0026-895X/01/5905-1324–1332$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:1324–1332, 2001

Vol. 59, No. 5
538/900679
Printed in U.S.A.

Carboxypeptidase D Is Up-Regulated in RAW 264.7 Macrophages and Stimulates Nitric Oxide Synthesis by Cells in Arginine-Free Medium

VAISHALI HADKAR and RANDAL A. SKIDGEL

Departments of Pharmacology and Anesthesiology, University of Illinois College of Medicine at Chicago, Chicago, Illinois

Received August 31, 2000; accepted February 26, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Membrane-bound carboxypeptidase D (CPD) is a B-type carboxypeptidase that specifically cleaves C-terminal Arg or Lys from peptides and proteins. RAW 264.7 cells contained significant membrane-bound CPD activity as shown by activity assays and immunoprecipitation. To determine whether CPD can increase nitric oxide (NO) synthesis by releasing precursor Arg, cells were activated in Arg-free medium with 50 U/ml interferon-γ (IFN-γ) and 0.1 μg/ml lipopolysaccharide (LPS) to up-regulate inducible NO synthase. Addition of the specific carboxypeptidase substrate, 200 μM furylacryloyl-Ala-Arg, stimulated NO production by 6-fold and this effect was blocked 83% by a specific inhibitor, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA). MGTA did not inhibit NO synthesis stimulated by added free Arg. Lys, an inhibitor of Arg transport, also blocked the effect of the carboxypeptidase substrate. In cells stimulated with IFN-γ and LPS in Arg-free medium, CPD activity increased 2- to 3-fold between 8 and 16 h after treatment, but did not change in cells stimulated in medium containing 0.4 mM Arg. The NO synthase inhibitor *N*-monomethyl-L-arginine blocked the inhibitory Arg effect and the NO donor *S*-nitroso-acetylpenicillamine mimicked it, indicating that high levels of NO block the up-regulation of CPD. Immunohistochemical staining and Western analysis revealed an increase in CPD protein, and Northern analysis showed increased CPD mRNA upon stimulation of cells in Arg-free medium. CPD was localized both on the plasma membrane and in the Golgi. These data suggest that CPD expression is enhanced during inflammatory processes and may stimulate NO production by cleaving Arg from peptide substrates.

The amino acid arginine is used by one of three types of a nitric-oxide synthase (NOS) to produce the biologically active gas nitric oxide (NO). The K_m value of purified NOSs for Arg lies between about 1 and 20 μM (Knowles and Moncada, 1994), whereas the Arg concentration in cells and plasma normally ranges from about 100 μM to at least 800 μM (McDonald et al., 1997; Wu and Morris, 1998; Closs et al., 2000). Because the Arg concentration is well above the K_m value for NOSs, it is generally considered that the Arg supply is not rate limiting for NO production. However, studies in a variety of different model systems have demonstrated that increasing extracellular Arg does increase NO production (Moncada and Higgs, 1995) and this has been termed the "arginine paradox" (McDonald et al., 1997).

Another factor that determines the dependence of NO production on Arg levels is the amount of NO produced. Whereas the constitutive NOSs (endothelial or neuronal NOS) require an increase in intracellular calcium for activation and produce low levels of NO upon stimulation, iNOS produces NO levels that can be orders of magnitude greater (Knowles and Moncada, 1994; Michel and Feron, 1997). iNOS has a tightly bound Ca^{2+}/calmodulin that doesn't dissociate at the low intracellular calcium level; thus, its activity is primarily regulated at either the transcriptional level or by altering the supply of substrate Arg (Michel and Feron, 1997). To maintain the high output of NO by this isoform, a higher supply of Arg is required. In addition, at wounds or other inflammatory sites where iNOS levels would be up-regulated, the supply of Arg can be depleted by arginase, which converts it to ornithine. Arginase levels in macrophages are up-regulated in response to LPS (Albina et al., 1988), and arginase is responsible for up to two-thirds of the Arg consumed by macrophages under these conditions (Granger et al., 1990). The resulting low level of Arg reduces the cytotoxicity of macrophages (Albina et al., 1988). That the supply of Arg can

These studies were supported by National Heart Lung and Blood Institute Grant HL60678 and National Institute of Diabetes and Digestive and Kidney Diseases Grant DK41431.

ABBREVIATIONS: NOS, nitric-oxide synthase; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; LPS, bacterial lipopolysaccharide; IFN-γ, interferon-γ; CP, carboxypeptidase; DMEM, Dulbecco's modified Eagle's medium; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; dansyl-Ala-Arg, 5-dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine; Fa-Ala-Arg, furylacryloyl-L-alanyl-L-arginine; BSA, bovine serum album; SSC, standard saline citrate; PBS, phosphate-buffered saline; L-NMA, *N*-monomethyl-L-arginine; NF-κB, nuclear factor-κB.

be a critical factor in NO production is also supported by the finding that induction of iNOS is accompanied by an increase in Arg transport in macrophages (Bogle et al., 1992). Studies have shown that inducible NOS activity is dependent on the concomitant induction of the high-affinity L-Arg transporter system y^+, and in the absence of extracellular Arg uptake by system y^+, inducible NOS activity is reduced to zero (Stevens et al., 1996). Thus, the level of extracellular Arg can be rate limiting for NO synthesis.

Another factor that can enhance NO synthesis, even in the presence of extracellular Arg, is the recycling of citrulline (produced by NOS) via the combined action of argininosuccinate synthetase and argininosuccinate lyase to produce Arg. The ability of cells to recycle citrulline to Arg for NO synthesis was first described in detail in endothelial cells and was also detected in a murine macrophage cell line (Hecker et al., 1990). This was shown to be a significant pathway for regulating NO synthesis in stimulated murine macrophages where coinduction of iNOS and argininosuccinate synthetase was demonstrated in response to LPS and IFN-γ (Nussler et al., 1994).

Arg is a semiessential amino acid, becoming an essential amino acid during growth and development or wound healing (Wu and Morris, 1998). However, little is known about the possibility that Arg derived from peptides and proteins can be used for NO synthesis. The release of a single amino acid from a peptide or protein requires the action of an exopeptidase. One class of enzyme that is likely to be involved in this type of process is basic or B-type metallocarboxypeptidases. These enzymes cleave a single amino acid at a time from the C terminus of peptides and proteins and exhibit strict specificity for C-terminal Arg or Lys (Skidgel, 1996; Skidgel and Erdös, 1998). Although the prototype is pancreatic carboxypeptidase B, this enzyme is found only in the digestive tract where it degrades dietary substrates (Skidgel, 1996). Two members of the regulatory carboxypeptidase family, carboxypeptidase (CP) M and CPD, are relatively widely distributed membrane-bound proteins that specifically cleave C-terminal arginine or lysine (Skidgel and Erdös, 1998). CPM is a 62-kDa membrane protein anchored via glycosylphosphatidylinositol, whereas CPD is a 180-kDa single chain glycoprotein with three homologous carboxypeptidase active site domains and a carboxyl-terminal hydrophobic transmembrane anchor (Song and Fricker, 1995; McGwire et al., 1997; Skidgel and Erdös, 1998)

In this study we used the mouse monocyte/macrophage-like cell line RAW 264.7 as a model system to test the hypothesis that B-type carboxypeptidases can generate the Arg substrate for iNOS to increase NO production, particularly under inflammatory conditions. We also determined whether the inflammatory stimuli that up-regulate NO production, could also up-regulate B-type carboxypeptidase activity.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (DMEM) and bacterial LPS (*Escherichia coli* 0127:B8) were obtained from Sigma (St. Louis, MO). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) was from Calbiochem (San Diego, CA). 5-Dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine (dansyl-Ala-Arg) substrate was synthesized and purified as described previously (Tan et al., 1995). Trifluoroacetic acid was from Pierce Chemical (Rockford, IL). Custom-made arginine-free DMEM, interferon-γ (murine recombinant), and TRIzol reagent for RNA isolation was from Life Technologies (Gaithersburg, MD). Furylacryloyl-Ala-Arg (Fa-Ala-Arg) was from Bachem Biosciences (King of Prussia, PA). [α-^{32}P]deoxycytidine triphosphate and the enhanced chemiluminescence Western blotting analysis kit were from Amersham Pharmacia Biotech (Piscataway, NJ). Goat anti-rabbit IgG (H+L)-horseradish peroxidase was purchased from Southern Biotechnology Associates (Birmingham, AL). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA). Common chemicals were from Fisher Scientific (Pittsburgh, PA).

Cell Culture. RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM or arginine-free DMEM and 10% heat-inactivated fetal bovine serum (to destroy any endogenous serum carboxypeptidase). For NO measurements, RAW 264.7 cells were cultured in 24-well cell culture dishes (1×10^6 cells/well) and were activated with IFN-γ (50 U/ml) and LPS (0.1 μg/ml) at 37°C. Cells were activated in DMEM with or without 0.4 mM Arg for 16 h. Medium was replaced with fresh Arg-free medium containing either added 200 μM Arg or the carboxypeptidase substrate Fa-Ala-Arg (200 μM), with or without the carboxypeptidase inhibitor MGTA (20 μM), or 10 mM lysine. NO production was determined after a further incubation of 8 h.

Measurement of NO Production. NO production was assessed by measuring its stable metabolite, nitrite. Nitrite was quantified colorimetrically after its reaction with Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dichloride, and 2% phosphoric acid) (Vodovotz et al., 1994). Culture medium (100 μl) was mixed with 100 μl of Griess reagent and after 10 min at room temperature the absorbance was measured at 540 nm and compared with a standard curve generated with known concentrations of sodium nitrite.

Measurement of Carboxypeptidase Activity. RAW 264.7 cells, with or without activation by IFN-γ and LPS in DMEM or arginine-free DMEM as described above, were harvested, homogenized, and fractionated by differential centrifugation (100*g* for 10 min, 10,000*g* for 25 min, and 100,000*g* for 1 h). Enzyme activity in the final 100,000*g* (P_3) membrane pellet was determined in a fluorometric assay with dansyl-Ala-Arg substrate as described previously (Tan et al., 1995). CPM activity was measured in 50 mM HEPES, pH 7.5, with 0.1% Triton X-100, and CPD activity was measured in 0.1 mM sodium acetate, pH 5.5, containing 0.1% Triton X-100 (McGwire et al., 1997).

Protein Determination. Protein concentrations were measured as described previously (Bradford, 1976) with bovine serum albumin (BSA) as the standard.

Immunoprecipitation of Carboxypeptidase Activity. Immunoprecipitation studies were carried out using polyclonal antiserum to rat full-length CPD, generously provided by Dr. Lloyd Fricker of the Albert Einstein College of Medicine (Bronx, NY). First, a 500-μl sample of the resuspended P_3 membrane fraction was solubilized with 500 μl of 20 mM Tris, pH 7.4, containing 0.3 M NaCl and 2% Triton X-100, at 4°C for 2 h. The solubilized sample was then centrifuged at 100,000*g* for 1 h to remove insoluble material. The supernatant was incubated with 10 μl of the antiserum against CPD (diluted 1:2 and heated at 56°C for 30 min to inactivate carboxypeptidase N in the serum) overnight at 4°C. Protein A-Sepharose (50 μl of a 10% suspension) was added and the incubation continued for an additional 30 min at 4°C followed by centrifugation at 1400*g* for 4 min to pellet the immune complexes. The supernatant was assayed for remaining CPD activity. Control experiments were performed the same way, except normal rabbit serum was used instead of the anti-CPD antiserum.

Western Blot Analysis. RAW 264.7 cells were cultured in Arg-free medium and incubated with or without IFN-γ and LPS for 16 h as described above. At the end of the incubation the cells were harvested and fractionated by differential centrifugation. Equal amounts of protein from the final membrane fraction (P_3) were

loaded on 7% polyacrylamide gels and separated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose and the membrane was incubated with blocking solution (phosphate-buffered saline containing 0.1% Tween 20, 1% bovine serum albumin, 5% nonfat dried milk) for 1 h at room temperature. Membranes were incubated with rabbit antiserum against rat-full length CPD (diluted 1:1,000) for 3 h, washed, and then incubated with goat anti-rabbit antiserum (1:30,000) conjugated to horseradish peroxidase for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence using the ECL-Western blotting analysis system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Quantitative comparison of the bands was performed by densitometry.

Northern Blot Analysis. RAW 264.7 cells were incubated with or without IFN-γ and LPS for 16 h in Arg-free medium as described above. Total RNA was extracted from the cells using TRIzol reagent as recommended by the manufacturer. Total RNA (20 μg/lane) was run on a denaturing 1% agarose-formaldehyde gel, separated by electrophoresis, and transferred to a nylon membrane by capillary diffusion followed by baking the membranes at 80°C for 1.5 h. To obtain a probe for hybridization with mouse mRNA, the GenBank expressed sequence tag database was screened using the human CPD sequence. A mouse clone (GenBank accession number AA 5289868), which has 87% homology to human CPD domain I, was identified and obtained from the American Type Culture Collection. The plasmid DNA was digested with *Eco*RI and *Hin*dIII and the cDNA purified by agarose gel electrophoresis. The mouse cDNA clone thus obtained was used as a specific probe for detection of mouse CPD mRNA and a probe for mouse β-actin was used to control for equal loading. The probes were labeled by the random primer method using [α-^{32}P]dCTP. Hybridization was carried out at 42°C for 18 h, and then the blots were washed two times at room temperature in 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) with 0.1% SDS followed by a wash in 1× SSC containing 0.1% SDS at 42°C for 30 min followed by a wash at 56°C with 0.2× SSC containing 0.1% SDS for 20 min. Blots were sealed in plastic bags and exposed to X-ray film for 24 h. Bands were detected by autoradiography and quantitated by densitometry correcting for RNA loading by normalization with β-actin.

Immunohistochemistry. RAW 264.7 cells were cultured on coverslips in Arg-free medium in the presence or absence of IFN-γ and LPS, for 16 h as described above. Cells used for immunocytochemistry were fixed for 20 min in 4% paraformaldehyde and then washed with 100 mM glycine to quench free aldehyde. After three washes with Ham's F-12 and 0.2% BSA, the cells were preincubated in Ham's F-12 containing 5% goat serum, 0.2% BSA, 0.01% sodium azide, and 0.1% Triton X-100, for 30 min at room temperature and then incubated with the primary antibody (1:200 anti-recombinant human CPD) or with preimmune serum overnight at 4°C. Cells were washed again with Ham's F-12 containing 0.2% BSA and then incubated with the secondary antibody (goat anti-rabbit Alexa 488) for 2 h at room temperature. After washes with Ham's F-12 containing 0.2% BSA, cells were mounted on glass slides using Vectashield mounting medium and observed under a Nikon digital fluorescence microscope or a Zeiss laser scanning confocal microscope.

Flow Cytometry. RAW 264.7 cells were harvested, washed with phosphate-buffered saline (PBS), and suspended in PBS containing 0.1% sodium azide (PBS-azide). Cells were incubated with the polyclonal antiserum against the C-terminal region of domain I of human recombinant CPD (1:100), or preimmune serum for 1 h at 4°C. Cells were washed three times with PBS-azide (1%) and then incubated for 1 h at 4°C with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate. Cells were washed three times with PBS-azide (1%) and analyzed on a FACSCalibur analyzer (Becton Dickinson, Mountain View, CA). Cells were identified and gated using forward scattering and side scattering of light. Data were analyzed using the Cell Quest Software (Becton Dickinson). Values acquired reflect the mean fluorescence intensity for 10,000 events.

Results

Carboxypeptidase Substrate Stimulates NO Production in Mouse Monocyte/Macrophages. Unstimulated RAW 264.7 cells produced very low levels of NO even in the presence of Arg (data not shown). RAW 264.7 cells were activated with IFN-γ and LPS for 16 h in Arg-free DMEM to up-regulate iNOS. In control studies, maximal NO synthase activity was achieved between 12 and 18 h after treatment (data not shown), consistent with previously reported results (Schmidt et al., 1992). Because the medium contained 10% fetal bovine serum, it was not completely Arg-free. Based on estimates of plasma levels of Arg in mammals, which range from 100 to 200 μM (Wu and Morris, 1998), it is likely that the cells were exposed to 10 to 20 μM Arg in this and subsequent experiments using Arg-free DMEM. This probably explains the low amount of NO generated by stimulated cells in the absence of added Arg (Fig. 1).

To test whether cellular B-type carboxypeptidases can generate sufficient Arg to stimulate NO synthesis, we used a specific carboxypeptidase substrate, Fa-Ala-Arg (Tan et al., 1995). As shown in Fig. 1, addition of 200 μM Fa-Ala-Arg resulted in a 6-fold increase in NO production. To prove that the substrate was being hydrolyzed by a B-type carboxypeptidase and not used directly by iNOS or cleaved by another type of enzyme, we used a B-type carboxypeptidase inhibitor, MGTA (Skidgel, 1996; Skidgel and Erdös, 1998). MGTA specifically inhibits only metallocarboxypeptidases that cleave

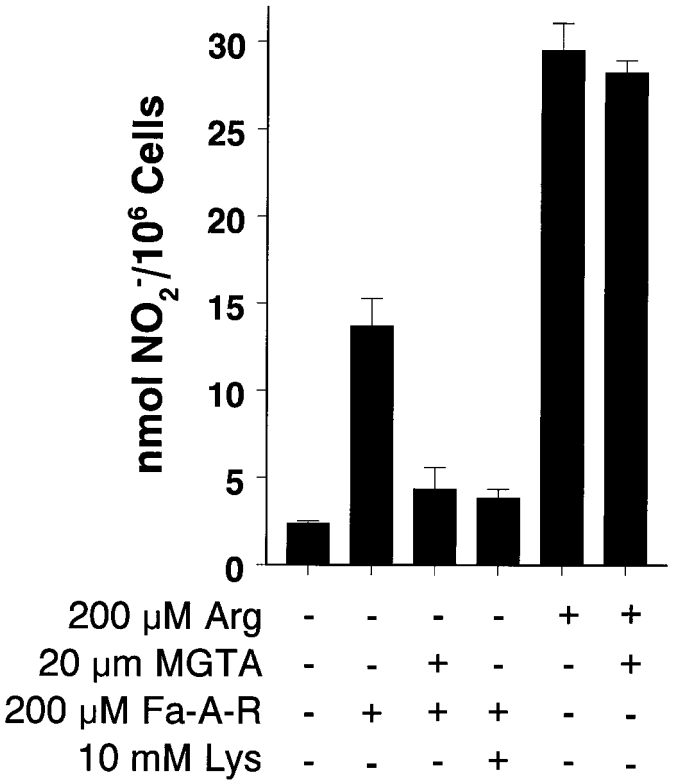

Fig. 1. Carboxypeptidase substrate stimulates NO production in RAW 264.7 cells. RAW 264.7 cells were activated with IFN-γ and LPS for 16 h in arginine-free DMEM as described under *Experimental Procedures*. Medium was replaced with fresh DMEM containing 200 μM carboxypeptidase substrate Fa-Ala-Arg or 200 μM Arg, with or without the carboxypeptidase inhibitor MGTA (20 μM) or with Fa-A-R and lysine (10 mM), an inhibitor of Arg transport. Results shown are the mean ± S.E.M. of three to four separate experiments.

C-terminal Arg or Lys, but it does not distinguish among members of this family (e.g., between CPM and CPD or CPE). MGTA (20 μM) inhibited by 86% the increase in NO production stimulated by 200 μM Fa-Ala-Arg (Fig. 1). As a control, MGTA was also applied in the presence of 200 μM Arg to confirm that its effect is not due to inhibition of iNOS or other nonspecific effects. In the presence of 200 μM Arg, there was a large increase in NO production, but MGTA did not inhibit the effect (Fig. 1).

To determine whether the hydrolysis occurred extracellularly, requiring uptake of the released Arg by the basic amino acid transporter, experiments were run in the presence of lysine. Lysine is a competitive inhibitor of the Arg transporter that does not directly inhibit iNOS and it is a commonly used tool for determining whether the source of Arg for NOS is extracellular (Bogle et al., 1992; Closs et al., 2000). As shown in Fig. 1, 10 mM Lys almost completely inhibited the increase in NO production stimulated by 200 μM Fa-Ala-Arg. This indicates that the substrate Fa-Ala-Arg is cleaved extracellularly by a carboxypeptidase and that the released Arg is taken up into the cells and used for NO synthesis.

Identification of Membrane-Bound Carboxypeptidase Activity. We previously showed that the major membrane-bound carboxypeptidase in another mouse macrophage cell line, J774A.1, was CPD (McGwire et al., 1997). To determine the nature of the carboxypeptidase in the RAW 264.7 cell line, cells were harvested, homogenized, and then fractionated by differential centrifugation. Carboxypeptidase activity was determined with dansyl-Ala-Arg substrate in the final P_3 membrane pellet. Dansyl-Ala-Arg was used in these assays instead of Fa-Ala-Arg for three reasons (Tan et al., 1995): 1) Because it has a fluorescent tag, it is more sensitive. 2) It can be used with membrane fractions in the presence of detergents. 3) The assay is faster with multiple samples. Dansyl-Ala-Arg was not used with intact cells because at high concentrations (1–3 mM) it had some nonspecific deleterious effects on the cells, whereas Fa-Ala-Arg did not. Assays were run at both pH 5.5 and 7.5 to differentiate the two major membrane-bound cellular carboxypeptidases; carboxypeptidase M, which has a pH optimum around 7.0 and CPD, which has a pH optimum of 6.2 (Song and Fricker, 1995; McGwire et al., 1997; Skidgel and Erdös, 1998). The carboxypeptidase activity was much higher at pH 5.5 (76.7 $\pm$ 2.3 nmol/h/mg; $n = 3$) than at pH 7.5 (7.1 $\pm$ 2.8 nmol/h/mg), consistent with its identity as CPD (Song and Fricker, 1995; McGwire et al., 1997; Skidgel and Erdös, 1998). To conclusively identify the enzyme, immunoprecipitation studies were carried out on solubilized membrane fractions with antiserum specific for CPD. Compared with the preimmune serum control, antiserum to CPD precipitated 94.0 $\pm$ 1.7% ($n = 3$) of the carboxypeptidase activity solubilized from the RAW 264.7 cells, showing that it is indeed CPD. The presence of CPD protein and mRNA was confirmed by Western blot, flow cytometry, immunohistochemistry, and Northern blot analysis (see below).

Up-Regulation of CPD Activity. Stimuli that up-regulate NO production also up-regulate many other proteins involved in inflammatory processes. To determine whether CPD activity can also be up-regulated, RAW 264.7 cells were cultured in DMEM or Arg-free DMEM in the presence or absence of IFN-γ and LPS for 24 h. Cells were harvested, homogenized, and fractionated by differential centrifugation and the carboxypeptidase activity was determined in the final membrane pellet. IFN-γ and LPS had no effect on carboxypeptidase activity in cells treated in normal DMEM, which contains 0.4 mM Arg, but in the Arg-free medium, CPD activity increased by almost 2-fold (Fig. 2). To determine the time course of the up-regulation, RAW 264.7 cells, cultured in Arg-free medium, were treated with IFN-γ and LPS for various time periods. As shown in Fig. 3, CPD activity steadily increased after treatment with IFN-γ and LPS to a maximum of about 3-fold between 8 and 16 h and began to decrease by 24 h. Thus, under low Arg conditions, carboxypeptidase activity is up-regulated in response to inflammatory mediators. The prolonged time course of the response is consistent with up-regulation at the transcriptional level.

Western Blot Analysis. Western blot analysis of membrane fractions of RAW 264.7 cells revealed a band of about 190 kDa, consistent with the reported size of CPD (Song and Fricker, 1995; McGwire et al., 1997) (Fig. 4). Densitometric scanning revealed that the intensity of the band increased 2.2 $\pm$ 0.1-fold ($n = 3$) in the cells treated with IFN-γ and LPS in Arg-free medium (Fig. 4). The increased protein synthesis is consistent with the measured increase in enzyme activity.

Northern Blot Analysis. Northern blot analysis revealed a band of about 8 kilobase pairs, consistent with that re-

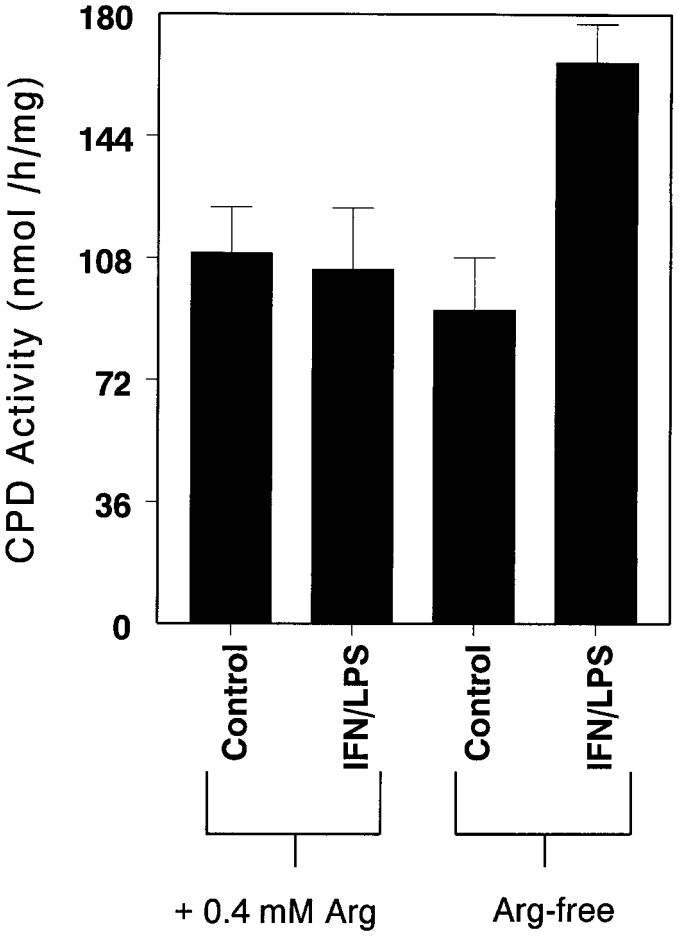

Fig. 2. Up-regulation of CPD activity in RAW 264.7 cells. RAW 264.7 cells were treated with 50 U/ml of IFN-γ and LPS (0.1 μg/ml) for 24 h in Arg-free DMEM or DMEM containing 0.4 mM Arg. Cells were harvested, homogenized, and fractionated by differential centrifugation and CPD activity was measured in the final membrane fraction. Results shown are the mean $\pm$ S.E.M. ($n = 3$).

ported for CPD (Tan et al., 1997) (Fig. 5). Densitometric analysis revealed a 10.1 ± 2.8-fold (n = 3) increase in the intensity of the band, corrected for β-actin, in the cells activated with IFN-γ and LPS in Arg-free medium. This indicates that the up-regulation is at the transcriptional level, although enhanced mRNA stability cannot be ruled out.

Immunofluorescence Microscopy. Immunohistochemical staining of RAW 264.7 cells with antiserum specific for CPD revealed bright intracellular perinuclear staining and also diffuse staining of the cell membrane (Fig. 6). This is consistent with the *trans*-Golgi and plasma membrane distribution of CPD seen in other cell types (Varlamov and Fricker, 1998). In contrast, the cells did not show any staining with preimmune serum (data not shown). Staining for CPD was more intense in cells treated with IFN-γ and LPS in Arg-free medium, consistent with the results reported above, but the pattern of distribution remained the same (Fig. 6B).

Flow Cytometry. To further confirm the presence of CPD on the cell surface, we performed flow cytometric analysis on nonpermeabilized cells using antibody raised against domain 1 of human recombinant CPD (Fig. 7). The mean fluorescence intensity (for 10,000 events) was determined in three separate experiments. There was a 5.23 ± 0.55-fold increase in mean fluorescence intensity for cells treated with the antiserum compared with those treated with preimmune serum. Thus, CPD is on the surface of RAW 264.7 cells, where it can hydrolyze the substrate Fa-A-R.

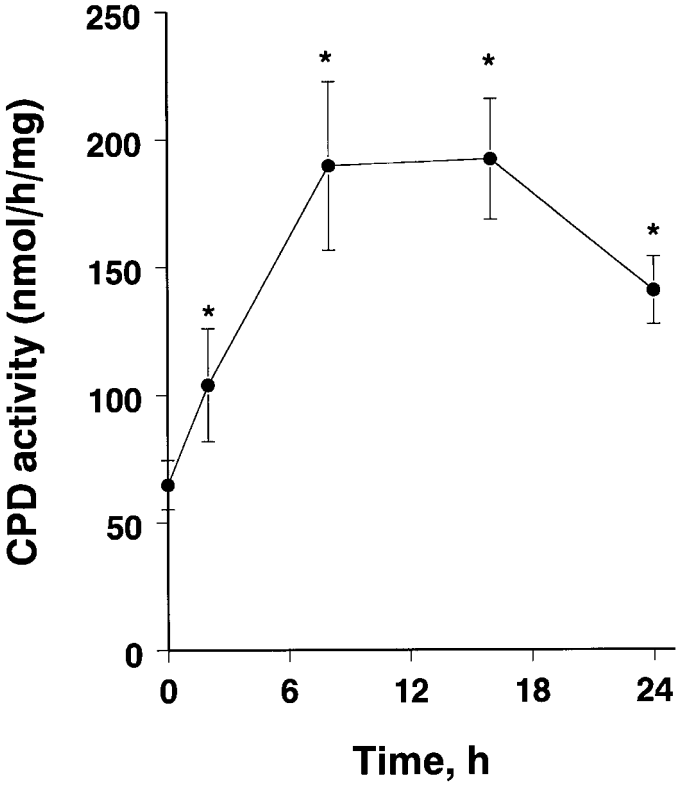

Fig. 3. Time course of up-regulation of CPD activity in RAW 264.7 cells. RAW 264.7 cells were cultured in Arg-free DMEM in the presence of IFN-γ (50 U/ml) and LPS (0.1 μg/ml) for various time periods. Cells were then harvested, homogenized, and fractionated by differential centrifugation. CPD activity was determined in the final membrane fraction. Results shown are the mean ± S.E.M. (n = 3). *$p < 0.05$ compared with the zero time control. CPD activity in the membrane fraction of control (untreated) cells was 72.7 ± 8.0 nmol/h/mg at zero time and 77.1 ± 16.4 nmol/h/mg at 24 h.

Inhibition of the Up-Regulation of CPD by Arg. We wondered whether the ability of Arg to block up-regulation of CPD in response to inflammatory mediators (Fig. 2) might be mediated through the production of NO. To investigate this possibility, cells were first cultured in normal DMEM containing 0.4 mM Arg and stimulated with IFN-γ and LPS for 24 h in the presence and absence of 2 mM *N*-monomethyl-L-arginine (L-NMA), the NOS inhibitor. As before, there was no change in carboxypeptidase activity with IFN-γ and LPS in medium containing 0.4 mM Arg (Fig. 8). However, in the presence of L-NMA, IFN-γ and LPS increased carboxypeptidase activity to a similar extent as that in Arg-free medium (Fig. 8). In Arg-free medium, IFN-γ and LPS stimulation increased CPD activity as before (Fig. 8). However, addition of an NO donor, 1 mM *S*-nitrosoacetylpenicillamine, blocked the up-regulation of carboxypeptidase activity by IFN-γ and LPS in Arg-free medium. To rule out the possibility that NO decreases CPD activity by direct inhibition (e.g., by *S*-nitrosation or nitration of Tyr residues), purified human recombinant CPD or membrane fractions from RAW 264.7 cells stimulated with IFN-γ and LPS or from control cells were incubated in 0.05 M HEPES buffer, pH 7.4, with or without 1 mM *S*-nitrosoacetylpenicillamine for 4 or 20 h, and then assayed for CPD activity with dansyl-Ala-Arg. The direct effects of 1 mM *S*-nitrosoacetylpenicillamine on CPD activity, expressed as percentage of control activity (incubated in buffer alone) are as follows (average of duplicate samples from two separate experiments): purified human CPD =

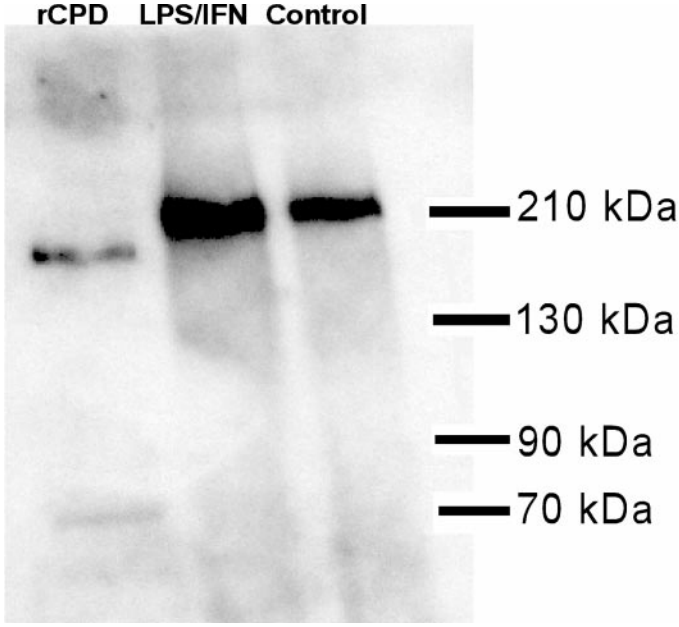

Fig. 4. Western blot analysis of CPD in control and stimulated RAW 264.7 cells. Equal amounts of protein from membrane fractions of unstimulated RAW 264.7 cells (control) and from cells activated with IFN-γ and LPS (LPS/IFN) for 16 h in Arg-free medium were separated by SDS-polyacrylamide gel electrophoresis and evaluated by Western blot analysis using antiserum against rat full-length CPD. In the first lane, human recombinant CPD (rCPD) generated in a baculovirus system was run as a positive control. The lower molecular mass of this band is due to the reduced glycosylation of recombinant proteins in insect cells compared with mammalian cells. Although the CPD band migrates at the same position as the 210-kDa marker, when calculated based on a curve using all the molecular mass standards, the apparent molecular mass of the CPD band is 190 kDa. The figure shown is representative of three separate experiments.

103% at 4 h, 96% at 20 h; membrane fractions from control RAW 264.7 cells = 86% at 4 h, 84% at 20 h; and membrane fractions from stimulated RAW 264.7 cells = 97% at 4 h, 94% at 20 h. Thus, the NO donor had little, if any, effect on CPD activity. These data indicate that in medium containing Arg, NO prevents the up-regulation of carboxypeptidases by IFN-γ and LPS, but under low Arg conditions, carboxypeptidase activity increases in response to inflammatory mediators.

Discussion

The above-mentioned data show that a basic carboxypeptidase, which specifically cleaves C-terminal Arg and Lys from peptides and proteins, can play a role in providing Arg for NO production. In the present study, we demonstrated that a carboxypeptidase substrate increases NO production in activated macrophages and that the increase is blocked by the specific carboxypeptidase inhibitor MGTA. That the effect is due to the extracellular release of Arg from the substrate is also indicated by the observation that an arginine transport inhibitor blocked the effect.

For carboxypeptidases to play a role in NO production, it would be necessary for a variation in Arg levels to alter the amount of NO produced. It is generally thought that normal plasma or cellular concentrations of Arg are sufficient to sustain maximal NOS activity in the presence of an appropriate stimulus. This is because the normal Arg concentration in cells and plasma is far above the K_m value for the NOSs, which indicates they should be saturated with substrate when activated (Knowles and Moncada, 1994; McDonald et al., 1997; Wu and Morris, 1998; Closs et al., 2000). However, addition of extracellular Arg increases NO production in a variety of different model systems. For example, administration of oral Arg to human subjects increased plasma Arg, increased the concentrations of exhaled NO (Kharitonov et al., 1995) and also decreased blood pressure (Smulders et al., 1997). The NO production of murine peritoneal macrophages was enhanced in a dose-dependent manner by supplemental Arg in the range of 80 μM to 1 mM and was associated with an increase in microbicidal activity toward *Trypanosoma cruzi* (Norris et al., 1995).

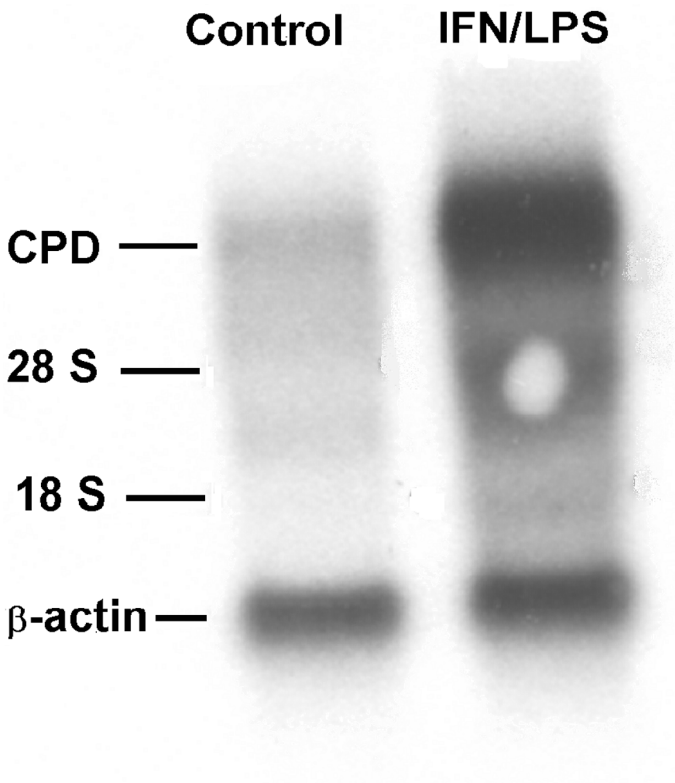

Fig. 5. Northern blot analysis of CPD mRNA in RAW 264.7 cells. Equal amounts of total RNA from unstimulated RAW 264.7 cells (control) or from cells activated with IFN-γ and LPS (IFN/LPS) for 16 h in Arg-free medium were separated on an agarose gel, transferred to a nylon membrane, and probed with radiolabeled CPD cDNA and with β-actin cDNA to control for equal loading. The figure shown is representative of three separate experiments.

Conversely, several experimental models have shown a link between Arg depletion and reduced NO synthesis. For example, although Arg is not considered an essential amino acid, experimental reduction of the dietary supply of Arg impaired constitutive and inducible NO synthesis in young rats even though plasma Arg levels remained above 100 μM (Wu et al., 1999). At wounds or other inflammatory sites, Arg is depleted and ornithine is increased as a consequence of arginase derived from macrophages or tumor cells (Albina et al., 1988) and the resulting low concentration of Arg reduces the cytotoxicity of macrophages. In addition, mouse peritoneal macrophages cultured in the presence of IFN-γ with or without LPS rapidly depleted the medium of L-Arg, abolishing NO production, which then resumed when the medium was replenished with Arg (Vodovotz et al., 1994).

Another way to regulate Arg supply, and thus NO production, is through cellular conversion of citrulline to Arg (Hecker et al., 1990). For example, it was shown that in RAW 264.7 cells stimulated with IFN-γ or LPS to up-regulate iNOS, citrulline enhanced NO production in the presence of 50 to 80 μM extracellular Arg (Nussler et al., 1994). This pathway may play a role in regulating NO production in inflammatory conditions because a major enzyme involved in the conversion, argininosuccinate synthetase, is up-regulated by LPS or IFN-γ in murine macrophages (Nussler et al., 1994).

One explanation of the so-called arginine paradox is that arginine is sequestered in pools that are unavailable to the NOS. Recent evidence supports this possibility in J774A.1 macrophages and EA.hy926 endothelial cells (Closs et al., 2000). In both cell lines, there existed an Arg pool (pool I, estimated at 2–3.5 mM) that was freely exchangeable with extracellular arginine as well as an intracellular pool (pool II, estimated at 160–600 μM) that could not be depleted or exchanged with extracellular Arg. Whereas endothelial NOS (NOS III) could use pool II to generate NO in the absence of extracellular Arg, iNOS (NOS II), in the macrophage cell line could not, and was dependent on extracellular or pool I Arg for NO synthesis (Closs et al., 2000). Based on these data, the ability of carboxypeptidases to generate extracellular Arg for NO synthesis would play a more critical role during inflammatory conditions, when iNOS (NOS II) is up-regulated.

Despite the importance of Arg supply on NO production during inflammation, only a few studies have addressed the possibility that Arg derived from protein or peptide sources could be used for NO synthesis. In one study using Arg-depleted endothelial cells, Arg production was inhibited 45% by a mixture of protease inhibitors (Hecker et al., 1990).

Moreover, when several Arg-containing dipeptides were incubated with cultured endothelial cells, they were cleaved to yield free Arg, consistent with their ability to potentiate NO release and NO-dependent responses in vivo (Hecker et al., 1990; Thiemermann et al., 1991). Arg-containing dipeptides (Arg-Arg, Arg-Phe and Ala-Arg) significantly stimulated NO synthesis in cytosolic and microsomal fractions of endothelial cells and activated murine J774 cells (Hecker et al., 1991), and incubation of Arg-Asp or angiotensin II with murine and rat peritoneal macrophages led to 9- or 4-fold increases in nitrite production (Hrabak et al., 1994). Despite these initial reports, no information is available on the type of enzyme(s) involved in this process. Based on protease specificity, the release of free Arg from peptides and proteins would require the action of an exopeptidase (i.e., an aminopeptidase or carboxypeptidase). Because of their ability to cleave only C-terminal Arg or Lys, membrane-bound B-type carboxypeptidases (such as carboxypeptidases M and D) are likely candidates to be involved in this process as shown in the present studies. Potential in vivo substrates containing C-terminal Arg are numerous (Skidgel, 1988, 1996), including: bradykinin; anaphylatoxins C3a, C4a, and C5a; enkephalin hexapeptides (i.e., Arg6- or Lys6-, Met-, or Leu-enkephalin); dynorphin $A_{1\text{-}13}$; dynorphin $A_{1\text{-}11}$; dynorphin $A_{1\text{-}9}$; fibrinopeptides 6A, 6D, A, and B; atriopeptin II; cardiodilatin$_{1\text{-}67}$; albumin propeptide; insulin C-peptide; hemoglobin; erythropoietin; and a variety of growth factors, including epidermal growth factor (McGwire and Skidgel, 1995). Other potential sources for B-type carboxypeptidase substrates are the numerous proteolytic cascades such as the fibrinolytic system, coagulation cascade, and the complement system, which consist of a series of normally inactive proenzymes that can be activated after injury or during inflammation (Barrett et al., 1998). Each step in the cascade requires the cleavage of an Arg-X bond, resulting in the generation of a new protein chain with a C-terminal Arg (Barrett et al., 1998). Thus, activation at

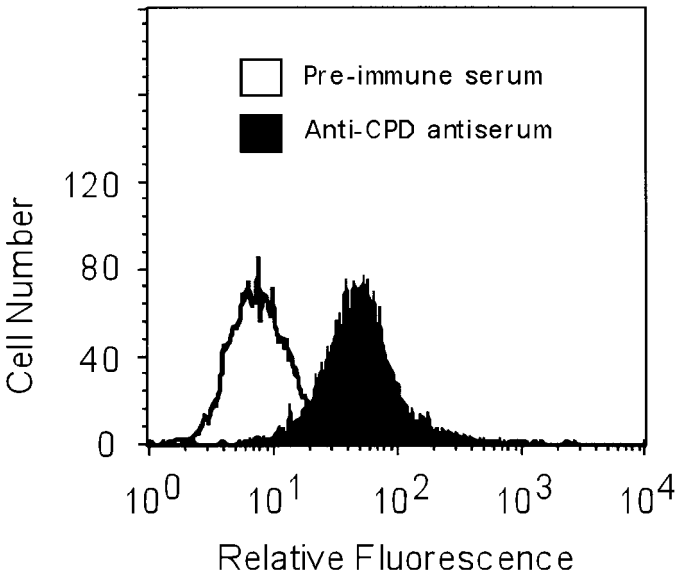

Fig. 7. Flow cytometric analysis for CPD on RAW 264.7 cells. Flow cytometric analysis was carried out using polyclonal antiserum against human CPD and fluorescein isothiocyanate-labeled secondary goat anti-rabbit IgG. Cells incubated with preimmune serum are represented by the open histogram and cells incubated with the antiserum are represented by the solid histogram. Representative tracing from three separate experiments.

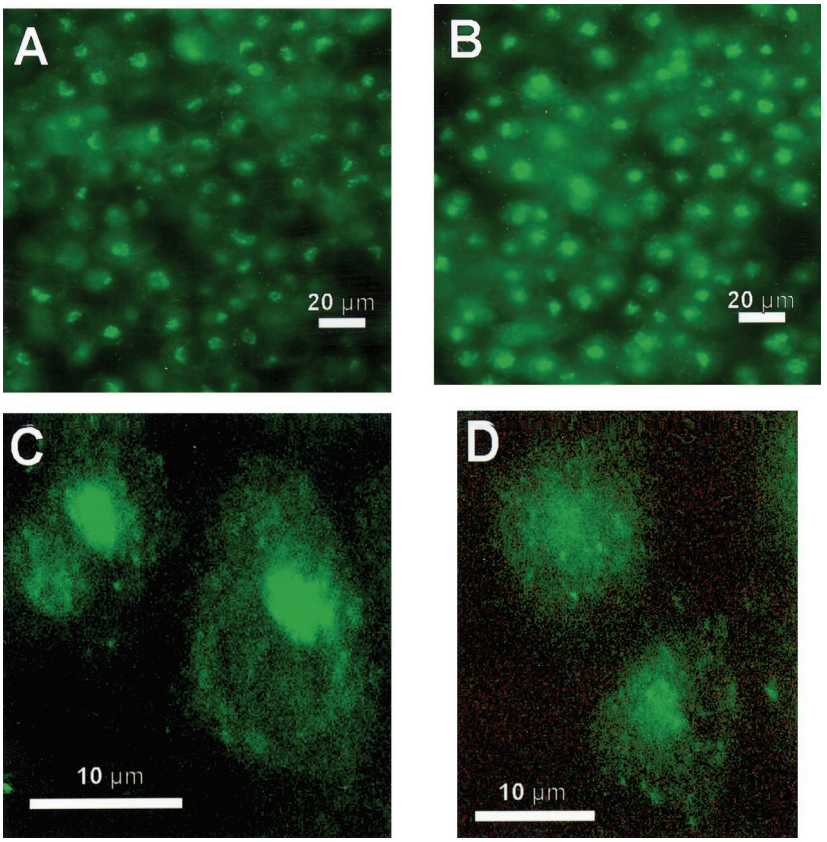

Fig. 6. Immunohistochemistry of CPD in RAW 264.7 cells. RAW 264.7 cells were cultured in Arg-free medium in the presence or absence of IFN-γ and LPS as described under *Experimental Procedures*. Cells were fixed, permeabilized with 0.1% Triton X-100 and stained with antiserum against recombinant human CPD followed by goat anti-rabbit Alexa 488 and observed using a Nikon digital fluorescence microscope (A and B) or a Zeiss laser scanning confocal microscope (C and D). A, control RAW 264.7 cells. B, RAW 264.7 cells stimulated with IFN-γ and LPS. C, confocal image of control RAW 264.7 cells showing perinuclear localization of CPD. D, confocal image of control RAW 264.7 cells showing surface localization of CPD.

each step would produce a potential carboxypeptidase substrate, which could then be cleaved to produce free Arg.

There are no known endogenous inhibitors that control the activity of the B-type carboxypeptidases as there are for most of the endoproteases (Barrett et al., 1998). Thus, the regulation of their activity will depend on their expression mediated at the transcriptional or translational level. The present data show that CPD can be up-regulated (as shown by increased mRNA, protein expression and enzyme activity) about 3-fold by LPS and IFN-γ. That CPD can play a role in NO production in inflammatory conditions is supported by the observation that up-regulation of the enzyme is stimulated by LPS and IFN-γ, mediators that also up-regulate iNOS (Schmidt et al., 1992). Because this effect was seen only in cells grown in Arg-free medium, it might be a mechanism by which increased Arg generation from protein and peptide substrates can be stimulated under low Arg conditions. This "arginine sensor" is modulated via NO because when NO synthesis was blocked, CPD activity was up-regulated by LPS and IFN-γ even in the medium containing Arg, whereas an NO donor blocked the up-regulation in Arg-free medium. This effect was not due to direct inhibition of CPD because an NO donor did not directly decrease the activity of purified human CPD or CPD in membrane fractions of RAW 264.7 cells. These data are consistent with the possibility that the regulation of CPD is an NF-κB-mediated response because it was shown previously that NO can inhibit NF-κB activation by increasing the expression, nuclear translocation, and stabilization of its inhibitory protein I-κB (Peng et al., 1995; Spiecker et al., 1997). NO thus blocks the NF-κB-mediated up-regulation of other proteins such as iNOS (Peng et al., 1995), vascular cell adhesion molecule-1 (De Caterina et al., 1995), intercellular adhesion molecule-1, E-selectin (Spiecker et al., 1997), and cyclooxygenase-2 (Habib et al., 1997).

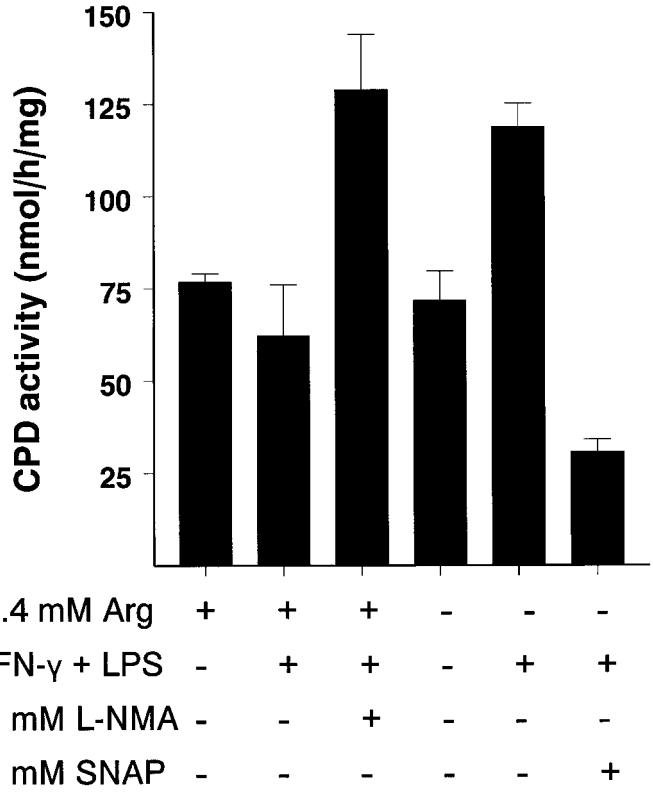

Fig. 8. NO blocks the up-regulation of CPD activity in response to inflammatory mediators. RAW 264.7 cells were incubated for 24 h with or without IFN-γ and LPS as before in normal (with 0.4 mM Arg) or Arg-free DMEM as indicated. The NOS inhibitor L-NMA was used to block NO synthesis in Arg-containing medium, and the NO donor *S*-nitrosoacetylpenicillamine was used to generate NO in Arg-free medium. After treatment, cells were lysed, fractionated by differential centrifugation and CPD activity was measured in the final membrane pellet. Results shown are the mean ± S.E.M. (n = 3).

In the kinin system, B-type carboxypeptidases such as CPD can either inactivate or alter the specificity of the peptides by cleaving the C-terminal Arg^9 from bradykinin (Skidgel, 1988). This eliminates binding to the constitutive bradykinin B_2 receptor, but the resulting des-Arg^9-bradykinin binds to the B_1 receptor. Although the B_1 receptor is not normally present in most tissues and cells, injury or inflammatory mediators up-regulate transcription of the B_1 receptor (DeBlois et al., 1991; Bhoola et al., 1992). A recent article reported the coinduction of B_1 receptors and B-type carboxypeptidase activity in aortas of pigs that had been treated with an infusion of LPS (Schremmer-Danninger et al., 1998). Our finding that CPD is up-regulated in response to inflammatory mediators is further evidence that coinduction of a membrane carboxypeptidase could provide a mechanism for increased generation of B_1 receptor agonists during inflammatory conditions that induce expression of the B_1 receptor itself.

These observations may have pathological significance in conditions such as septic shock. The hypotension and vascular hyporesponsiveness seen in septic shock is attributed to excess NO production by iNOS (Anggard, 1994). NO can also play a beneficial role in septic shock by inhibiting platelet aggregation and leukocyte adhesion, thus preventing thrombosis and microvascular stasis (Anggard, 1994). Under these conditions, complete blockade of NO production would abrogate both the beneficial and deleterious actions of NO. One possible approach in the management of septic shock would be to modulate NO production by regulating the levels of the substrate L-Arg. Inhibition of carboxypeptidases could represent a potential strategy in limiting NO production in septic shock under conditions in which the Arg supply and Arg synthesis/recycling from citrulline is rate limiting.

Acknowledgments

We thank Dr. Lloyd D. Fricker of the Albert Einstein College of Medicine for providing antiserum to rat CPD, Joseph Schober for help with the flow cytometry, Dr. Richard Minshall for help with the confocal microscopy, and David Schacht for technical assistance.

References

Albina JE, Mills CD, Barbul A, Thirkill CE, Henry WLJ, Mastrofrancesco B and Caldwell MD (1988) Arginine metabolism in wounds. *Am J Physiol* **254:**E459–E467.

Anggard E (1994) Nitric oxide: mediator, murderer and medicine. *Lancet* **343:**1199–1205.

Barrett AJ, Rawlings ND and Woessner JF (1998) *Handbook of Proteolytic Enzymes*, Academic Press, London.

Bhoola KD, Figueroa CD and Worthy K (1992) Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* **44:**1–80.

Bogle RG, Baydoun AR, Pearson JD, Moncada S and Mann GE (1992) L-arginine transport is increased in macrophages generating nitric oxide. *Biochem J* **284:**15–18.

Bradford MM (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.

Closs EI, Scheld JS, Sharafi M and Forstermann U (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol* **57:**68–74.

DeBlois D, Bouthillier J and Marceau F (1991) Pulse exposure to protein synthesis

inhibitors enhances vascular responses to des-Arg9-bradykinin: possible role of interleukin-1. *Br J Pharmacol* **103:**1057–1066.
De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MAJ, Shin WS and Liao JK (1995) Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* **96:**60–68.
Granger DL, Hibbs JBJ, Perfect JR and Durack DT (1990) Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J Clin Invest* **85:**264–273.
Habib A, Bernard C, Lebret M, Creminon C, Esposito B, Tedgui A and Maclouf J (1997) Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J Immunol* **158:**3845–3851.
Hecker M, Sessa WC, Harris HJ, Anggard EE and Vane JR (1990) The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci USA* **87:**8612–8616.
Hecker M, Walsh DT and Vane JR (1991) On the substrate specificity of nitric oxide synthase. *FEBS Lett* **294:**221–224.
Hrabak A, Idei M and Temesi A (1994) Arginine supply for nitric oxide synthesis and arginase is mainly exogenous in elicited murine and rat macrophages. *Life Sci* **55:**797–805.
Kharitonov SA, Lubec G, Lubec B, Hjelm M and Barnes PJ (1995) L-arginine increases exhaled nitric oxide in normal human subjects. *Clin Sci* **88:**135–139.
Knowles RG and Moncada S (1994) Nitric oxide synthases in mammals. *Biochem J* **298:**249–258.
McDonald KK, Zharikov S, Block ER and Kilberg MS (1997) A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the arginine paradox. *J Biol Chem* **272:**31213–31216.
McGwire GB and Skidgel RA (1995) Extracellular conversion of epidermal growth factor (EGF) to des-Arg53- EGF by carboxypeptidase M. *J Biol Chem* **270:**17154–17158.
McGwire GB, Tan F, Michel B, Rehli M and Skidgel RA (1997) Identification of a membrane-bound carboxypeptidase as the mammalian homolog of duck gp180, a hepatitis B virus-binding protein. *Life Sci* **60:**715–724.
Michel T and Feron O (1997) Nitric oxide synthases: which, where, how, and why? *J Clin Invest* **100:**2146–2152.
Moncada S and Higgs EA (1995) Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J* **9:**1319–1330.
Norris KA, Schrimpf JE, Flynn JL and Morris SMJ (1995) Enhancement of macrophage microbicidal activity: supplemental arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of *Trypanosoma cruzi*. *Infect Immunol* **63:**2793–2796.
Nussler AK, Billiar TR, Liu ZZ and Morris SMJ (1994) Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line. Implications for regulation of nitric oxide production. *J Biol Chem* **269:**1257–1261.
Peng H-B, Libby P and Liao JK (1995) Induction and stabilization of I-kB by nitric oxide mediates inhibition of NF-κB. *J Biol Chem* **270:**14214–14219.
Schmidt HH, Warner TD, Nakane M, Forstermann U and Murad F (1992) Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages [published erratum appears in Mol Pharmacol (1992) **42:**174]. *Mol Pharmacol* **41:**615–624.
Schremmer-Danninger E, Öffner A, Siebeck M and Roscher AA (1998) B1 bradykinin receptors and carboxypeptidase M are both upregulated in the aorta of pigs after LPS infusion. *Biochem Biophys Res Commun* **243:**246–252.
Skidgel RA (1988) Basic carboxypeptidases: regulators of peptide hormone activity. *Trends Pharmacol Sci* **9:**299–304.
Skidgel RA (1996) Structure and function of mammalian zinc carboxypeptidases, in *Zinc Metalloproteases in Health and Disease* (Hooper NM ed) pp 241–283, Taylor and Francis Ltd., London.
Skidgel RA and Erdös EG (1998) Cellular carboxypeptidases. *Immunol Rev* **161:**129–141.
Smulders RA, Aarsen M, Teerlink T, De Vries PM, Van Kamp GJ, Donker AJ and Stehouwer CD (1997) Haemodynamic and biochemical responses to L-arginine and L-lysine infusions in normal subjects: L-arginine-induced vasodilatation cannot be explained by non-specific effects of cationic amino acids. *Clin Sci* **92:**367–374.
Song L and Fricker LD (1995) Purification and characterization of carboxypeptidase D, a novel carboxypeptidase E-like enzyme, from bovine pituitary. *J Biol Chem* **270:**25007–25013.
Spiecker M, Peng HB and Liao JK (1997) Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of IκBα. *J Biol Chem* **272:**30969–30974.
Stevens BR, Kakuda DK, Yu K, Waters M, Vo CB and Raizada MK (1996) Induced nitric oxide synthesis is dependent on induced alternatively spliced CAT-2 encoding L-arginine transport in brain astrocytes. *J Biol Chem* **271:**24017–24022.
Tan F, Deddish PA and Skidgel RA (1995) Human carboxypeptidase M. *Methods Enzymol* **248:**663–675.
Tan F, Rehli M, Krause SW and Skidgel RA (1997) Sequence of human carboxypeptidase D reveals it to be a member of the regulatory carboxypeptidase family with three tandem active site domains. *Biochem J* **327:**81–87.
Thiemermann C, Mustafa M, Mester PA, Mitchell JA, Hecker M and Vane JR (1991) Inhibition of the release of endothelium-derived relaxing factor in vitro and in vivo by dipeptides containing N^G-nitro-L-arginine. *Br J Pharmacol* **104:**31–38.
Varlamov O and Fricker LD (1998) Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. *J Cell Sci* **111:**877–885.
Vodovotz Y, Kwon NS, Pospischil M, Manning J, Paik J and Nathan C (1994) Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophages with IFN-gamma and bacterial lipopolysaccharide. *J Immunol* **152:**4110–4118.
Wu G, Flynn NE, Flynn SP, Jolly CA and Davis PK (1999) Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. *J Nutr* **129:**1347–1354.
Wu G and Morris SMJ (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* **336:**1–17.

Send reprint requests to: Randal A. Skidgel, Ph.D., Department of Pharmacology (m/c 868), University of Illinois College of Medicine, 835 S. Wolcott, Chicago, IL 60612. E-mail: rskidgel@uic.edu