Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells

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Morris, Sidney M., Jr., Diane Kepka-Lenhart, and Li-Chun Chen. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E740–E747, 1998.—Activated macrophages avidly consume arginine via the action of inducible nitric oxide synthase (iNOS) and/or arginase. In contrast to our knowledge regarding macrophage iNOS expression, the stimuli and mechanisms that regulate expression of the cytosolic type I (arginase I) or mitochondrial type II (arginase II) isoforms of arginase in macrophages are poorly defined. We show that one or both arginase isoforms may be induced in the RAW 264.7 murine macrophage cell line and that arginase expression is regulated independently of iNOS expression. For example, 8-bromo-cAMP strongly induced both arginase I and II mRNAs but not iNOS. Whereas interferon-γ induced iNOS but not arginase, 8-bromo-cAMP and interferon-γ mutually antagonized induction of iNOS and arginase I mRNAs. Dexamethasone, which did not induce either arginase or iNOS, almost completely abolished induction of arginase I mRNA by 8-bromo-cAMP but enhanced induction of arginase II mRNA. Lipopolysaccharide (LPS) induced arginase II mRNA, but 8-bromo-cAMP plus LPS resulted in synergistic induction of both arginase I and II mRNAs. In all cases, increases in arginase mRNAs were sufficient to account for the increases in arginase activity. These complex patterns of expression suggest that the arginase isoforms may play distinct, although partially overlapping, functional roles in macrophage arginine metabolism.

arginase; adenosine 3',5'-cyclic monophosphate; interferon-γ; lipopolysaccharide

**MATERIALS AND METHODS**

Materials. GeneScreen membranes, [α-32P]dCTP, and [14C]guanidinodiacetic acid were purchased from NEN Life Science Products. Random-primer DNA labeling kits were obtained from Ambion. Dulbecco’s modified Eagle’s medium (DMEM) and murine interferon-γ were purchased from Life Technologies. Pefabloc and 8-bromo-cAMP were from Boehringer Mannheim. Lipopolysaccharide (LPS; Escherichia coli serotype 111:B4), dexamethasone, peptatin A, leptin, and dexamethasone were supplied by Sigma Chemical. All other reagents were of the highest grade commercially available.

Cell culture. The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection and routinely passaged in DMEM containing 10 mM HEPES (pH 7.5), 2 mM glutamine, and 10% fetal calf serum (low endo-
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Toxin; HyClone Laboratories). Cells were switched to fresh medium at the beginning of all experiments to analyze arginase expression. Unless indicated otherwise, selected agents were added to the culture medium to give the following final concentrations: LPS (2 µg/ml), murine interferon-γ (100 U/ml), dexamethasone (1 µM), or 8-bromo-cAMP (0.5 mM).

Isolation and analysis of RNA. Total cellular RNA was isolated according to Chomczynski and Sacchi (5), and Northern blotting was performed as described previously (37). Cloned probes included full-length cDNAs encoding rat type I arginase (25), murine type II arginase (GenBank accession no. AF032466; see Ref. 47), murine iNOS (31), and a genomic DNA clone for murine 18S rRNA (23). After hybridization for specific mRNAs, the membranes were stripped and rehybridized with a labeled DNA probe for 18S rRNA to correct for any minor variations in sample loading. Results of Northern blotting were quantified by analysis on a Molecular Dynamics STORM PhosphorImager. Hybridized membranes also were exposed to Kodak X-AR or Biomax MS X-ray film at −75°C in the presence of intensifying screens.

Arginase and nitrite assays. All arginase assays were performed on lysates of cells that had been treated with the indicated agents for 24 h. Cultured cells were lysed in 0.1% Triton X-100 containing 2 mM Pefabloc, 2 mg/ml peptatin A, and 10 µg/ml leupeptin. Lysates were centrifuged at 12,000 g for 10 min, and supernatants were used for assays. Protein concentration was determined by the bicinchoninic acid assay (Pierce Chemical). Arginase activity in cell lysates was measured by the conversion of [14C]guanidinoarginine to [14C]urea, using a combination of the methods of Russell and Ruegg (45) and Spector et al. (51). Activation of cell lysates and initiation of the arginase assay were exactly as described by Russell and Ruegg (45). After a 30-min incubation at 37°C, the reaction was terminated by heating at 100°C for 3 min, and the reaction mixture was incubated for an additional 45 min at 37°C after addition of potassium phosphate buffer and jack bean urease as described previously (51). The urease reaction was terminated by addition of 2 N HCl, and incubation was continued at 37°C for 30 min. Liberated [14C]CO2, trapped as Na2[14C]CO3, was quantified by scintillation counting. One unit of arginase activity is defined as the amount of enzyme that produces one micromole of urea per minute at 37°C. Aliquots of conditioned medium were assayed for nitrite by the method of Griess as described previously (13).

Immunoblot analyses. For analyses of iNOS and interferon regulatory factor-1 (IRF-1) expression, whole cell extracts were prepared as described previously (26). Arginase I expression was determined in the same cell lysates used for the arginase activity assays. Samples of adult mouse liver and kidney were directly homogenized in SDS sample buffer to provide reference material for the arginase I immunoblots. Immunoblotting procedures were as described previously (26). Immunoblots were developed with the enhanced chemiluminescence kit (Amersham) according to the manufacturer’s directions. Primary antibodies used included a monoclonal antibody to murine iNOS (Transduction Laboratories) and a polyclonal antibody to murine IRF-1 (Santa Cruz Biotechnology). Dr. David Ash generously provided purified recombinant rat arginase I (4), which was used to raise antibodies in a chicken (Aves Laboratories). Dilutions of the primary antibodies used for immunoblots were 1:2,000 (iNOS), 1:2,000 (IRF-1), and 1:50,000 (arginase I). The secondary antibody for the arginase I immunoblots was peroxidase-conjugated rabbit anti-chicken IgY (Jackson ImmunoResearch Laboratories). Control experiments demonstrated the absence of any signal with the preimmune chicken antibody, comigration of the arginase I band in the mouse liver extract with purified recombinant rat arginase I, and blockage of the signal in the mouse liver sample by preincubating the arginase I antibody with excess purified recombinant arginase I.

RESULTS

cAMP and dexamethasone. We first examined the effects of a cAMP analog and dexamethasone, two agents that do not induce iNOS expression in macrophages but that induce arginase activity in a variety of cell types. For example, cAMP analogs induce arginase activity in murine peritoneal macrophages (6, 20, 29) and cultured hepatocytes (40). Dexamethasone induces arginase also in hepatocytes and hepatoma cell lines (8, 14, 40) but not in rat macrophages (17). 8-Bromo-cAMP was a potent inducer of arginase in RAW 264.7 cells, increasing activity by 88-fold (Fig. 1). Dexamethasone alone had no effect on arginase activity, but a combination of dexamethasone and 8-bromo-cAMP reduced induction of arginase activity to 40% of the value obtained with 8-bromo-cAMP alone. None of these treatments had any effect on NO production, as indicated by the absence of any change in the low background level of nitrite.

Whereas previous studies have clearly demonstrated expression of arginase I in RAW 264.7 cells (12, 30, 55), there has been no convincing evidence for the expression of arginase I. However, as we previously found cAMP to be a potent inducer of arginase I expression in hepatocytes (40), we sought to determine whether...
8-bromo-cAMP might also induce arginase I expression in RAW 264.7 cells. Immunoblot analysis using an antibody specific for arginase I revealed strong expression of arginase I in cAMP-treated RAW 264.7 cells, and this induction was largely abolished by dexamethasone (Fig. 1). Arginase I in both the mouse liver and RAW 264.7 samples migrated as a closely spaced doublet of 35- and 38-kDa species, as reported previously for mouse liver (52), rat hepatocytes (49), and murine peritoneal macrophages (30). No arginase I was detected in the mouse kidney extract, which served as a negative control.

To compare the responses of arginase I and arginase II, we measured arginase I and arginase II mRNA levels in the RAW 264.7 cells as a function of time posttreatment. Although arginase I mRNA was consistently undetectable in untreated control cells, a low level of arginase II mRNA was usually detectable in control cells. 8-Bromo-cAMP induced both arginase I and arginase II mRNAs, but the induction kinetics of the arginase mRNAs differed greatly (Fig. 2). Induction of arginase II mRNA was apparent by 6 h, with near-maximal induction occurring by 10 h. In contrast, induction of arginase I mRNA was delayed, with little induction apparent until 18 h, indicating that the response of arginase I mRNA to 8-bromo-cAMP is indirect. Dexamethasone alone had no effect on either arginase I or arginase II mRNA, but it strongly affected the responses of these mRNAs when combined with 8-bromo-cAMP. Induction of arginase I mRNA was almost completely abolished, consistent with the immunoblot (Fig. 1), but the response of arginase II mRNA was clearly enhanced (Fig. 2). Despite the additional increase in arginase II mRNA levels, total arginase activity was still less than half the activity in cells treated with 8-bromo-cAMP alone, suggesting that arginase I represents the majority of the activity in cAMP-treated cells. iNOS mRNA was not induced in cells treated with 8-bromo-cAMP, dexamethasone, or a combination of these agents, consistent with the nitrite data in Fig. 1.

Interferon-γ. As described previously (55), interferon-γ did not induce arginase activity in RAW 264.7 cells. Interferon-γ and 8-bromo-cAMP proved to be mutually antagonistic in their effects on arginase activity and NO production (Fig. 3). Indeed, the combination of these agents reduced induction of arginase or NO production by 8-bromo-cAMP or interferon-γ, respectively, by ~75–85%. Immunoblot analysis indicated that interferon-γ completely blocked induction of arginase I by 8-bromo-cAMP, suggesting that the residual arginase activity might represent arginase II. Interferon-γ and dexamethasone, which had no effect on arginase activity when added singly, also had no effect on arginase activity when added together (data not shown).

The mutual antagonism between interferon-γ and 8-bromo-cAMP on arginase activity and NO production was apparent at the level of individual mRNAs. cAMP-dependent induction of arginase I mRNA was completely abolished by interferon-γ, confirming the results of the immunoblot (Fig. 3), but induction of arginase II mRNA was only slightly reduced (Fig. 4).
The 8-bromo-cAMP-dependent inhibition of iNOS mRNA induction by interferon-γ (IFN-γ) (Fig. 4) agreed very well with the inhibition of NO production in these cells (Fig. 3). Significant inhibition of iNOS induction occurred at 8-bromo-cAMP concentrations as low as 50 µM (Fig. 5). However, this inhibition is gene specific, as interferon-γ-dependent induction of IRF-1 was unaffected by 8-bromo-cAMP. Because IRF-1 itself is required for induction of iNOS mRNA by interferon-γ (22, 33), these results demonstrate that inhibition of iNOS expression by 8-bromo-cAMP is not simply a consequence of reduced IRF-1 expression. Because induction of IRF-1 by interferon-γ requires activation of the transcription factor STAT1 (38, 43, 44), our results further indicate that 8-bromo-cAMP did not block STAT1 activation, in contrast to the cAMP-dependent inhibition of STAT1 activation found in some other cell types (7, 19, 24).

LPS. LPS induced arginase activity about sixfold (Fig. 6). Taking into account the differences in unit definition of arginase activity, the specific activity of arginase after 24 h of LPS treatment was virtually identical to that reported by Wang et al. (55). A combination of LPS and 8-bromo-cAMP, each of which alone was capable of inducing arginase, synergistically induced arginase more than 180-fold above the value in untreated cells (Fig. 6). This combination of stimuli was the most potent inducer of arginase activity in these experiments. The induction of arginase I by 8-bromo-cAMP was significantly enhanced by the addition of LPS, even though LPS alone failed to induce arginase I (Fig. 6). Remarkably, arginase I levels in RAW 264.7 cells treated with LPS plus 8-bromo-cAMP approached the levels found in adult liver, which is normally the site of the highest arginase I expression. Induction of arginase activity by LPS was largely abolished by dexamethasone, reducing it to a level only twofold above control values.

LPS alone induced arginase II mRNA but not arginase I mRNA (Fig. 7), confirming previous reports (10, 30). The combination of LPS and 8-bromo-cAMP synergistically induced arginase II mRNA but with induction kinetics very different from those obtained with LPS or 8-bromo-cAMP alone. Maximum induction of arginase II mRNA by LPS (~6-fold) or 8-bromo-cAMP (~16-fold) occurred at 18 h, whereas maximum induction in response to both agents (~50-fold) occurred at 6 h (Fig. 7). Even though arginase I mRNA was not induced by
LPS alone, there was a synergistic response when LPS and 8-bromo-cAMP were combined.

Dexamethasone alone had no effect on levels of arginase II mRNA, but induction of arginase II mRNA by LPS plus dexamethasone was greater than that by LPS alone (Fig. 8). In contrast, dexamethasone inhibited the induction of arginase activity by LPS (Fig. 6). In these experiments, arginase activity consisted solely of arginase II as neither arginase I protein nor mRNA was detectable in cells treated with dexamethasone, LPS, or a combination of these two agents.

Dexamethasone did not affect induction of iNOS mRNA by LPS (Fig. 8). This clearly differed from the response seen in the murine macrophage J 774.2 line, where dexamethasone inhibited LPS-dependent induction of iNOS (56). A possible clue as the basis for the cell-specific effects of dexamethasone on iNOS induction may lie in the discovery that dexamethasone-dependent inhibition in J 774.2 cells is mediated by lipocortin 1 (56). Thus our failure to observe a similar inhibition may be due to impaired expression of lipocortin 1 in RAW 264.7 cells.

DISCUSSION

This study demonstrates that both arginase isoforms can be coexpressed in RAW 264.7 cells, which resemble murine peritoneal macrophages (30) in this regard. The ability of rodent macrophages to express both arginases appears to be species specific as only arginase I is expressed in rat macrophages (30, 49).

The summary profiles of arginase mRNA induction clearly show that expression of the arginase isoforms in RAW 264.7 cells is not coordinate (Fig. 9). Arginase II mRNA was induced under all six conditions in which arginase activity increased in RAW 264.7 cells. In contrast, induction of arginase I mRNA in RAW 264.7 cells was quite restricted and always required the presence of 8-bromo-cAMP. Arginase I in RAW 264.7 cells could not be detected in RAW 264.7 cells treated with LPS alone, in agreement with the results of other groups (12, 30). Thus RAW 264.7 cells differ somewhat from murine peritoneal macrophages, in which arginase I is strongly induced by LPS alone (30). However, our results and those of the other groups conflict with the results of Wang et al. (55), who reported that arginase I might account for up to 10% of the total arginase activity in LPS-treated RAW 264.7 cells. The basis for the conflicting results is not known but could possibly reflect a low level of nonspecific immunoinactivation or immunoprecipitation by the anti-arginase I antibody employed by Wang et al. (55). Arginase I expression in RAW 264.7 cells was not completely unresponsive to LPS; once arginase I was induced by 8-bromo-cAMP, its expression was further induced by the addition of LPS. These results suggest that some basal level of arginase I expression in either murine peritoneal macrophages or RAW 264.7 cells may be a precondition for its induction by LPS. We were unable to confirm the results of Gotoh et al. (10), who reported that arginase I mRNA was not detectable in RAW 264.7 cells treated with LPS plus dibutyryl-cAMP. We consistently found that arginase I mRNA was strongly in-
The increases in arginase activity. This suggests that nase mRNA levels appear to be sufficient to account for involve other factor(s) as yet unidentified.

Interferon-\(\gamma\)-induced iNOS expression apparently involves factors other than arginase I. Antagonism of cAMP-dependent induction of iNOS in RAW 264.7 cells. To the best of our knowledge, these results suggest that the indirect mechanism involving induction of the transcription factor C/EBP\(\beta\) (11), our results suggest that this indirect mechanism is not operative in RAW 264.7 cells. The response of arginase I mRNA to combinations of these agents also exhibited striking differences between these cell types. The combination of dexamethasone plus cAMP resulted in a strongly synergistic induction of arginase I mRNA in rat hepatocytes, but dexamethasone almost completely antagonized induction of arginase I mRNA by 8-bromo-cAMP in RAW 264.7 cells. To the best of our knowledge, these results are the first to indicate that regulation of arginase I expression by dexamethasone and cAMP occurs by mechanisms that are cell-type specific.

Although combining 8-bromo-cAMP with LPS resulted in a 30-fold greater level of arginase activity than in RAW 264.7 cells treated with LPS alone, the much higher level of arginase expression did not dimin-

Fig. 9. Relative induction of arginase mRNAs in RAW 264.7 cells. Data for arginase I and arginase II mRNAs were averaged for the experiments shown in Figs. 1–8. Results are shown for 18 h posttreatment with the indicated agents as this was the time of maximal mRNA induction under most conditions. For each mRNA, abundances are expressed relative to abundance in cells treated with 8-bromo-cAMP (cAMP). Arginase I mRNA was undetectable in control cells, and average arginase II mRNA levels were <3% of the value in cells treated with 8-bromo-cAMP.

In the course of these studies, we noted that 8-bromo-cAMP strongly antagonized the induction of iNOS by interferon-\(\gamma\). Highly variable effects of cAMP on iNOS expression have been reported previously for macrophages and macrophage cell lines (39, 50), as well as for other cell types (e.g., see Refs. 2, 9, 15, 18, 27, 28, 42).

For example, cAMP analogs alone induced NO synthesis in rat peritoneal macrophages (50) but not in the ANA-1 (39) or RAW 264.7 murine macrophage cell lines (Figs. 3 and 5). cAMP analogs had a marked biphasic effect on interferon-\(\gamma\)-induced NO synthesis in ANA-1 cells, enhancing NO production at low concentrations but inhibiting NO synthesis at high concentrations (39). In contrast, the same range of 8-bromo-cAMP concentrations consistently inhibited interferon-\(\gamma\)-dependent induction of iNOS in RAW 264.7 cells. Because our results ruled out inhibition of STAT1 activation or IRF-1 induction as the basis for the block in iNOS induction, the inhibitory effects of cAMP on interferon-\(\gamma\)-induced iNOS expression apparently involve other factor(s) as yet unidentified.

In general, the magnitude of the increases in arginase mRNA levels appear to be sufficient to account for the increases in arginase activity. This suggests that transcription of the arginase genes is probably the major control point for arginase induction in macrophages. For cases in which both arginases are expressed, these conclusions should not be considered definitive until arginase II antibodies or isoform-specific inhibitors become available to more precisely determine the mass of each isoform and its individual contribution to total arginase activity. Under some conditions, however, arginase II is expressed in the absence of arginase I, allowing a more straightforward analysis of the data. For example, both arginase activity and levels of arginase II mRNA increased by about sixfold in LPS-treated RAW 264.7 cells, a strong indication that regulation of arginase expression occurred entirely at the pretranslational level in this case. However, there also was strong evidence that arginase II expression can be regulated at a translational or posttranslational step; whereas cAMP coordinately induced arginase activity and arginase II mRNA, combining dexamethasone with LPS further enhanced the induction of arginase II mRNA but inhibited the LPS-dependent increase in arginase activity (cf. Figs. 6 and 8). Because only arginase II was expressed under these conditions, these results suggest that dexamethasone decreased the translational efficiency of arginase II mRNA, decreased the half-life of arginase II protein, or triggered some posttranslational modification of arginase II that reduced its catalytic efficiency.

The new data on arginase I expression reported here led us to ask whether cAMP and dexamethasone have the same effect on arginase I expression in macrophages as they do in hepatocytes (40), the only other cell type in which arginase I expression has been extensively studied. Comparison of arginase I mRNA responses in the two cell types revealed striking differences. Induction of arginase I mRNA by cAMP in rat hepatocytes was rapid, indicative of a direct response, whereas its induction by cAMP in RAW 264.7 cells was quite delayed, characteristic of an indirect response mechanism. Dexamethasone alone induced arginase I mRNA in rat hepatocytes, but it had no effect on arginase I mRNA expression in RAW 264.7 cells. Because induction of hepatic arginase I mRNA by dexamethasone occurs by an indirect mechanism involving induction of the transcription factor C/EBP\(\beta\) (11), our results suggest that this indirect mechanism is not operative in RAW 264.7 cells. The response of arginase I mRNA to combinations of these agents also exhibited striking differences between these cell types. The combination of dexamethasone plus cAMP resulted in a strongly synergistic induction of arginase I mRNA in rat hepatocytes, but dexamethasone almost completely antagonized induction of arginase I mRNA by 8-bromo-cAMP in RAW 264.7 cells. To the best of our knowledge, these results are the first to indicate that regulation of arginase I expression by dexamethasone and cAMP occurs by mechanisms that are cell-type specific.
ish cellular NO production. This result is consistent with the notion that production of N-hydroxyarginine, an intermediate in NO synthesis and a potent inhibitor of arginase, inhibits arginase activity, as shown previously for rat alveolar macrophages (16) and endothelial cells (3). In the case of activated murine peritoneal macrophages, however, arginase apparently can reduce arginine concentrations to levels that become rate-limiting for cellular NO production (54). The difference in results for RAW 264.7 cells and murine peritoneal macrophages may reflect the fact that murine macrophages express considerably higher levels of arginase than do RAW 264.7 cells (30).

It should be emphasized that the role of arginase in inflammation may not simply be to regulate arginine availability for enzymes such as iNOS but to regulate other pathways by producing ornithine. Because ornithine is a precursor for synthesis of polyamines and proline, arginase may enhance cell proliferation and collagen synthesis, thereby promoting wound healing and tissue remodeling during recovery from injury and infection (1, 46). This notion is supported, for example, by the fact that both cAMP and LPS, which induce arginase in RAW 264.7 cells, also induce ornithine decarboxylase in murine macrophage cells (41, 48).

Whereas most studies of arginine-metabolizing enzymes in cultured macrophages have exposed the cells only to individual cytokines or other agents, it should be borne in mind that, in vivo, macrophages are simultaneously exposed to bacterial endotoxins or inflammatory cytokines in the presence of glucocorticoids or cAMP-elevating agents such as prostaglandins or epinephrine. We show here that glucocorticoids and cAMP can dramatically modify macrophage responses to LPS and interferon-γ, indicating that the regulation of arginine metabolism during inflammation in vivo is probably more complex than we currently appreciate. Although the functional significance of distinct arginase isoforms located in different subcellular compartments of the macrophage has not been determined, the differences in subcellular localization may play a role in regulating the metabolic fate of ornithine. For instance, ornithine aminotransferase, which is involved in the conversion of ornithine to proline or glutamate, is mitochondrial, whereas ornithine decarboxylase, which is required for conversion of ornithine to polyamines, is cytosolic. Thus ornithine produced by arginase I may be preferentially used for proline or glutamate synthesis, whereas ornithine produced by arginase II may be preferentially used for synthesis of proline or glutamate. Because there are no isoform-specific arginase inhibitors, elucidation of the physiological roles of the arginase isoforms must be determined by manipulating arginase gene expression by recombinant DNA techniques, a task that will be facilitated by the availability of both arginase I and arginase II cDNA clones.

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