Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages?

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Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages? Am J Physiol Regulatory Integrative Comp Physiol 279: R2237–R2242, 2000.—Because arginase hydrolyzes arginine to produce ornithine and urea, it has the potential to regulate nitric oxide (NO) and polyamine synthesis. We tested whether expression of the cytosolic isoform of arginase (arginase I) was limiting for NO or polyamine production by activated RAW 264.7 macrophage cells. RAW 264.7 cells, stably transfected to overexpress arginase I or β-galactosidase, were treated with interferon-γ to induce type 2 NO synthase or with lipopolysaccharide or 8-bromo-cAMP (8-BrcAMP) to induce ornithine decarboxylase. Overexpression of arginase I had no effect on NO synthesis. In contrast, cells overexpressing arginase I produced twice as much putrescine after activation than did cells expressing β-galactosidase. Cells overexpressing arginase I also produced more spermidine after treatment with 8-BrcAMP than did cells expressing β-galactosidase. Thus endogenous levels of arginase I are limiting for polyamine synthesis, but not for NO synthesis, by activated macrophage cells. This study also demonstrates that it is possible to alter arginase I levels sufficiently to affect polyamine synthesis without affecting induced NO synthesis.

nitrile oxide synthase; ornithine decarboxylase; putrescine; RAW 264.7

THE AMINO ACID L-arginine is subject to various metabolic fates in its role as precursor for synthesis of nitric oxide (NO), urea, polyamines, proline, glutamate, creatine, agmatine, and proteins (reviewed in Ref. 37). The committed step in the conversion of arginine to polyamines, proline, or glutamate is catalyzed by arginase, which converts L-arginine to L-ornithine and urea. Arginase occurs as two distinct isozymes in mammals; arginase I is cytosolic and normally is expressed almost exclusively in hepatocytes, whereas arginase II is mitochondrial and is expressed at low levels in many cell types (14, 24). The different subcellular location of the two isozymes is thought to play a role in determining whether ornithine becomes converted to polyamines or to glutamate and proline (22, 37).

Recent work has demonstrated that arginase expression, including that of arginase I, can be strongly induced by a variety of cytokines and other stimuli in several nonhepatic cell types, especially macrophages (7, 20, 23, 25, 28). Because arginase I is located in the cytosol, its hydrolysis of arginine to produce ornithine could potentially affect other cytosolic enzymes such as the nitric oxide synthase (NOS) enzymes or ornithine decarboxylase (ODC) that use arginine or ornithine as substrates. In particular, there is considerable interest in the possibility that arginase I may limit NO production by inducible NOS (iNOS), suggested by reports that inhibition of arginase activity resulted in increased NO production after activation of rat alveolar macrophages (12), murine macrophages, (32), and a murine macrophage cell line (5). However, the arginase(s) expressed in these studies were not identified. Thus the impact of arginase I activity on NO production by activated macrophages remains to be clarified.

Arginase I may also play a role in regulating synthesis of polyamines by macrophages in conditions such as wound healing (29). Previous studies have demonstrated that ODC, generally regarded as the rate-limiting enzyme for polyamine synthesis, is rapidly induced in macrophages and macrophage cell lines by stimuli such as lipopolysaccharide (LPS) or a cAMP analog (26, 31, 34), thus raising the possibility that production of the substrate ornithine via arginase may be an important determinant of polyamine synthesis rates in activated macrophages.

With this background in mind, our objective was to test the hypothesis that arginase I expression is a limiting factor in the synthesis of NO or polyamines by activated macrophages. The RAW 264.7 murine macrophage cell line was selected as the model system for this study because iNOS and ODC can be readily induced in these cells by defined stimuli, and expression of the endogenous arginas in response to these stimuli has been well-characterized (25). To the best of

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our knowledge, the present study is the first to use gene transfection to manipulate expression specifically of arginase I to study its impact on synthesis of NO and polyamines in activated macrophages. This approach avoids potential pitfalls associated with the use of arginase inhibitors or mixtures of cytokines and other agents to induce arginase, as these interventions may have effects on the treated cells in addition to altering arginase activity or expression. Within the range of arginase I expression achieved in our experiments, we found that arginase I was limiting for synthesis of putrescine and spermidine but was not a determinant of induced NO synthesis by activated murine macrophage cells.

MATERIALS AND METHODS

Reagents. Rabbit anti-β-galactosidase was purchased from Life Technologies. All other reagents were obtained from suppliers identified previously (25).

Expression plasmids. The β-galactosidase expression plasmid (pEF1/Myc-His/lacZ) containing the Escherichia coli lacZ gene under control of the human EF-1α promoter was purchased from Invitrogen. The cDNA for rat arginase I (16) was cloned into the pEF1/Myc-His expression plasmid (Invitrogen) with the use of appropriate restriction sites; no additional epitope sequences were fused to the arginase-coding sequence in this construct.

Cell culture. RAW 264.7 cells were transfected by exposure to a mixture of Lipofectin (Life Technologies) and plasmid DNA (14:1) in serum-free DMEM for 4 h. The cells were returned to DMEM containing 10% fetal calf serum for 24 h before selection in medium containing 1 mg/ml G418. After the first passage, stably transfected cells were maintained in serum-supplemented DMEM containing 500 μg/ml G418, which was always omitted from the medium 2 days before initiating any experimental studies. To initiate all metabolic experiments described here, cells were transferred to DMEM containing 10% dialyzed fetal calf serum (HyClone). LPS (E. coli serotype 111:B4) or 8-bromo-cAMP (8-BrcAMP) was added to confluent cultures (10^6 cells per well in 6-well culture plate) at final concentrations of 2 μg/ml and 0.5 mM, respectively (25), and incubation was continued for the times indicated.

Assays of arginase activity. Preparation of cell extracts and determinations of arginase-specific activity were as described (25). One milliunit of activity represents hydrolysis of 1 nmol of arginine per minute at 37°C. Arginine hydrolysis by intact cells was determined for cells cultured in a 96-well plate. To each well was added 0.05 μCi of [14C]guanidino-arginine in 150-μl complete DMEM medium containing 10% dialyzed fetal calf serum. Twenty-four hours later, the medium was collected, boiled for 3 min to inactivate enzymes, and conversion of labeled arginine to labeled urea was determined as for the in vitro arginase assay. Results are expressed as percent-labeled arginine hydrolyzed to labeled urea.

Western blotting. Whole cell extracts were prepared in Laemmli sample buffer and used for Western blotting as reported (17, 25).

Measurement of polyamines and nitrite. For determination of polyamines, the entire contents of each well (cells plus medium) were extracted with perchloric acid. After neutralization and removal of insolubles, polyamines (putrescine, spermidine, and spermine) in the extracts were resolved by HPLC and quantified as described previously (38). Blanks consisted of complete medium without cells that were incubated and extracted parallel with the cultured cells. Blank values for each polyamine were subtracted from the polyamine values obtained for the cultured cells. As an index of NO production, nitrite concentrations in conditioned media of cell cultures were determined by the Griess reaction (10). Data were analyzed by the two-tailed paired t-test, with the use of the statistical utilities in the Prism program (GraphPad Software).

RESULTS

To obtain cell lines expressing varying levels of arginase activity, RAW 264.7 cells were stably transfected to express either rat arginase I (Arg I-RAW) or bacterial β-galactosidase (lacZ-RAW), the latter representing the control for expression of an unrelated cytosolic protein from the EF-1 plasmid. Expression of these proteins was confirmed by Western blot (Fig. 1). Arg I-RAW migrated as a single band slightly faster than mouse arginase I, which always appears as a closely spaced doublet on Western blots (25). Elevated activity of arginase I in the Arg I-RAW cells was confirmed by in vitro assay and by measuring conversion of labeled arginine to labeled urea by cultured cells. Hydrolysis of arginine to ornithine and urea in cultures of Arg I-RAW cells was 7.2-fold greater than in cultures of lacZ-RAW cells (Fig. 1). It is important to recognize, however, that measurement of arginine hy-
drolysis to urea in cultured cells underestimates the difference in intracellular arginase activity between the Arg I-RAW and lacZ-RAW cells because of the presence of arginase in fetal calf serum (13). In fact, the arginase activity in cultures of the lacZ-RAW cells is due almost entirely to arginase in the fetal calf serum present in the culture medium (Fig. 1). The specific activity of arginase in extracts of Arg I-RAW cells (17.6 mU/mg) was considerably higher than that of the lacZ-RAW cell extracts (0.1 mU/mg), consistent with the Western blot results.

To examine the impact of varying arginase I expression on induced NO synthesis, lacZ- and Arg I-RAW cells were treated with interferon-γ. This stimulus was chosen because it strongly induces iNOS in RAW 264.7 cells without inducing arginase (25). There were no differences in NO production between the two stably transfected cell lines over an eightfold range in arginine concentration (Fig. 2). This was true even at 50 μM arginine, a concentration at which cellular arginine uptake is not maximal because it is below the $K_m$ of the $y^+$-transport system (6, 15).

We next determined the impact of arginase I expression on polyamine production. The stably transfected RAW 264.7 cell lines were incubated in the presence or absence of LPS and 8-BrcAMP because these agents cause rapid induction of ODC in these cell lines (31, 34). Because LPS also induces expression of iNOS in RAW 264.7 cells, these experiments were conducted at short times after induction to avoid iNOS-dependent inhibition of arginase or ODC activity (2–4). Changes in expression of endogenous arginases are minimal during these brief periods of stimulation (25).

Putrescine concentrations were not significantly different between cultures of unstimulated lacZ-RAW and Arg I-RAW cells at 4 or 6 h (Fig. 3), indicating that arginase I is not limiting for putrescine production in unstimulated RAW 264.7 cells. When cells were treated with LPS or 8-BrcAMP to induce ODC, putrescine production increased in both lacZ-RAW and Arg I-RAW cells. However, putrescine production was appreciably higher in cells treated with 8-BrcAMP than with LPS; at 4 h, the levels were 2.1- and 1.8-fold higher in 8-BrcAMP-treated than in LPS-treated lacZ-RAW and Arg I-RAW cells, respectively, and at 6 h the levels were 1.8- and 1.4-fold higher, respectively. Regardless of the stimulus, there was a significantly greater production of putrescine in Arg I-RAW than in lacZ-RAW cells (Fig. 3). For example, relative to unstimulated controls, 6 h of LPS treatment resulted in 5.4- and 15.9-fold higher putrescine levels in lacZ-RAW and Arg I-RAW cells, respectively, and 6 h of treatment with 8-BrcAMP resulted in 9.7- and 22.1-fold higher levels of putrescine in lacZ-RAW and Arg I-RAW cells, respectively.

We also measured levels of polyamines generated by enzymes downstream of ODC. Spermidine levels were not different for unstimulated lacZ-RAW and Arg I-RAW cells at either time point (Fig. 4). Although spermidine levels in Arg I-RAW cells after 6 h of LPS treatment were higher than in the corresponding lacZ-RAW cells, LPS treatment resulted in no statistically significant increase in spermidine concentrations in either lacZ-RAW or Arg I-RAW cells. However, treatment of Arg I-RAW cells with 8-BrcAMP resulted in a 1.5-fold increase in spermidine levels, relative to unstimulated cells, at 4 and 6 h, but no statistically significant change was observed for lacZ-RAW cells (Fig. 4). Thus, when present, increases in spermidine levels were smaller and occurred later than increases.
in putrescine. The fact that we observed an increase in spermidine levels in 8-BrcAMP-treated cells but not in LPS-treated cells may reflect the fact that putrescine levels increased more rapidly and to a greater degree after 8-BrcAMP treatment than after LPS treatment (Fig. 3). At the time points examined here, there were no significant differences in spermine concentrations for lacZ-RAW and Arg I-RAW cells treated with either agent (not shown).

**DISCUSSION**

Information on the relationship between arginase expression, especially of arginase I, and NO synthesis in activated macrophages is limited. Chang et al. (5) reported that inhibition of arginase in the LPS-activated J774A.1 murine macrophage line by >5 mM l-norvaline resulted in increased NO production, but the arginase isoform(s) expressed in the J774A.1 cells were not identified [for comparison, only arginase II is expressed in the J774A.1 cells].

Gotoh and Mori (9) showed that NO production in RAW 264.7 cells treated with LPS + interferon-γ + dexamethasone + dibutyryl cAMP was less than in RAW 264.7 cells treated with LPS + interferon-γ, correlating with increased arginase expression in the former treatment. In this case, however, only arginase II was expressed, and it was not established that the reduced NO production was due only to increased arginase II expression rather than to some other factor, such as a decrease in arginine uptake. Moreover, these results did not match those of Fligger et al. (8), who found no correlation between arginase activity and NO production in murine macrophages and murine macrophage cell lines. The differing results obtained by Gotoh and Mori versus Fligger et al. may reflect differences in the stimuli these groups used to activate the macrophages. More relevant to the current study are the findings of Hey et al. (12) and Tenu et al. (32), who found that inhibition of arginase in activated rat alveolar macrophages or murine peritoneal macrophages, respectively, resulted in increased usage of arginine by iNOS. Although the arginase isoform(s) expressed in these studies were not identified, results of other studies indicate that the activated rat alveolar macrophages probably expressed only arginase I (30) and that the activated murine peritoneal macrophages probably expressed both arginases I and II (21).

Our results, which show no impact of elevated arginase I expression on induced NO synthesis, are consistent with those of Fligger et al. (8) but not with those of Hey et al. (12) and Tenu et al. (32). We wish to note, however, that even though the difference in intracellular arginase activity between the two stably transfected lines in our study was greater than 100-fold, this large difference was due more to the low arginase activity in the lacZ-RAW cells than to a very high level of arginase activity in the Arg I-RAW cells. Our results suggest that a relatively high-threshold level of arginase I activity is required to observe a reduction in NO production, and the arginase I expression in the Arg I-RAW cells simply may have been below this threshold. This proposition is consistent with the mathematical model of Tenu et al. (32) that describes the competition between arginase and iNOS. According to this model, arginase activities in the experiments by Hey et al. (12) and Tenu et al. (32) exceeded the threshold, accounting for differences in their results and those of the present study and of Fligger et al. (8). Taken together, our results and those of previous studies favor the concept that it is not simply the fold-change in arginase activity that is critical for regulation of NO synthesis but rather the level of arginase activity relative to that of iNOS. Further studies using additional cell types are needed to more fully test this notion.

Although regulation of ODC expression in RAW 264.7 cells has been the subject of several studies, there is relatively little information regarding regulation of polyamine production in these cells. The increase in putrescine production and the absence of any increase in spermidine or spermine levels after LPS stimulation of RAW 264.7 cells are consistent with previous findings (34). Tjandrawinata et al. (34) saw no effect of 8-BrcAMP on putrescine production within 4 h, but the difference between their results and ours is likely due to differences in culture conditions. Effects of cAMP analogs on spermidine or spermine production by RAW 264.7 cells have not been reported previously. In any case, the increased putrescine production by 8-BrcAMP-treated cells in the present study is...
entirely consistent with the previously reported induction of ODC by these agents. It is apparent that endogenous levels of arginase in RAW 264.7 cells, together with the arginase present in serum, suffice for putrescine synthesis by unstimulated cells but are insufficient to support maximal rates of putrescine production after induction of ODC.

Taken together with the present results, the occurrence of elevated arginase activities in wounds (1, 29) and tumor cells (e.g., Refs. 11, 18, 19, 27) suggests that arginase I could be a regulator of cellular proliferation. Indeed, increases in arginase I expression are positively correlated with increased proliferation of vascular smooth muscle cells (36). However, elevated arginase expression by itself was not sufficient to boost the proliferation rate of RAW 264.7 cells, a transformed cell line that normally grows rapidly, because Arg I-RAW cells did not proliferate faster than lacZ-RAW cells (not shown). This agrees with the finding that addition of 1 mM ornithine to the culture medium of unstimulated RAW 264.7 cells did not alter their proliferation rate (33). Our results are consistent with the notion that elevated levels of arginase I may be necessary but not sufficient to enhance cell-proliferation rates.

To the best of our knowledge, the present study is the first to demonstrate that the level of arginase I expression can be a determinant of polyamine production in activated macrophages. This is also likely to be true for other cell types. Accordingly, agents that alter arginase I activity or expression should be considered in strategies for modulating polyamine synthesis in clinical and research settings. Importantly, the present study demonstrates that it is possible to alter arginase I levels sufficiently to affect polyamine synthesis without having any effect on induced NO synthesis. Studies are currently underway to determine the impact of altered arginase I and II expression on various aspects of arginine metabolism in additional cell types.

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REFERENCES


