.8)/2 mM β -mercaptoethanol/4% SDS/0.2% Bromophenol Blue/20% glycerol].

SDS/PAGE and Western-blot analysis

Samples for Western-blot analysis were heated for 1 min at 90 °C in SDS/PAGE loading buffer and electrophoresed on 12% polyacrylamide gels as described by Laemmli [20]. After electrophoresis, the gels were transferred to nitrocellulose sheets for 1 h at 100 V on a Bio-Rad Mini Trans Blot cell in transfer buffer [20 mM Tris/HCl (pH 8.3)/154 mM glycine/20% methanol]. The nitrocellulose was then blocked with either the blocking reagent for the detection of phosphotyrosine or with 5% non-fat milk in TBST (TBS plus 0.1% Tween-20) for the detection of i-NOS protein. Blots were probed for either phosphotyrosine or i-NOS protein by incubating the blots overnight at 4 °C with HRP-conjugated mouse anti-phosphotyrosine (1:2000 dilution

in blocking reagent) or affinity-purified rabbit anti-mouse i-NOS (1:1000 dilution in TBST) respectively. Blots probed with anti-i-NOS antibodies were washed three times in TBST followed by a 45 min incubation in HRP-conjugated donkey anti-rabbit IgG (1:10000 dilution in 5% non-fat milk in TBST). Both antibody-probed membranes were then washed three times in TBST followed by antibody detection by ECL (enhanced chemiluminescence) substrate addition and exposure to ECL Hyperfilm according to the manufacturer's suggestions. When blots were reprobbed, they were first stripped of the previously bound antibodies by incubating the blots for 30 min at 70 °C in stripping buffer [62.5 mM Tris/HCl (pH 6.8)/2% SDS/100 mM β -mercaptoethanol]. The efficiency of probe removal was verified by incubating stripped membranes with ECL substrate and exposing to ECL Hyperfilm.

PCR analysis of induced i-NOS mRNA

Total RNA was isolated using TRIzol Reagent according to the manufacturer's instructions and stored in 25 μ l of diethyl pyrocarbonate-treated water at -80 °C. Total RNA (2.4 μ g) was used for cDNA synthesis with Superscript reverse transcriptase according to the manufacturer's instructions, except that 0.62 unit of Prime RNase inhibitor was added to the reaction. Samples were stored at -80 °C for later use.

PCR reactions were carried out in a Peltier thermal cycler (PTC-200, MJ Research) and were performed using 5 μ l of cDNA for the amplification of murine i-NOS gene product or 1 μ l of cDNA for the amplification of β -actin gene product. The cDNA was added to 50 μ l of the following reaction mixture: PCR buffer [10 mM Tris/HCl (pH 8.3)/50 mM KCl/1.5 mM $MgCl_2$], 200 μ M dNTP, 200 nM murine i-NOS or 59 nM β -actin oligonucleotide primer pairs, and 1.25 units of AmpliTaq polymerase. The sequence for the oligonucleotide primers were based on the published sequences for β -actin [21] and i-NOS [5,6]. Primer pairs for β -actin were: 5'-GTGGGCGCTCT-AGGCACCAA-3' (nucleotides +25 to +45) for the forward sense primer and: 5'-CTCTTTGATGTCACGCACGATTTC-3' (nucleotides +564 to +541) for the reverse antisense primer. Primer pairs for murine i-NOS were: 5'-CTCAGCCCAACAA-TACAAGATGACCCTAAG-3' (nucleotides +290 to +319) for the forward sense primer and: 5'-AGAGTGAGCTGGT-AGGTTCTCTGTTGTTTCT-3' (nucleotides +714 to +685) for the reverse antisense primer. For amplifying murine i-NOS sequences, the PCR process was carried out with 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s and a final incubation at 72 °C for 7 min. For amplifying β -actin sequences, the PCR process was carried out with 25 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s followed by a final incubation at 72 °C for 7 min. Reaction products (30 μ l for murine i-NOS and 15 μ l for β -actin) were electrophoresed on a 2%-agarose gel in buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.4), stained with ethidium bromide, and photographed.

RESULTS

Induction of i-NOS by IFN- γ and LPS

To characterize the time course of i-NOS protein expression and activity, cells were stimulated with IFN- γ and LPS over a 72 h period and the levels of nitrite, mRNA and protein were measured. The induction of i-NOS mRNA relative to control cells (0 h) was apparent 4 h after treatment and remained elevated

Figure 1 Time course of the induction of i-NOS mRNA expression

For measurement of mRNA, 10^6 cells in 500 μ l were seeded into 12-well plates and grown for 16 h. The medium was then replaced with 500 μ l of fresh culture medium containing IFN- γ (10 units/ml) and LPS (100 ng/ml) and incubated at 37 °C for the indicated times. Preparation of mRNA and PCR amplification of i-NOS target mRNA was accomplished as described in the Materials and methods section. The PCR product gel pictured is a representative result from one of three separate experiments.

Figure 2 Time course of i-NOS protein expression

Cells were seeded into a 75 cm² flask at 10^7 cells in 10 ml of culture medium and grown for 16 h. The cells were then given 10 ml of fresh medium containing IFN- γ (10 units/ml) and LPS (100 ng/ml) and incubated at 37 °C for the indicated times. SDS/PAGE was performed on either immunoprecipitated i-NOS samples or 50 μ g of total protein extract, transferred to nitrocellulose and probed with antibodies to i-NOS as described in the Materials and methods section. Western-blot analysis of the appearance of i-NOS protein was performed in three separate experiments, and a representative blot photographed for presentation.

at the 6, 16 and 24 h test periods (Figure 1). In three separate experiments the small variations in β -actin yields were observed to reflect slight variations in mRNA concentration readings and cDNA yields rather than to any specific induction effects and were insufficient to account for the appearance of measured i-NOS PCR products (results not shown). The appearance of i-NOS protein was measurable only after 6 h, with peak protein expression occurring at 16 h followed by a decline at 24 h (Figure 2). The levels of nitrite elevation in the medium lagged that of both mRNA and protein, with nitrite levels undetectable at 6 h. Significant elevation of nitrite in the medium did, however, occur by 16 h (Figure 3). Although the level of accumulated nitrite was highest at 72 h, the maximum rate of accumulation occurred at approx. 24 h. For comparison, the levels of nitrite production from cells stimulated with either IFN- γ or LPS alone were measured over the same period. The nitrite accumulation observed for IFN- γ - or LPS-stimulated cells was detectable beginning at 16 h (results not shown), although the total levels of accumulated nitrite were less than that of the co-stimulated cells at all the time points tested.

Tyrosine phosphorylation of i-NOS

To determine if i-NOS was tyrosine-phosphorylated under the conditions used for the induction of i-NOS expression, the Western blot in Figure 2 previously probed with antibodies to i-

Figure 3 Time course of nitrite induction by IFN- γ and LPS

To determine the time course of i-NOS induction in the Raw 264.7 cell line, the levels of nitrite produced were measured from the sample medium over a 72 h period. Cells were seeded into 96-well plates at 2.5×10^4 cells/well in 100 μ l of culture medium. After 16 h, the medium was replaced with 100 μ l of fresh culture medium in the absence (○) or presence of LPS (●; 100 ng/ml), IFN- γ (□; 10 U/ml), or both LPS and IFN- γ at the same concentrations (■). At the indicated times, medium was removed and stored at -70°C until assayed for nitrite content. The time course of nitrite accumulation in the medium was measured from four separate experiments and a single representative experiment depicted in the graph. Data points represent triplicate values $\pm$ S.D. * $P \leq 0.05$ versus the zero time point (Student's independent t test).

Figure 4 Tyrosine phosphorylation of i-NOS

The blot in Figure 2 demonstrating the time course of i-NOS protein expression was stripped of bound antibodies as described in the Materials and methods section and reprobed with a HRP-conjugated mouse anti-phosphotyrosine antibody.

NOS was stripped of antibodies and reprobed with a HRP-conjugated anti-phosphotyrosine antibody. The results showed that the immunoprecipitated i-NOS is tyrosine phosphorylated over the 6–24 h period tested and that phosphorylation coincided with the appearance of protein in the cells (Figure 4). Moreover, cell extracts loaded directly on to the gel from the same incubation times as the immunoprecipitated samples contain a major tyrosine phosphoprotein migrating the same distance as the immunoprecipitated i-NOS. In addition, the time course of appearance for this protein in the cell extracts coincided with the time course for i-NOS induction. Under the conditions employed, the anti-phosphotyrosine antibody binds slightly to the large amount of immunoglobulin used to immunoprecipitate the i-NOS, and occurs even when IgG is loaded on to the gels in the absence of cell extract (results not shown). Specificity was verified by preincubating the anti-phosphotyrosine antibody with O-

Figure 5 Effects of vanadate on i-NOS tyrosine phosphorylation

Cells were seeded into a 75 cm² flask at 10^7 cells in 10 ml of culture medium and grown for 24 h. The cells were then given 10 ml of fresh medium containing IFN- γ (10 units/ml) and LPS (100 ng/ml) and incubated at 37°C for a further 16 h, followed by a 30 min addition of the following components (final concentrations are indicated): no additions (lane 1), fresh LPS (100 ng/ml) and IFN- γ (10 units/ml) in combination (lane 2), 1 μ M PMA (lane 3), 100 μ M vanadate (lane 4) or 1 μ M okadaic acid (lane 5). Total cell lysates were prepared with 1% Triton X-100 lysis buffer and the i-NOS immunoprecipitated from 300 μ g of total cell protein extract. The upper panel is from a probing of the blot with HRP-anti-phosphotyrosine. The lower panel is the same blot that has been stripped and reprobed with antibodies to i-NOS. The blots are from a representative experiment performed three separate times.

phospho-L-tyrosine to eliminate i-NOS recognition (results not shown).

Effects of vanadate, PMA and okadaic acid on the tyrosine phosphorylation of i-NOS

To investigate the responsiveness of the cell to factors that may regulate i-NOS tyrosine phosphorylation, cells were co-stimulated with LPS and IFN- γ for 16 h, followed by the addition of the tyrosine phosphatase inhibitor vanadate, the serine/threonine phosphatase inhibitor okadaic acid, or the protein kinase C activator PMA for 30 min. Western blots of immunoprecipitated i-NOS from these cell extracts were probed with antibodies to phosphotyrosine or anti-i-NOS (Figure 5). Neither PMA nor okadaic acid affected the state of i-NOS tyrosine phosphorylation in these cells. In contrast, vanadate treatment resulted in a significant enhancement of tyrosine phosphorylation that could not be accounted for by an increase in total protein levels, since re-probing the filters with antibodies to i-NOS showed no detectable changes in i-NOS protein levels.

Vanadate potentiation of i-NOS activity

The effects of vanadate on the enzymic activity of i-NOS were assessed by measuring the conversion of L-arginine into citrulline from the cytosol and membrane fractions (Figure 6). Cells previously exposed to LPS and IFN- γ for 16 h were subsequently exposed to vanadate over a period of 5–60 min, followed by cell-extract preparation and separation into cytosol and membrane fractions. In both cell fractions the enzyme activity of i-NOS was found to increase to maximum levels within 5 min of exposing intact cells to vanadate and to remain elevated for at least 60 min after vanadate exposure. Four experimental trials were performed and the data from a single representative trial depicted in the graph. Variability of the maximum vanadate-induced values relative to untreated samples in the four separate trials ranged from 175–280% for cytosol fractions and 420–690% for particulate fractions (results not shown). The increase in NOS

Figure 6 Effects of vanadate on i-NOS activity

Cells were plated in 75 cm² flasks and grown for 24 h. Cells were then given 10 ml of fresh medium containing 100 ng/ml LPS and 10 units/ml IFN- γ and grown for 16 h. Vanadate was then added to the medium to a final concentration of 100 μ M for the indicated times and the cells harvested, hypotonically lysed, and separated by ultracentrifugation into particulate and soluble fractions. Citrulline production was measured over a 30 min period on 50 μ g of protein from the cytosol (●) and particulate fractions (○). A representative sample set from four independent trials is depicted in the graph. Samples were assayed in triplicate and results are depicted as means $\pm$ S.D. * $P \leq 0.05$ versus the zero time point. Significance is based on Student's independent t test.

Figure 7 Specificity and Ca²⁺-dependence of i-NOS activity in cytosol and membrane fractions before and after treatment with vanadate

Samples were prepared as described in Figure 6 and assayed for citrulline formation over a 15 min incubation period from 50 μ g of protein extract. Samples assayed in the absence of added Ca²⁺ included 1 mM EGTA in the buffer. L-NMMA (1 mM) was included where indicated. The data presented are from a representative experiment from four independent trials with each experimental condition performed in triplicate and are depicted as means $\pm$ S.D. * $P \leq 0.05$ versus the same conditions in the absence of L-NMMA. ** $P \leq 0.05$ versus the same conditions in the absence of vanadate. Significance is based on Student's independent t test.

activity from the extracts was specific, since the inclusion of L-NMMA inhibited the formation of citrulline in both vanadate-treated and untreated cells (Figure 7). Moreover, the citrulline formation was shown to be from i-NOS and not from the brain or endothelial NOS, since the enzyme's increase in activity remained independent of Ca²⁺ during the assay. No activity was measurable in cells not prestimulated with LPS and IFN- γ (results not shown).

DISCUSSION

NO has been shown to be an important mediator of the cytotoxic actions of macrophages on a variety of cell types, including cells

transformed by viruses [22], tumour cells [23], oligodendrocytes [24] and neurons [25,26]. The regulation of macrophage NO production is thought to be predominantly at the level of i-NOS gene expression [27], the induction of which is dependent on tyrosine kinase activity [14–17]. Little is known, however, about post-translational events that may regulate i-NOS cellular localization or activity. We therefore investigated whether tyrosine kinases phosphorylate i-NOS in the cell and whether tyrosine phosphorylation might be associated, either directly or indirectly, with changes in enzyme activity.

Our results showed that i-NOS is indeed post-translationally modified by cellular tyrosine kinases that are active concomitantly with induced protein expression after co-stimulation of the cells with LPS and IFN- γ (Figures 2 and 4). Moreover, treatment of the cells with vanadate further elevated the level of i-NOS tyrosine phosphorylation (Figure 5), suggesting that tyrosine phosphatases, as well as tyrosine kinases, are dynamically involved in the regulation of i-NOS phosphorylation. Protein kinase C activation by treatment of the cells with PMA did not mimic the effects of vanadate, nor did treatment of the cells with okadaic acid, which, at the concentrations used, inhibits the activity of protein phosphatase 2A, type 3, and, to a minor extent, type 1 [28]. Thus the effects of vanadate on i-NOS tyrosine phosphorylation appear not to involve these serine/threonine phosphatases or protein kinase C.

Vanadate is a general tyrosine phosphatase inhibitor [29] and its use to enhance the activities of tyrosine kinases has been reported for several proteins. For example, Atkinson et al. reported that phospholipase C- γ 1 is tyrosine-phosphorylated upon treatment of permeabilized mast cells with vanadate [30,31]. In those studies, vanadate-induced tyrosine phosphorylation of phospholipase C- γ 1 was reported not to occur in the absence of cell permeabilization. In the work presented here, vanadate-induced tyrosine phosphorylation of i-NOS occurred in the absence of cell permeabilization. The reason for the difference in cell-permeabilization requirements is unclear, but may be specific to the cell types used. Unlike the phospholipase C- γ 1 study on mast cells, the experiments presented here employed a macrophage cell line. The importance of this difference may centre around cellular membrane activity. Monocytes and macrophages are known to have high rates of pinocytosis activity, an attribute useful when purifying monocytes and macrophages by osmotic-shock techniques [32]. In our study, the induction of i-NOS tyrosine phosphorylation and activity in non-permeabilized RAW 264.7 cells was possibly a result of rapid vanadate uptake into the cells via pinocytosis.

To ascertain whether the tyrosine phosphorylation of i-NOS was associated with changes in the activity of i-NOS, we treated intact cells at various times with vanadate and measured the enzyme activity in membrane and cytosol cell fractions. Treatment of the cells with vanadate resulted in a rapid increase in i-NOS enzyme activity in both membrane and cytosol cell fractions (Figure 6), which could be explained either by the rapid production of new protein or by an alteration in the specific activity of the enzyme. Since vanadate induced nearly a two-fold increase in activity within 5 min of exposure and had indistinguishable effects on i-NOS protein levels up to at least 30 min of exposure, we propose that tyrosine kinases and phosphatases may be involved in post-translationally regulating NO production in these cells. Although elevation of i-NOS tyrosine phosphorylation is associated with increased enzyme activity, it is not possible from these data alone, to conclude that tyrosine phosphorylation has a direct effect on i-NOS function. It is possible that tyrosine phosphorylation regulates the activity of other proteins that, in turn, regulate i-NOS activity. Future work

identifying the involved kinases and/or other proteins that affect the activity of purified i-NOS is needed to conclusively demonstrate the details of such processes.

The observation that i-NOS is tyrosine-phosphorylated raises the question as to whether tyrosine kinases may be involved in regulating the compartmentalization of i-NOS in the cytosol and membrane. Tyrosine phosphorylation is known to mediate the translocation of several proteins from the cytosol to the membrane. An example of this is the specific protein-protein interactions that occur via the src homology 2 and 3 (SH2 and SH3 respectively) regions found in a number of cytosol proteins. Phospholipase C- γ 1, for example, associates with the cell membrane via an SH2 domain that binds to a tyrosine-phosphorylated C-terminal region of the epidermal-growth-factor receptor [33]. The GRB2 protein contains both SH2 and SH3 domains and serves as a docking protein between tyrosine phosphorylated membrane receptors such as epidermal-growth-factor and platelet-derived-growth-factor receptors and tyrosine phosphorylated cytoplasmic proteins such as ras [34]. It has not been shown whether macrophage i-NOS associates with such docking proteins, but i-NOS is known to associate with membrane components in the cell [35–37]. Although no recognizable src homology domains are present in i-NOS, that does not preclude translocation to the membrane or other cellular compartments via associations with proteins such as GRB2 that do contain them.

To investigate the possibility that tyrosine-phosphorylation may regulate the translocation of i-NOS to the cell membrane, we treated cells for various times with vanadate to increase the level of tyrosine-phosphorylated i-NOS in the cell. Enzyme assays of the soluble and particulate subcellular fractions, however, demonstrated elevated activity in both fractions, and thus we were not able to determine if there was a net protein transfer from cytosol to membrane compartments (Figure 6). Western-blot analysis with i-NOS antibodies was also inconclusive (results not shown).

In summary, we have demonstrated that the macrophage i-NOS is post-translationally modified by tyrosine kinases concomitantly with the induction of gene expression in the cell. Moreover, tyrosine phosphorylation of i-NOS is greatly enhanced by treatment of the cells with the tyrosine phosphatase inhibitor vanadate, an effect that could not be mimicked by the activation of protein kinase C or by the inhibition of serine/threonine phosphatases. In addition, the enhancement of i-NOS tyrosine phosphorylation by vanadate treatment correlated with a rapid increase in enzyme activity in the absence of observable changes in protein levels, implicating the involvement of tyrosine kinases directly or indirectly in the post-translational regulation of i-NOS activity. The tyrosine kinases and phosphatases involved in these processes are unknown, but their identification will be important in elucidating the mechanisms by which macrophage i-NOS activity is regulated once the protein is expressed in the cell.

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