Distinct arginase isoforms expressed in primary and transformed macrophages: regulation by oxygen tension

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SINCE THE DESCRIPTION of arginase (L-arginine-urea hydrolase, EC 3.5.3.1) in tumors and granulation tissue by Edlbacher and Merz (16), two independent paradigms propose a role for this enzyme in the regulation of specific and nonspecific immunity. According to the first paradigm, macrophage arginase consumes and depletes extracellular L-arginine to concentrations that are rate limiting for the proliferation or survival of lymphocytes, microorganisms, or tumor cells (12, 13).

The second, most recent, proposal indicates that it is nitric oxide (NO) or its derivatives, produced by macrophages from L-arginine by the inducible form of NO synthase (NOS), that mediate the macrophage’s suppressor phenotype and its antimicrobial and antitumor properties (1, 2, 6, 11). It has been hypothesized, in this connection, that arginase modulates NO production in macrophages by reducing the intracellular availability of L-arginine to inducible NOS (33). In explaining the physiological role of arginase in tumors, wounds, and other sites of inflammation, both models agree in identifying the macrophage as the cell expressing arginase in the aforementioned immune responses.

Although the presence of arginase in macrophages has been known for 20 years (28), controversy remains as to the precise isoform of the enzyme that is expressed in these cells. At least two major isozymes of arginase have been described, an hepatic AI isoform and an extrahepatic AII isoform. These isozymes are encoded by different genes and do not appear to emerge from alternative splicing or posttranslational modification of the same gene product (14). Early work by Chen and Broome (9) indicated profound similarities between arginases purified from murine peritoneal macrophages and liver in terms of molecular weight, kinetic characteristics, and Mn2+ requirements. However, it has been generally accepted that macrophage arginase is distinct from the AI isoform present in the cytosol of liver cells (25), a conclusion drawn mainly from the lack of detection of AI in macrophages using antibodies generated against hepatic arginase (7). It has been proposed that, by possessing comparable immunologic and molecular properties, macrophage arginase is similar or identical to the AII, kidney-type mitochondrial isoform of the enzyme (22). Work by Wang et al. (41) and Gotoh et al. (22) identified AII as the isoform expressed by lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, a murine macrophage cell line. More recently, Sonoki et al. (38) reported the presence of AI in rat peritoneal macrophages.

Previous work from this laboratory indicated that 1) arginase is the predominant, if not exclusive, pathway of L-arginine metabolism in maturing wounds (5); 2) macrophages contain most cell-associated arginase in wounds (4); and 3) the metabolism of L-arginine through arginase by wound-derived macrophages is greatly enhanced during anoxic culture (3). Motivated by these findings, experiments were performed to identify the arginase isoforms expressed in primary and transformed rodent macrophages and to investigate the molecular mechanisms for the O2-dependent modulation of L-arginine metabolism in these cells.

Results to be shown demonstrate the differential expression of distinct arginase isoforms among macrophage populations. Macrophages obtained from healing wounds or from the peritoneal cavity of rats expressed only AI mRNA and protein. Murine peritoneal macrophages, in contrast, expressed mRNA for both the AI and AII isoforms. Both isoforms were induced after LPS stimulation. Because of the frequent use of cell lines for
the study of l-arginine metabolism and NO production by macrophages (22, 29, 32, 41), three commonly employed transformed murine cell lines, RAW264.7, P388D0, and J774A.1, were included in these studies. Neither AI nor AII mRNA was constitutively present in these cells. RAW 264.7 cells, but not P388D0 or J 774A.1 cells, expressed AII mRNA after LPS stimulation. AI mRNA was not detected in any of the cell lines after LPS treatment.

In reference to the increased l-arginine metabolism through arginase in O2-deprived cultures, results to be presented demonstrate that AI and the l-arginine transporter mCAT-2 (31) were induced in hypoxic or anoxic culture, whereas AII mRNA was decreased under these conditions.

MATERIALS AND METHODS

Animals

Male Fischer rats (150–200 g, VAF-Plus, Charles River Laboratories, Wilmington, MA) and male B6D2F1 mice (Taconic, Germantown, NY) were used in all experiments. Animals were certified free of common rodent pathogens by the suppliers and were housed in an isolation environment on arrival at the laboratory. The animals were monitored by Brown University/Rhode Island Hospital (Providence, RI) veterinary personnel.

Wound Model, Cells, and Cell Culture

The experimental wound used was the subcutaneously implanted polyvinyl alcohol (PVA) sponge model that has previously been described (4). Briefly, 10 circular sterile PVA sponges (Unipoint Industries, High Point, NC) measuring \( \sim \)1 cm in diameter and 5 mm in thickness were implanted subcutaneously in the dorsum of anesthetized rats through a midline skin incision. The sponge wounds were retrieved 10 days after wounding, and wound-derived macrophages were harvested from the sponges as described (4). Peritoneal macrophages were obtained from nonwounded rats and mice by peritoneal lavage (1, 4). The murine macrophage cell lines RAW264.7, P388D0, and J774A.1 were obtained from American Type Culture Collection (Rockville, MD). Cells were incubated overnight in culture medium [RPMI-1640 (Life Technologies, Grand Island, NY) with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) or 1% fetal calf serum (HyClone Laboratories, Logan, UT), \( 5 \times 10^{-5} \text{ M} \) 2-mercaptoethanol, 10 mM 3-(N-morpholino)propanesulfonic acid, and antibiotics, pH 7.4] at a density of \( 2 \times 10^6 / \text{ml} \). Culture medium contained \( < 3 \text{pg} / \text{ml} \) endotoxin as detected by a chromogenic assay (QLC1000, Wittaker M. A. Bioproducts, Walkersville, MD). Culture medium was supplemented with 10 pg/ml LPS (Esherichia coli serotype 0128:B12, Sigma) or Escherichia coli serotype 0128:B12, Sigma) or \( 10 \mu g / \text{ml} \) LPS (Esherichia coli serotype 0128:B12, Sigma) or \( 100 \mu g / \text{ml} \) bovine chromogen assay (QCL1000, Wittaker M. A. Bioproducts, Walkersville, MD). Culture medium was supplemented with 10 pg/ml LPS (Esherichia coli serotype 0128:B12, Sigma) or \( 100 \mu g / \text{ml} \) bovine sperm DNA (Sigma) when so indicated.

Macrophages were cultured under normoxic, hypoxic, or anoxic conditions. For hypoxic and anoxic cultures, cells were plated in 6-cm Permanox culture plates (Nunc, Naperville, IL), and these were placed in gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA). The chambers were then gassed for 2 h using certified gases containing 1 or 0% \( \text{O}_2 \)-5% \( \text{CO}_2 \)-balance \( \text{N}_2 \). The final \( \text{Po}_2 \) levels in culture media were 8.3 and 0 Torr, respectively. The gassing ports were then closed, and the chambers were placed in a 37°C temperature-controlled incubator overnight. Normoxic cultures were incubated at 37°C in 5% \( \text{CO}_2 \) in room air.

RNA Isolation

Total RNA was isolated from 15–20 \( \times \)10⁶ cells or 1 g of kidney tissue using the reagent Ultraspec (Biotecx, Houston, TX) according to manufacturer's recommendations. Total RNA from liver tissues was provided by Dr. Nancy Thompson (Dept. of Medical Oncology, Brown University, Providence, RI).

Probes for Northern Blot Analysis

AI. The probe used to detect AI was prepared by polymerase chain reaction (PCR). Sense and antisense primers used for PCR amplification of rat macrophage arginase were 5’-CCATAGAGATTACGGAGCG-3’ and 5’-CAGTTCTT-CAGGAGAAAGGC-3’, which correspond to residues 7–13 and 140–146 of the reported hepatic arginase amino acid sequence (27). These primers were synthesized by the Clinical Molecular Biology Department at Rhode Island Hospital (Providence, RI). The PCR product was amplified using cDNA from rat wound-derived macrophages. The cDNA was generated with a First-Strand Synthesis Kit (Pharmacia, San Francisco, CA) from total RNA, according to manufacturer's directions. The PCR product was subcloned into a pCR-II plasmid vector (Invitrogen, San Diego, CA), and plasmid DNA was isolated with a Qiagen plasmid kit according to manufac-turer's directions (Qiagen, Chatsworth, CA). The insert was analyzed by restriction mapping and nucleotide sequencing by Seqwright (Houston, TX).

AII. A plasmid (pGEM-rAII-1) containing a 248-base pair (bp) fragment of rat AII cDNA shown to detect AII mRNA in RAW264.7 cells was employed (22).

mCAT-2. A pBluescript plasmid (clone 20.5.1) containing cDNA to mCAT-2 was a gift from Dr. Carol MacLeod (University of California, San Diego, La Jolla, CA). Bal I digestion of the plasmid yielded a 2-kilobase (kb) cDNA insert that recognizes two transcripts of \( 4.5 \) and 8.5 kb (18, 30).

18S ribosomal RNA. A pcUCB830 plasmid containing the mouse 18S ribosomal (r) cDNA (34) was obtained from American Type Culture Collection. Spl I and BamH I digestion of the plasmid yielded a 752-bp cDNA insert that detects 18S rRNA in rat and mouse macrophages (unpublished results). Measurement of 18S ribosomal RNA was used as a control for variations in the amount of RNA loaded in each lane.

Northern Blot Analysis

Total RNA (20 pg) was fractionated by 1% agarose-0.66% formaldehyde gel electrophoresis (39), transferred to a nylon membrane, and immobilized by ultraviolet crosslinking. AI, AII, mCAT-2, and 18S rRNA were detected with the cDNA probes described above radiolabeled with \([^{32}P]\)dCTP by random priming (Pharmacia). The membrane was hybridized in hybridization buffer [50% deionized formamide, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), 100 pg/ml dextran sulfate, 58 mg/ml sodium chloride, 10 pg/ml sodium dodecyl sulfate (SDS), 2 mg/ml BSA, 2 mg/ml polyvinylpyrrolidone, 2 mg/ml Ficoll (400,000 mol wt), 1 mg/ml sodium pyrophosphate, and 0.25 mg/ml denatured salmon sperm DNA] containing radiolabeled probe (10 ng/ml) for 18–24 h. The membrane was washed for two 30-min periods with 2 \( \times \) saline-sodium citrate (SSC) at room temperature followed by two 30-min periods with 2 \( \times \) SSC-1.0% SDS at 65°C. The membrane was autoradiographed at \(-80°C, AI, AII, mCAT-2, and 18S rRNA were quantitated by densiometry using a Protein Databases desktop densitometer and Quantity 1D gel analysis software (PDI, Huntington Station, NY).
Western Blot Analysis

Postnuclear supernatants of cell lysates (30 µg of protein/ lane) were size fractionated in 15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (3). Membranes were incubated for 1 h in buffer A [phosphate-buffered saline (PBS, pH 7.5)-0.3% Tween 20] and then for 1 h with a rabbit antibody (Ab) against human hepatic AI previously shown to detect AI in rat macrophages (38). The Ab was diluted 1/1,000 in buffer B [PBS (pH 7.5)-0.05% Tween 20]. The membranes were washed with buffer B and incubated with a goat anti-rabbit Ab conjugated with alkaline phosphatase (1/1,000) for 1 h in buffer B. AI was detected on reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Immunoreactive AI was quantitated by densitometry as described above. Protein was determined with the Bicinchoninic Acid Protein Assay Reagent A (Pierce Chemical, Rockford, IL).

Arginase Activity

Arginase activity was measured in cell lysates with a radiometric assay using L-[guanido-14C]arginine according to Russell and Ruegg (36). Arginase activity gives rise to [14C]urea and unlabeled ornithine. Radiolabeled urea was separated from L-[guanido-14C]arginine by ion-exchange chromatography (36).

L-Arginine Metabolism by Cultured Cells

Macrophages were incubated overnight under normoxic, hypoxic, or anoxic conditions. Culture supernatants were then removed, and media containing 0.5 µCi/ml L-[guanido-14C]arginine and the NOS inhibitor NG-monomethyl-L-arginine (0.5 mM, Calbiochem, La Jolla, CA) were added to the cells. Culture supernatants were harvested and analyzed for [14C]urea by high-performance liquid chromatography after 2 h of culture at 37°C in 5% CO2 in air. No radiolabeled L-citrulline, the product of the NOS reaction, was found in supernatants. Flux of L-arginine through arginase was calculated from the accumulation of radiolabeled urea and the initial L-arginine specific radioactivity in culture media.

Data Presentation and Analysis

Data shown are representative of at least two independent experiments. Quantitative data are means ± SD from quadruplicate samples in a representative experiment. Statistical analysis was performed using analysis of variance.

RESULTS

AI mRNA Expression in Rat Wound-Derived Macrophages

A cDNA probe for Northern blot analysis of rat macrophage arginase was generated by PCR and cloned. The PCR fragment was generated with cDNA from rat wound-derived macrophages using primers designed to amplify a region of liver arginase that encodes for three histidine residues, which are highly conserved among all known arginase amino acid sequences (25). These histidine residues function both as ligands for the binuclear Mn2+ reaction center and as general base catalysts (8). The cloned fragment was radiolabeled and used in a Northern blot to detect mRNA isolated from rat wound-derived macrophages, liver, and kidney. Figure 1 shows that wound-derived macrophages expressed a transcript identical in size to that found in rat liver (~1.6 kb). No mRNA transcript was detected in kidney RNA preparation. Sequencing of the cloned fragment confirmed that the detected mRNA was AI, because the sequence of the 440-bp cloned fragment was identical to the corresponding region of rat liver arginase cDNA (27).

Arginase mRNA Expression in Primary Cultured and Transformed Macrophages

AI and AII mRNA expression was measured in the different macrophage populations indicated in Fig. 2 after overnight culture in the absence or presence of LPS. AI mRNA was detected only in primary cultures of rat and mouse peritoneal macrophages and induced by LPS treatment seven- and ninefold, respectively. AII mRNA was expressed in mouse peritoneal macrophages and induced approximately threefold by treat-
Arginase Expression and Regulation in Macrophages

Methods. Densitometric values for AI and AII normalized to 18S rRNA were as follows: AI: 9.2 for rat peritoneal macrophages, 63.4 for rat peritoneal macrophages + LPS, 2.5 for mouse peritoneal macrophages, and 23.1 for mouse peritoneal macrophages + LPS; AII: 2.1 for mouse peritoneal macrophages, 5.8 for mouse peritoneal macrophages + LPS, and 5.1 for RAW264.7 + LPS. mRNA was not detected in remaining cells or culture conditions.

AI Protein Expression in Macrophages

Immunoreactive AI was also measured in cell lysates obtained in the experiment described above (Fig. 3). The anti-AI polyclonal Ab immunoreacted with two proteins of ~35 and 38 kDa in rat hepatocyte lysates, as previously shown by Sonoki et al. (38). Immunoreactive proteins of identical molecular mass were detected in mouse peritoneal macrophage cell lysates and increased twofold after treatment with LPS. A single protein, 35 kDa in size, was detected in rat peritoneal macrophages and was increased twofold after LPS treatment. In agreement with the Northern blot analysis, immunoreactive AI was not detected in cell lysates from the murine macrophage cell lines (results not shown).

Induction of Arginase by Anoxia or Hypoxia

This laboratory previously reported that L-arginine metabolism through arginase in rat wound-derived macrophages increases substantially during anoxic culture (3). Data in Fig. 4 demonstrate that increases in L-arginine metabolism through arginase in these cells after anoxic culture is associated with a greater than twofold increase in immunoreactive AI protein. Similar to hepatocytes, two proteins of ~35 and 38 kDa, were detected by the Ab in rat wound-derived macrophage lysates. In addition, results shown in Fig. 4 indicate that cobalt chloride, a compound that mimics the effects of hypoxia on the expression of some hypoxia-inducible genes (20, 21), increased immunoreactive AI to the same extent as anoxia.

To investigate the molecular level of arginase regulation by O2 and also to explore whether absolute anoxia is required for enzyme induction, AI and AII mRNA were measured by Northern blot analysis in rat wound-derived macrophages and mouse peritoneal macrophages cultured under hypoxic (1% O2) or anoxic conditions. As shown in Fig. 5, hypoxia and anoxia increased AI mRNA expression in rat wound-derived macrophages 2.5- and 7.5-fold, respectively. AI mRNA expression was also induced by hypoxia and anoxia (4.5-fold) in mouse peritoneal macrophages. In marked contrast to in cultured macrophages, induction of AI mRNA was not observed in murine cells decreased by hypoxia and anoxia by 50 and 85%, respectively (Fig. 5).

Arginase Activity vs. Metabolic Flux in Cultured Macrophages

Arginase activity was measured in lysates from rat wound-derived and rat and mouse peritoneal macrophages cultured overnight in normoxic, hypoxic, or anoxic conditions. In the same experiments and at the end of the overnight culture, identically treated intact cells were incubated with L-[guanido-14C]arginine, and the accumulation of its metabolite [14C]urea in culture supernatants was measured after 2 h. Arginase activity in cell lysates increased over values found in normoxic cultures in both hypoxic and anoxic cultures and did so to a similar extent (Table 1). The metabolism of L-arginine through arginase by the intact cells was greater in cells cultured in anoxia compared with those cultured under normoxic or hypoxic conditions.
tally and in agreement with Schneider and Dy (37) and others (4), arginase activity was not detected in culture supernatants harvested after overnight culture (results not shown).

Regulation of Arginine Transporter mRNA by Anoxia and Hypoxia

To determine whether transport of L-arginine into the cell could be preferentially induced by anoxia, mCAT-2 mRNA was measured in rat wound-derived macrophages and mouse peritoneal macrophages after overnight culture under normoxic, hypoxic, or anoxic conditions. mCAT-2 encodes an Na$^{+}$-independent cationic amino acid transporter that has been shown to be inducible in RAW264.7 cells by interferon-γ (IFN-γ) and LPS (31). As stated in MATERIALS AND METHODS, the radiolabeled probe detects two mRNA transcripts of ~4.5 and 8.5 kb. Both of these transcripts were increased in rat wound-derived and mouse peritoneal macrophages by hypoxic and anoxic culture, with the changes being more prominent for the 8.5-kb transcript. This transcript was increased after hypoxic or anoxic culture 2- and 5.5-fold, respectively, in rat wound-derived macrophages and 3.5- and 2-fold, respectively, in mouse peritoneal macrophages.

DISCUSSION

Current results extend previous findings demonstrating the presence of arginase in rodent macrophages (reviewed in Ref. 25). Moreover, they identify the expression of mRNA for both isoforms of arginase, AI and AII, in murine peritoneal macrophages, the exclusive presence of AI in rat macrophages, and disparity in arginase expression among murine macrophage cell lines.

In addition to the lack of AI mRNA expression in the transformed macrophage cell lines and the detection of AII mRNA only in LPS-stimulated RAW264.7 cells, striking differences were found in the level of arginase activity detected in lysates from the different cell types. These activities ranged from being minimally detectable in unstimulated RAW264.7, P388D1, and J774A.1 cells (results not shown) to a high of 40.3 ± 2.3 nmol/min per 10$^6$ cells in anoxic cultured murine macrophages. The greatest arginase activity among the transformed macrophage cell lines was measured in LPS-treated RAW264.7 cells and was 2.35 ± 0.51 nmol/min per 10$^6$ cells. If, indeed, arginase serves in macrophages as a modulator of inducible NOS activity through the depletion of extra- or intracellular L-arginine, then none of the transformed cells studied here appears well suited to model for events occurring in primary macrophages. Incidentally, these macrophage cell lines are the most frequently cited in the literature in investigations of the metabolism of L-arginine in macrophages.

This laboratory previously highlighted the impact of the hypoxic or anoxic environment of the healing wound on the activities of different enzymes of arginine metabolism (3). Results from those studies demonstrated that rat wound-derived macrophages cultured in an anoxic environment contain more immunoreactive inducible NOS than those cultured in room air and that anoxia fully suppresses inducible NOS activity probably through deprivation of its substrate O$_2$. In addition, a 15-fold increase in the amount of L-arginine metabolized to urea through arginase was observed in wound-derived macrophages cultured overnight under anoxic conditions (3). Examination of the promoter region for rat liver AI (35) revealed the presence of sequences that have been identified as binding sites for...
similar to that employed by other O2-responsive genes, greater in cells cultured under anoxic conditions than through arginase was substantially and consistently confirmed arginase as an hypoxia- or anoxia-inducible gene (Figs. 4 and 5). The increase in immunoreactive AI metabolism by intact cells reveals that arginase activity (nmol urea/min per 10^6 cells) was measured in cell lysates as described in MATERIALS AND METHODS. Formation of [14C]urea from L-[guanido-14C]arginine by intact cells was measured over a 2-h period after overnight culture as described under MATERIALS AND METHODS (nmol urea/min per 10^6 cells). α–c Within each cell type, values followed by different letters are different (P < 0.05, analysis of variance).

These observations led to experiments that confirmed arginase as an hypoxia- or anoxia-inducible gene (Figs. 4 and 5). The increase in immunoreactive AI in cultures treated with cobalt chloride suggests that AI is induced through a pathway involving an O2 sensor similar to that employed by other O2-responsive genes, including erythropoietin, vascular endothelial growth factor, glucose transporter 1, and others (17, 19). In contrast to AI, AII mRNA was actually decreased by hypoxia and anoxia.

With regard to the mechanisms conveying the hypoxic or anoxic signal, preliminary results suggest the involvement of at least one stress-activated protein kinase pathway. This is so because addition of the specific inhibitor of the p38 mitogen-activated protein kinase (MAPK), SB-203580 (23, 42), but not of the MAPK-extracellular regulated protein kinase inhibitor, PD-98059 (15), fully prevented the increase in arginase activity found in hypoxic cultures of wound-derived macrophages (unpublished observations). Work is in progress to complete the elucidation of the second messenger pathways mediating the hypoxic or anoxic response in these cells.

Additional experiments were performed to correlate O2-dependent changes in arginase activity in cell lysates to the actual metabolism of L-arginine by intact cells in culture. A quantitative comparison of results shown in Table 1 for arginase activity in cell lysates and L-arginine metabolism by intact cells reveals that arginase activity greatly exceeds metabolic flux. This discrepancy stems, at least partially, from the conditions employed in the arginase assay, which maximize enzyme activity by providing optimal substrate concentration, alkaline pH, and Mn^2+ (25). It was, however, of interest to observe that, in contrast to findings with arginase activity in cell lysates, L-arginine metabolism through arginase was substantially and consistently greater in cells cultured under anoxic conditions than in those cultured in hypoxic conditions. These results suggested that the flux-generating step in L-arginine metabolism through arginase in these cells may be external to the enzyme. In this regard, a family of basic amino acid transporters has recently been identified (reviewed in Ref. 31). One of these transporters, mCAT-2, has been shown to be present in macrophages and induced by IFN-γ and LPS. Examining the published promoter region of the mCAT-2 gene disclosed the presence of the putative HIF-1 binding site (18). To test the hypothesis that the higher rate of L-arginine metabolism through arginase in cells cultured in anoxic conditions could have resulted from the preferential induction of mCAT-2, its mRNA expression was measured in cells cultured in normoxic, hypoxic, or anoxic conditions. Data in Fig. 6 demonstrate that mCAT-2 mRNA was induced by culture in reduced O2. These results indicate the inducibility of mCAT-2 by low PO2 but do not entirely explain the enhanced processing of L-arginine through arginase in anoxic cultured cells.

These findings demonstrate considerable variability in the expression of the AI and AII isoforms of arginase in rodent macrophages. In addition, they also add AI

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<th>Method</th>
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<th>Peritoneal Macrophages</th>
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<td></td>
<td>Arginase activity</td>
<td>Urea production</td>
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<td>22.12 ± 2.0^a</td>
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Values are means ± SD. Macrophages were cultured overnight under normoxic, hypoxic (1% O2), or anoxic conditions. Arginase activity (nmol urea/min per 10^6 cells) was measured in cell lysates as described in MATERIALS AND METHODS. Formation of [14C]urea from L-[guanido-14C]arginine by intact cells was measured over a 2-h period after overnight culture as described under MATERIALS AND METHODS (nmol urea/min per 10^6 cells). α–c Within each cell type, values followed by different letters are different (P < 0.05, analysis of variance).
and mCAT-2 to the previous description of inducible NOS, tumor necrosis factor-α, and interleukin-6 as hypoxia- or anoxia-inducible genes in macrophages (3). In doing so, these observations contribute to the concept that macrophages perceive O2 deprivation as a generalized activating signal (3).

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