Arginase II Downregulates Nitric Oxide (NO) Production and Prevents NO-mediated Apoptosis in Murine Macrophage-derived RAW 264.7 Cells

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Abstract. Excess nitric oxide (NO) induces apoptosis of some cell types, including macrophages. As NO is synthesized by NO synthase (NOS) from arginine, a common substrate of arginase, these two enzymes compete for arginine. There are two known isoforms of arginase, types I and II. Using murine macrophage-like RAW 264.7 cells, we asked if the induction of arginase II would downregulate NO production and hence prevent apoptosis. When cells were exposed to lipopolysaccharide (LPS) and interferon-γ (IFN-γ), the inducible form of NOS (iNOS) was induced, production of NO was elevated, and apoptosis followed. When dexamethasone and cAMP were further added, both iNOS and arginase II were induced, NO production was much decreased, and apoptosis was prevented. When the cells were transfected with an arginase II expression plasmid and treated with LPS/IFN-γ, some cells were rescued from apoptosis. An arginase I expression plasmid was also effective. On the other hand, transfection with the arginase II plasmid did not prevent apoptosis when a NO donor SNAP or a high concentration (12 mM) of arginine was added. These results indicate that arginase II prevents NO-dependent apoptosis of RAW 264.7 cells by depleting intracellular arginine and by decreasing NO production.

Key words: arginase • NO • NO synthase • apoptosis • arginine

NITRIC oxide (NO)1 is an important multifunctional biomolecule involved in a variety of physiological and pathologic processes, including regulation of blood vessel dilatation and immune response, and functions as a neurotransmitter (Christopherson and Bredt, 1997; Harrison, 1997; Mayer and Hemmens, 1997; Nathan, 1997). In pathologic processes, NO functions as a bactericidal or tumoricidal agent; however, excess NO production is thought to be the cause of several diseases, such as septic shock (Stuehr and Marletta, 1985), autoimmune disease (Farrell et al., 1992), cerebral infarction (Dawson et al., 1996), and diabetes mellitus (Corbett and McDaniel, 1992). NO-mediated apoptosis is often observed in such cases (Kröncze et al., 1997). NO has several cytotoxic effects, including reactions with proteins and nucleic acids. The main targets of NO in proteins are the SH group (Molina y Vedia et al., 1992) and Fe of active sites (Hibbs et al., 1988), especially Fe²⁺ (Kim et al., 1995) in heme. In the nucleus, NO has been shown to cause mutations of genes (Routledge et al., 1994; Juedes and Wogan, 1996), and inhibition of DNA repair enzymes (Kwon et al., 1991; Lepoivre et al., 1991), and to mediate DNA strand breaks (Nguyen et al., 1991; Fehsel et al., 1993). In several types of cells including macrophages (Albina et al., 1993; Sarih et al., 1993), chondrocytes (Blanco et al., 1995), neurons (Bonfoco et al., 1995), and islet β cells (Kaneto et al., 1995), NO has been shown to mediate apoptosis (Dimmelker and Zeiher, 1997).

Morphologically, chromatin condensation, nuclear fragmentation, and formation of apoptotic body are characteristic changes of apoptosis (Kerr, 1995). In the cascade of inducing these morphological changes, IL-1β-converting enzyme (ICE)-like proteases or caspases play a key role (Miura et al., 1993; Nicholson et al., 1995). Activation of caspases leads to activation of a specific endonuclease (Enari et al., 1998). Digestion of DNA at the linker portions of nucleosomes by endonuclease causes fragmentation of DNA (Gaido and Cidlowski, 1991; Peitsch et al., 1995). Many agents including NO synthase (NOS)-inducing agents have been shown to induce activation of caspases, and apoptosis follows (Hale et al., 1996).

NO is synthesized from arginine by NOS, generating citrulline. The availability of arginine is one of the rate limit-
ing factors of cellular NO production. When the inducible type NOS (iNOS) is induced, the activity is so high that the supply of arginine is important. This amino acid can be obtained from blood circulation, intracellular protein degradation, or by regeneration from citrulline through the “citrulline-NO cycle” (Hecker et al., 1990; Hattori et al., 1994; Nussler et al., 1994; Nagasaki et al., 1996). As arginine is a common substrate for NOS and arginase, arginase may compete with NOS for arginine, and downregulate production of NO. Arginase exists in two known isoforms. Liver-type arginase (arginase I) is expressed almost exclusively in the liver and catalyzes the last step of urea synthesis. However, we found that arginase I is highly induced in rat macrophages activated with bacterial lipopolysaccharide (LPS; Sonoki et al., 1997). On the other hand, nonhepatic type (arginase II) is expressed in some extrahepatic tissues (Grody et al., 1989). Recently, we (Gotoh et al., 1996) and others (Vockley et al., 1996; Morris et al., 1997) cloned human arginase II cDNA and localized the arginase II gene to chromosome 14q24.1-24.3 (Gotoh et al., 1997). Activity (Wang et al., 1995) and mRNA (Gotoh et al., 1996) of arginase II, as well as those of iNOS, are induced by LPS in murine macrophage-like RAW 264.7 cells. On the other hand, treatment of RAW cells with LPS and interferon-γ (IFN-γ) induces apoptosis (Messmer et al., 1996).

Here, we report that the LPS/IFN-γ-dependent apoptosis of RAW cells is prevented by dexamethasone (Dex) and cAMP, and that this antiapoptotic effect depends on induction of arginase II that leads to suppression of iNOS-dependent production of NO in these cells.

Materials and Methods

Plasmids

pCAGGS-rAI, a mammalian expression plasmid for rat arginase I, was constructed by inserting the EcoRI-PstI fragment of pARGr-2 (Kawamoto et al., 1987) into the EcoRI site of the plasmid pCAGGS (Niwa et al., 1991) after linker attachment. The rat arginase II cDNA clone was isolated by PCR, using mRNA from the small intestine of a male Wistar rat. PCR was carried out using rat arginase II primers corresponding to nucleotides 86–1211 (GenBank, accession number U90887). The obtained product was inserted into the HincII site of pGEM-3Zf(+) (Promega Corp.), yielding pGEM-rAI-2.

Materials

Antiserum against human arginase II was raised in a rabbit by injecting Escherichia coli–expressed and the purified mature portion of human arginase II (residues 25–354). A monoclonal antibody against mouse iNOS was obtained from Transduction Laboratories.

Cell Culture and DNA Transfection

Mouse macrophage-like RAW 264.7 cells were grown in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. Transfection of RAW cells with plasmids was carried out using TransIT-LT1 Polyamine (PanVera Corp.) according to the protocol provided by the manufacturer.

Treatment of RAW Cells

RAW cells were treated in medium with or without 1 μM Dex and/or 1 mM dibutyryl cAMP for 24 h, and then treated with various combinations of E. coli LPS (serotype 0127:B8, Sigma Chem. Co., 150 μg/ml) and mouse IFN-γ (100 U/ml) for indicated periods. A NO scavenger carbonyl-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, 500 μM] or a NO donor SNAP (5-nitroso-N-acetyl-l- penicillamine, 1 mM) was added to the medium concomitantly with the addition of LPS and/or IFN-γ.

RNA Blot Analysis

Total RNA from RAW cells was prepared by the guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). After electrophoresis in formaldehyde-containing agarose gels, RNAs were transferred to nylon membranes. Hybridization was performed using digoxigenin-labeled antisense RNAs as probes for rat iNOS, or rat arginase II. The antisense RNAs were synthesized using as templates pcDNAIi-rNOS (Nagasaki et al., 1996), or pGEM-rAI-2, digested by appropriate restriction enzymes. Chemiluminescence signals derived from hybridized probes were detected on x-ray films using DIG luminescence detection kits (Boehringer Mannheim GmbH), and were quantified using a MacBAS bio-image analyzer (Fuji Photo Film Co.).

Immunoblot Analysis

RAW cells were homogenized in 20 mM Hepes-KOH (pH 7.5) containing 1% Triton X-100, 20% glycerol, and 1 mM dithiothreitol. After centrifugation, the supernatants were used as cell extracts for immunoblot analysis. The cell extracts were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using ECL kits (Newcom Amsham), according to the protocol provided by the manufacturer. The results were quantified using a MacBAS bio-image analyzer.

Immunocytochemical Staining

RAW cells were cultured on coverslips and transfected with the human arginase II expression plasmid or stimulated with LPS (150 μg/ml), Dex (1 μM), and dibutyryl cAMP (1 mM), as described above. The cells were fixed with 4% paraformaldehyde for 40 min at room temperature and treated with phosphate-buffered saline containing 1% Triton X-100. The cells were then incubated with antiserum against human arginase II and then with goat anti-rabbit IgG conjugated with FITC (Vector Labaratories) as the second antibody. The stained cells were observed under a fluorescence microscope.

Detection of Apoptosis

RAW cells were treated as described above. To analyze morphological changes of nuclei, the cells were fixed, stained with 8 μg/ml of the Hoechst dye 33258 for 5 min, and washed with phosphate-buffered saline. The stained cells were observed under a fluorescence microscope. DNA fragmentation was detected by the terminal transferase-mediated dUTP-biotin nick end labeling method (TUNEL method), using in situ apoptosis detection kits (Takara) according to the protocol provided by the manufacturer. To detect DNA ladder formation, we used apoptosis ladder detection kits (Wako Pure Chemical Industries) according to the protocol provided by the manufacturer. DNA was isolated from the cells, resolved on agarose gels, stained with SYBR Green I, and visualized by UV transillumination.

Other Methods

Arginase activity was measured as described in Schimke (1962). Concentration of NO<sub>2</sub>− plus NO<sub>3</sub>− in culture supernatants was measured using the Griess reagent with the NO<sub>2</sub>-nitroso-DL-penicillamine, 1 mM) and a NO donor SNAP (5-nitroso-N-acetyl-l-penicillamine, 1 mM) was added to the medium concomitantly with the addition of LPS and/or IFN-γ.

Results

LPS/IFN-γ-Induced NO-mediated Apoptosis of RAW Cells

When mouse macrophage-like RAW 264.7 cells were treated with LPS/IFN-γ for 24 h, morphological changes characteristic of apoptosis occurred (Fig. 1 A). Round-shaped cells and apoptotic bodies were observed in phase-
contrast images, and chromatin condensation and nucleus fragmentation were seen in the case of Hoechst dye 33258 staining. Most cells were positive for TUNEL staining. These apoptotic changes were strongly prevented by adding a NO scavenger carboxy-PTIO (Akaike et al., 1993; Fig. 1 B). These results show that LPS/IFN-γ induces apoptosis of RAW cells, and this apoptosis is mediated by NO. LPS alone was much less effective in inducing apoptosis (data not shown).

**Induction of Arginase II Prevents iNOS-dependent Apoptosis of RAW Cells**

NO is synthesized from arginine, a common substrate for NOS and arginase. We reported that iNOS and arginase II mRNAs are coinduced in RAW cells by LPS (Gotoh et al., 1996). On the other hand, when these cells were treated with LPS plus IFN-γ, the induction of iNOS mRNA was augmented, whereas the induction of arginase II was strongly suppressed (Gotoh et al., 1996).

We then asked whether LPS/IFN-γ-induced apoptosis would be prevented by arginase II induction. RAW cells were cultured in the presence or absence of Dex/cAMP for 24 h, and then treated with LPS or IFN-γ, or their combination for 6 or 12 h (Fig. 2 A). Fig. 2, B and C, shows effects of various combinations of reagents on iNOS and arginase II mRNAs. iNOS mRNA of ~4.5 kb was not evident in the untreated cells but was induced by LPS, and this induction was enhanced by IFN-γ or Dex/cAMP, and even more strongly by their combination. Dex/cAMP was without effect. Arginase II mRNA of ~1.8 kb that was low in untreated cells was induced by LPS or Dex/cAMP and even more so by their combination. IFN-γ strongly prevented the induction of arginase II by LPS, whereas it had little effect on the induction by LPS plus Dex/cAMP. In other words, inhibition of LPS induction of arginase II mRNA by IFN-γ was alleviated by Dex/cAMP. cAMP alone had some effect in such cases, whereas Dex alone was ineffective (Fig. 2 D).

Fig. 2, E and F, shows immunoblot analysis of arginase II protein in RAW cells treated with Dex/cAMP for 24 h and then with LPS and/or IFN-γ for 12 h. Arginase II protein was induced by LPS and this induction was prevented by IFN-γ. It was also induced by Dex/cAMP, and LPS plus Dex/cAMP appeared to have an additive effect. The induction of arginase II protein by LPS plus Dex/cAMP was somewhat reduced by the further addition of IFN-γ. The results of arginase II protein were in accord with those of arginase II mRNA (Fig. 2, B and C).

We then examined the effect of Dex and cAMP on LPS/IFN-γ-induced apoptosis of RAW cells (Fig. 2 G). When LPS/IFN-γ, a portion of the cells was apparently dead and detached from coverslips and the number of attached cells decreased. (B) RAW cells were cultured for 24 h with 150 μg/ml LPS and 100 U/ml IFN-γ. At the same time of the addition of LPS/IFN-γ, a NO scavenger carboxy-PTIO (300 μM) was added to the medium where indicated. After fixation, the cells were analyzed for apoptosis by the TUNEL method. Phase-contrast images (a and c) and fluorescence images (b and d) of the same fields are shown. Original magnifications: ×400. Bars, 10 μm.
cells were treated with LPS/IFN-γ, iNOS but not arginase II was induced. Under these conditions, apoptotic changes were observed. These changes were effectively prevented by Dex/cAMP; under these conditions both iNOS and arginase II were induced. Dex or cAMP alone was much less effective than their combination in arginase II induction in the presence of LPS/IFN-γ (Fig. 2D), and was not effective in preventing apoptosis.
Fig. 2 H shows results of agarose gel electrophoresis of DNA. The formation of a DNA ladder, characteristic of apoptotic cells, was observed when the cells were treated with LPS/IFN-γ. This ladder formation was prevented by Dex/cAMP. Dex or cAMP had little effect in preventing ladder formation. All these results suggest strongly that apoptosis is prevented by the induction of arginase II.

**Transfection of Arginase Expression Plasmids Prevents iNOS-dependent Apoptosis of RAW Cells**

To examine the effect of arginase on apoptosis even more directly, transfection experiments were done. In a control experiment, RAW cells were treated with LPS plus Dex/cAMP to induce arginase II. After fixation, the cells were immunostained with an antibody against arginase II (Fig. 3). Little immunoreactivity was observed in untreated cells. On the other hand, when the cells were treated with LPS plus Dex/cAMP, the cells became positive for arginase II immunoreactivity. Thread-like structures were stained, results reflecting localization of arginase II in the mitochondria (Gotoh et al., 1996). When the cells were transiently transfected with the arginase II plasmid, some cells that were apparently transfected with the plasmid were strongly immunostained. Thread-like mitochondrial structures were visible in most of the positive cells, whereas whole cells were stained in some positive cells, probably due to a high overexpression of the enzyme.

Fig. 4 shows the effect of arginase II expression on apoptosis of RAW cells. Apoptotic changes were observed by the addition of LPS/IFN-γ. When the arginase II plasmid was transfected, some cells were protected from apoptotic change. Transfection of an expression plasmid for arginase I, a cytosolic enzyme, was also effective in protecting cells from LPS/IFN-γ-dependent apoptosis. Transfection of the insertless plasmid was without effect. These results support our hypothesis that arginase prevents apoptosis by depleting intracellular arginine and thus decreasing production of NO. Both mitochondrial arginase II and cytosolic arginase I were effective.

To gain support for this hypothesis, we examined the effect of a NO donor SNAP on apoptosis of RAW cells. SNAP alone induced apoptosis of the cells. SNAP also canceled the antiapoptotic effects of Dex/cAMP or arginase II plasmid (data not shown). Therefore, the antiapoptotic effect of arginase relates to downregulation of NO production by the enzyme. In fact, when a high concentration of arginine (12 mM) was added to the culture medium, antiapoptotic effects of the arginase II plasmid were not observed, and all the cells underwent apoptosis (data not shown). 12 mM arginine alone induced no apoptotic change of the cells.

**Arginase II Suppresses NO Production in RAW Cells**

We next examined the effect of arginase II induction on NO production in RAW cells (Fig. 5). NO production measured by NO₂ plus NO₃ in the culture medium was not detected in untreated cells. When the cells were treated with LPS/IFN-γ, iNOS protein was induced and NO production was markedly increased. On the other hand, when Dex plus cAMP were included with LPS/IFN-γ, iNOS protein was further induced, whereas arginase activity was highly induced, and NO production was markedly decreased. These results suggest strongly that the induced arginase II competes with iNOS for arginine and suppresses NO production.

**Discussion**

Arginine is a common substrate for NOS and arginase. In RAW cells, LPS/IFN-γ treatment induces iNOS and NO-mediated apoptosis. We obtained evidence that the induced arginase downregulates NO production by depleting arginine and hence preventing apoptosis. First, when arginase II was induced by Dex/cAMP in activated cells, iNOS induction was not suppressed, but apoptosis was prevented. Second, when the expression plasmid for arginase I or II was transiently transfected, some cells were prevented from apoptosis. Third, a NO donor or a high concentration of arginine canceled out this antiapoptotic effect of arginase. Fourth, NO production was suppressed under conditions where the iNOS expression remained high and arginase II was induced.

In cases of septic shock, iNOS is strongly induced and a large amount of NO is produced. This high-output production of NO is thought to lead to various symptoms, especially hypotension (Moncada and Higgs, 1993). Glucocorticoids and epinephrine are prescribed in cases of shock state. There are reports that glucocorticoids inhibit NF-κB activity through the induction of IkB synthesis (Auphan et al., 1995; Scheinman et al., 1995) and suppress expres-
sion of inflammatory genes, including the iNOS gene. Our present results suggest that the combination of Dex, a synthetic glucocorticoid, and cAMP, a second messenger of epinephrine, may also contribute to the antishock effect by inducing arginase II and downregulating NO production. If such is the case, arginase would be a good target for therapy of septic shock and other conditions due to the overexpression of NO. NOS inhibitors usually inhibit all types of NOS and inhibition of constitutive types of NOS may cause side effects. On the other hand, the demand of iNOS for its substrate arginine is much higher than those of constitutive NOSs because of its very high activity. The induction of arginase may lead to selective inhibition of the high output NO production by iNOS.

We reported that iNOS and arginase I, not arginase II, are coinduced by LPS in cultured rat peritoneal macrophages and in the lung and spleen in vivo (Sonoki et al., 1997). The induction of arginase I is slower than that of iNOS. Based on these findings, we suggested that iNOS is first induced, NO is produced, and then arginase is induced and the sustained overproduction of NO that is toxic to surrounding cells is prevented. In rat aortic endothelial cells, both constitutive and LPS-inducible arginase activity were reported to be present (Buga et al., 1996). The constitutive presence of arginase I and induction of arginase II by LPS were evident, as determined using immunoprecipitation experiments. iNOS is known to be highly induced in rat hepatocytes by the combination of tumor necrosis factor, IL-1, IFN-γ, and LPS (Geller et al., 1993). Treatment of rats with glucocorticoids and cAMP induces arginase I in hepatocytes (Morris et al., 1987). It remains to be seen whether expression of arginase I is affected by treatment with LPS in combination with glucocorticoids and cAMP.

Induction of arginase I and II differs between rats and mice. As reported previously (Sonoki et al., 1997) and as mentioned above, only arginase I is induced by LPS in rat peritoneal macrophages and in the lung and spleen in vivo. On the other hand, only arginase II is induced in mouse macrophage-like RAW cells (Wang et al., 1995; Gotoh et al., 1996). Thus, there appears to be a differential regulation of expression of these two isoforms in various cell types in

Figure 4. Effect of transfection of arginase expression plasmids. RAW cells were transfected with insertless pCAGGS (e and f), human arginase II expression plasmid pCAGGS-hAII (g–j), or rat arginase I expression plasmid pCAGGS-rAII (k and l) 24 h after transfection. LPS (150 μg/ml) and IFN-γ (100 U/ml) were added to the medium and cultured for 12 h. The cells were then fixed and stained with Hoechst dye 33258 except i and j that were analyzed for apoptosis by the TUNEL method after fixation. Phase-contrast images (a, c, e, g, i, and k) and fluorescence images (b, d, f, h, j, and l) of the same fields are shown. Original magnifications: ×400. Bars, 10 μm. A portion of the cells was detached from coverslips by treatment with LPS/IFN-γ (c–l). Arrows indicate cells rescued from apoptosis. The percentage of total cells which were determined to be apoptotic is shown on the bottom of each panel.
various species. Despite this, our results show both forms are effective in preventing NO-mediated apoptosis.

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