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Macrophage Activation by Culture in an Anoxic Environment[1]

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The extracellular amino acid composition of experimental wounds in rats during peak macrophage infiltration bears the imprint of the elevated arginase activity present in wound fluid: L-arginine is found in this space in concentrations markedly lower, and L-ornithine in concentrations markedly higher, than those that are detectable in plasma. No evidence, in the form of L-citrulline or NO_2^- accumulation, can be found at this time for nitric oxide synthase (NOS) activity. Wound-derived macrophages, however, metabolize L-arginine through both arginase and NOS in culture. Given the requirements of NOS for O_2 and the reduced O_2 tension in wounds, experiments were performed to determine the role of O_2 availability on the metabolism of L-arginine by wound-derived macrophages. Results demonstrated that, beyond inhibiting NOS, culture of wound-derived macrophages in an anoxic environment provided an activation signal, markedly increasing total L-arginine metabolism, arginase activity, NOS protein content, and the release of TNF-α and IL-6. Neither resident nor *Corynebacterium parvum*-elicited peritoneal macrophages responded to anoxic culture with increases in L-arginine utilization, arginase activity or, in the case of resident macrophages, in NOS protein content. The enhanced TNF-α and IL-6 release induced by anoxia in wound-derived macrophages was also found in resident peritoneal macrophages. Anoxia appears to act, then, as an inducer of activation-associated traits in macrophages obtained from different sites. *The Journal of Immunology,* **1995, 155: 4391–4396.**

Previous results from this laboratory defined the local metabolism of L-arginine in experimental wounds (1, 2). In this regard, it was established that this amino acid is catabolized in wounds through at least two distinct enzymes: nitric oxide synthase (NOS)[3] and arginase. The differential flux of L-arginine through these pathways was shown to conform to a strict temporal pattern, with the expression of NOS confined to the initial 24 to 72 h following injury. NOS activity found at this time was associated with the arrival of polymorphonuclear leukocytes, cells shown to produce abundant NO in culture, into the wound (1).

Results obtained in those studies also indicated that NOS activity is not detectable in the wounds during the subsequent period of predominant macrophage infiltration, and that arginase constitutes the only high throughput pathway of L-arginine metabolism expressed at that time (1). This conclusion was based on the lack of accumulation of the NOS products L-citrulline or NO_2^-, and the increasing activity of arginase and substantial appearance of L-ornithine, a product of L-arginine metabolism through arginase, in wound fluids over time (1).

In contrast with the aforementioned evidence against the expression of NOS in macrophage-infiltrated wounds, macrophages harvested from these wounds express significant NOS activity in culture, being able to produce as much or more NO_2^- as resident or *Corynebacterium parvum*-activated peritoneal macrophages (1, 3). Taken together, these results suggested that NOS expression by macrophages in wounds in vivo was suppressed through substrate deprivation, resulting from the local consumption of L-arginine by extracellular arginase (1, 3).

Experiments reported here were performed to investigate the potential role of O_2 availability as an additional regulator of NOS in wounds. This approach was suggested by the O_2 requirements of NOS (4, 5) and by the low O_2 tension in wounds (6–9). In regard to the latter, Silver reported O_2 tensions ranging between 0 and 2 torr in the center of rabbit ear wound chambers, and showed that a rise in O_2 tension in the wound occurs only after it is revascularized (8). Work by Niinikoski, Heughan, and Hunt, in turn, using implanted cellulose sponge cylinders as wound models, demonstrated pO_2 to remain below 20 torr within the wounds for 15 to 20 days (9). Marked reductions in O_2 tension have been recorded even in incisional wounds, where revascularization takes place at a faster pace than in dead space wounds (7).

Results to be shown demonstrate that, indeed, NOS activity is suppressed in an anoxic environment and, moreover, that culture under reduced O_2 tension is perceived by wound-derived macrophages as an activating signal leading to increased arginase activity (10), immunoreactive NOS content, and cytokine release. In contrast, resident peritoneal macrophages studied under identical conditions only responded to anoxia with increased cytokine release. These results suggest that anoxia is a selective inducer of activation-associated traits in macrophages harvested from different sites.

Additionally, results show that overnight culture promoted the accumulation of immunoreactive NOS protein in wound-derived and resident peritoneal macrophages. In the case of wound-derived macrophages, anoxic culture resulted in a further increase in NOS protein content. These results suggest that NO may regulate the NOS content of cells.

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[3] Abbreviations used in this paper: NOS, nitric oxide synthase; *N*-MMA, N^G-monomethyl-L-arginine; DPI, diphenylene iodonium.

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Materials and Methods

Polyvinyl alcohol sponge implantation

Male Fischer rats (150–200 g, VAF-Plus; Charles River Laboratories, Wilmington, MA) were used in all experiments. VAF-Plus animals are certified free of common rat pathogens by the supplier, and housed in an isolation environment upon their arrival at the laboratory. The animals were monitored by Brown University/Rhode Island Hospital (Providence, RI) veterinary personnel. Sterile circular polyvinyl alcohol sponges (Unipoint Industries, High Point, NC), measuring approximately 1 cm in diameter and 0.4 cm in thickness, were implanted s.c. through a 7-cm midline incision in the dorsum of each animal (10 sponges per animal) under i.p. anesthesia (pentobarbital, 5 mg/100 g body weight; Abbott Laboratories, North Chicago, IL) (1, 2).

Wound cell harvesting

The animals were sacrificed with CO_2 10 days after sponge implantation, and cells contained in the sponges were harvested exactly as described previously (1, 2). Wound-derived macrophages were isolated from the sponge cell preparation by adherence to plastic and recovered with ice-cold Ca- and Mg-free HBSS. Adherent cells represented approximately 35% of total cells. Purity was greater than 90%, as determined by Wright-Giemsa staining and by immunofluorescence using an anti-rat macrophage Ab (11). Cell viability at the time of harvest was >95% by trypan blue exclusion.

In vivo activation and harvesting of peritoneal macrophages

Peritoneal cells were obtained from naive rats or from animals injected i.p. 7 days earlier with 2 mg *C. parvum* (Wellcome Laboratories, Research Triangle Park, NC). These cells were harvested by sterile peritoneal lavage using HBSS supplemented with 1% FCS (HyClone Laboratories, Logan, UT) and antibiotics. The cells so obtained were washed, resuspended in culture medium, and plated at 10^6/ml, as indicated below. Nonadherent cells were removed by repeated washing after 2-h incubation at 37°C. Cell purity and viability were determined, as mentioned above for wound-derived macrophages. Adherent cells constituted 79 ± 5% and 91 ± 2% of plated resident and *C. parvum*-elicited cells, respectively, and were >95% macrophages in both cases.

Cell culture

Culture medium consisted of MEM (Life Technologies, Inc., Grand Island, NY) supplemented to 1 mM L-arginine, 10% FCS, 10 mM 3-(*N*-morpholino)-propanesulfonic acid, 2.5×10^{-5} M 2-ME, and antibiotics. All media and additives contained <3 pg/ml endotoxin, as assayed by a chromogenic assay (QCL 1000; Whittaker M.A. Bioproducts, Walkersville, MD). In experiments performed to investigate the metabolism of L-arginine, culture media contained [*guanido*-^{14}C]L-arginine (0.1 μCi/ml; DuPont NEN, Cambridge, MA). In addition, and when so indicated, culture media were supplemented with murine rIFN-γ (10 U/ml; Genzyme Corp., Cambridge, MA) and LPS (1 μg/ml, from *Escherichia coli* 055:B5; Difco Laboratories, Detroit, MI), and/or N^G-monomethyl-L-arginine (*N*-MMA, 0.5 mM; Calbiochem, La Jolla, CA) or diphenylene iodonium (DPI, 10 μM; Cayman Chemical, Ann Arbor, MI).

Cultures were performed in Permanox 4-well Lab-Tek chambered slides or 60-mm culture plates (Nunc, Inc., Naperville, IL). Wound macrophages were added directly to the containers at 10^6/ml. As indicated before, resident or *C. parvum*-elicited peritoneal macrophages were added at the same density and selected by adherence.

The chambered slides or culture plates were placed in gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA). The chambers were then gassed continuously for 60 min using certified gases containing: 0%, 1%, 5%, 10%, or 21% O_2, all with 5% CO_2 and balance N_2. The gassing ports were then closed and the chambers placed in a 37°C temperature-controlled incubator overnight.

Preliminary experiments determined the optimal gassing period to obtain a stable pO_2 in the culture media. For this purpose, the electrode of an Instech Laboratories (Horsham, PA) O_2 analyzer was fitted in a gas-tight manner through a perforation in a modular incubator chamber and immersed in the culture media. The resultant pO_2 in culture media after 60-min gassing for the respective gasses were: 0, 8.3, 36.2, 71.2, and 148 torr, respectively. These gas tensions were maintained during the overnight culture. Cell viability at all O_2 tensions by the end of 24-h culture was >85%, as measured by the release of lactic dehydrogenase into culture supernatants. There were no significant differences in the pH of culture supernatants sampled after 24-h culture under the mentioned O_2 tensions.

L-arginine metabolite analysis

Radioactive urea, L-arginine, and L-citrulline contained in deproteinized culture supernatants at the end of incubation were separated using a Bio-LC amino acid analyzer (Dionex, Sunnyvale, CA) and on-line peak detection or fraction collection. Flux through arginase and NOS was calculated from the radioactivity in urea and L-citrulline, respectively, and the L-arginine-specific radioactivity in the media at the beginning of culture. The concentration of nitrites (NO_2^-) in culture supernatant, an additional indicator of the flux of L-arginine through NOS, was determined using Griess reagent (3).

TNF-α assay

Immunoreactive TNF-α present in culture supernatants was measured using a kit from Genzyme Corp.

IL-6 assay

Bioactive IL-6 in culture supernatants was measured, as described previously, using the IL-6-dependent 7TD1 mouse-mouse hybridoma (strain CRL 1851; American Type Culture Collection, Rockville, MD) (12).

Immunoblot analysis of nitric oxide synthase expression

Postnuclear supernatants of cell lysates, obtained from freshly harvested or overnight cultured cells incubated in room air or 0% O_2, were size fractionated in 7.5% SDS-PAGE loaded with 15 μg cell protein per lane. Proteins were transferred to a nitrocellulose membrane and incubated overnight in blocking buffer containing 10 mM Tris-HCl, pH 7.5, 1% BSA, 100 mM NaCl, and 0.1% Tween 20. Blots were probed with a mouse mAb against macrophage NOS (Transduction Laboratories, Lexington, KY), diluted 1/500 in blocking buffer for 1 h at room temperature, followed by washing for 30 min in blocking buffer. Ab was detected using alkaline phosphatase-conjugated goat anti-mouse Ig (Sigma Chemical Co., St. Louis, MO), and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad Laboratories, Inc., Hercules, CA). A contour plot of each band was generated, and OD of the area was determined using a Protein Databases, Inc. (PDI) desktop densitometer and Quantity One 1D gel analysis software (PDI, Huntington Station, NY).

Data presentation

Unless otherwise indicated, data shown are means ± 1 SD of quadruplicate samples from a representative of at least three identical experiments. When not shown in the figures, the value of the SD was smaller than that of the symbols representing the value of the means. Statistical analysis of results was performed by ANOVA-Newman-Keuls or Mann-Whitney's U test, as appropriate. The null hypothesis was rejected if $p < 0.05$.

Results

Hypoxia suppresses L-arginine metabolism through NOS and enhances its flux through arginase in wound-derived macrophages

Figure 1 shows the effects of a range of environmental O_2 tensions on the metabolism of L-arginine by wound-derived macrophages in culture. Data in Figure 1 and Table I demonstrate that L-arginine is catabolized by both arginase and NOS (as shown by the accumulation of radioactive urea or L-citrulline, respectively, in culture supernatants) when cells are cultured at room O_2 tension (21% O_2, media $pO_2 \cong 148$ torr).

Reducing the O_2 tension in the media below that resulting from exposure to 5% O_2 (media pO_2 <37 torr) caused a decrease in NOS activity, as measured by the production of radiolabeled L-citrulline from L-arginine. Virtually no L-arginine was metabolized through NOS in anoxic cultures. In turn, the processing of L-arginine through arginase under anoxic conditions increased more than 15-fold (Fig. 1 and Table I).

The elevated consumption of L-arginine through arginase in anoxic cultures did not result from the release of this enzyme from the cells. There was no detectable arginase activity in culture supernatants nor increases in the release of lactic dehydrogenase from the cells under any of the conditions tested (data not shown).

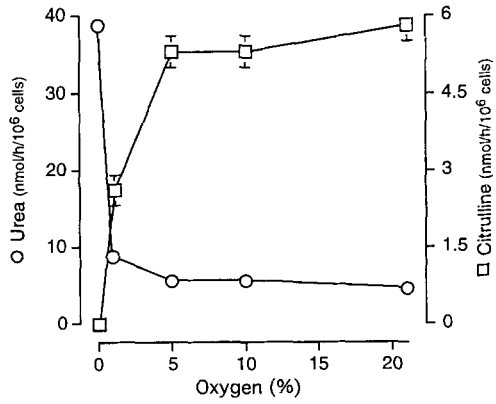


FIGURE 1. Impact of O_2 tension on the metabolism of L-arginine by wound-derived macrophages. Cells, obtained as described in *Materials and Methods*, were cultured overnight in media containing 1 mM L-arginine and 0.1 μCi/ml [*guanido*-^{14}C]L-arginine in gas-tight chambers. The concentration of O_2 in the environment is shown in the horizontal axis and corresponded to the media pO_2 indicated in *Materials and Methods*. The production of urea and L-citrulline by the end of culture was calculated from the appearance of radioactivity in these compounds and the specific radioactivity of L-arginine at the beginning of the cultures.

The increased metabolism of L-arginine through arginase by wound-derived macrophages in anoxic cultures does not result solely from the inhibition of NOS

It is conceivable that the increased flux of L-arginine through arginase in anoxic cultures results directly from the inhibition of NOS by O_2 deprivation. In this construct, L-arginine spared from catabolism through NOS would become available for metabolism through arginase. This appears to have been the case when NOS activity was suppressed by the inclusion of the enzyme inhibitors *N*-MMA (13) or DPI (14) in aerobic cultures (21% O_2) (Table I). Indeed, more L-arginine was metabolized through arginase in the presence of the enzyme inhibitors than in their absence. However, the total amount of L-arginine that was catabolized in cultures including *N*-MMA or DPI remained comparable with that found in cultures not containing these compounds. In contrast, anoxic culture resulted in a greater than fourfold increase in total L-arginine utilization and a greater than 15-fold increase in its processing through arginase, indicating an absolute increase in arginase activity.

Interestingly, total L-arginine flux through arginase and NOS was less in cells exposed to *N*-MMA than in those cultured without inhibitors or with DPI. These results are congruent with the observation that *N*-MMA cannot only inhibit NOS, but also competes with L-arginine for transport into the cell (15).

IFN-γ and lipopolysaccharides increase arginase and nitric oxide synthase activities in wound-derived macrophages, but are less effective in this regard than anoxic culture

To establish the effect of classical activation signals on the metabolism of L-arginine by wound macrophages, these cells were cultured aerobically in the presence of IFN-γ and LPS at concentrations determined previously to maximally stimulate NOS activity. Table I shows that these additions enhanced the catabolism of L-arginine, albeit to a lesser extent than anoxic culture. Arginine metabolism in cells treated with IFN-γ and LPS occurred predominantly through NOS, with no detectable increase in the flux of L-arginine through arginase. The addition of *N*-MMA or DPI to IFN-γ- and LPS-containing cultures, however, revealed that arginase activity was also increased by the activating signals, since the suppression of NOS allowed for enhanced urea production. The presence of IFN-γ and LPS during anoxic cultures failed to further increase arginase activity.

Table I. *L-Arginine metabolism by wound-derived macrophages; effects of O_2 availability, NOS inhibitors, and IFN-γ/LPS*[a]

Oxygen (%)	NOS Inhibitor	IFN-γ + LPS	Urea	Citrulline
21			2.5 ± 0.2 a	7.9 ± 0.6 a
21	N-MMA		5.8 ± 0.2 b	0.4 ± 0.1 b
21	DPI		8.4 ± 1.4 c	0.1 ± 0.1 c
21		+	3.0 ± 0.2 a	12.9 ± 0.4 d
21	N-MMA	+	11.5 ± 0.3 d	0.7 ± 0.1 e
21	DPI	+	18.3 ± 0.3 e	0.1 ± 0.1 c
0			45.2 ± 0.9 f	0.1 ± 0.1 c
0		+	42.9 ± 0.7 f	0.1 ± 0.1 c

[a] Wound-derived macrophages were cultured overnight in the presence of 1 mM L-arginine and 0.1 μCi/ml [*guanido*-^{14}C]-L-arginine as indicated in *Materials and Methods*. The NOS inhibitors N-MMA (0.5 mM) or DPI (10 μM), and/or IFN-γ (10 U/ml) and LPS (1 μg/ml) were added when so indicated. Cultures were conducted in room air or 0% oxygen. Product formation data are nmol/h/10^6 cells. For each product, values followed by different letters are different ($p < 0.05$).

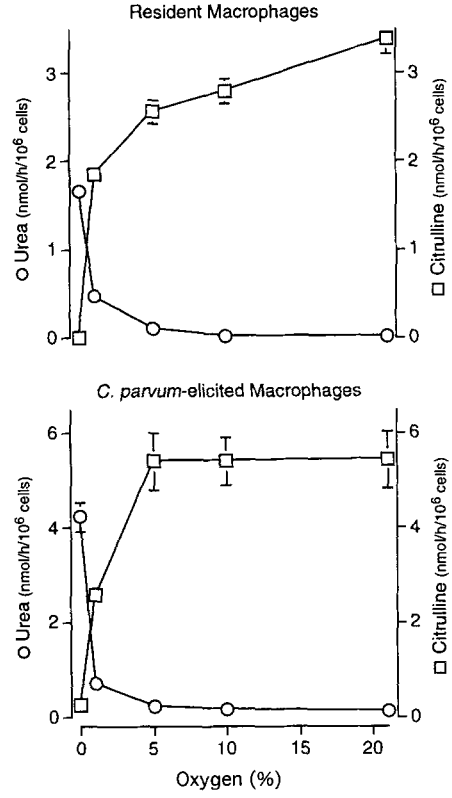


FIGURE 2. Modulation of arginase and NOS activity in resident or *Corynebacterium parvum*-elicited peritoneal macrophages by O_2 availability. Resident or *C. parvum*-elicited peritoneal macrophages were cultured in conditions identical with those indicated in the legend to Figure 1. Flux of L-arginine through arginase or NOS was calculated as indicated in *Materials and Methods*.

Anoxic culture does not increase the metabolism of L-arginine in resident or Corynebacterium parvum*-elicited peritoneal macrophages*

To investigate whether the effects of anoxia or IFN-γ and LPS on L-arginine metabolism also occurred in macrophages not derived from wounds, similar experiments to those just described were

Table II. *Metabolism of L-arginine by resident and* C. parvum*-elicited peritoneal macrophages*[a]

Oxygen (%)	N-MMA	IFN-γ + LPS	Resident Macrophages		*C. parvum*-Elicited Macrophages	
			Urea	Citrulline	Urea	Citrulline
21			0.1 ± 0.1 a	1.4 ± 0.1 a	0.1 ± 0.1 a	5.4 ± 0.5 a
0			1.4 ± 0.1 b	0.1 ± 0.1 b	4.2 ± 0.2 b	0.2 ± 0.2 b
21	+		0.4 ± 0.3 a	0.3 ± 0.1 b	0.3 ± 0.1 a	0.6 ± 0.2 c
21		+	0.2 ± 0.1 a	4.1 ± 0.2 c	0.1 ± 0.1 a	5.5 ± 0.5 a
21	+	+	0.7 ± 0.2 c	0.4 ± 0.1 b	0.3 ± 0.1 a	0.5 ± 0.2 b,c

[a] Resident and *C. parvum*-elicited peritoneal macrophages were cultured as indicated in the *Materials and Methods* in the conditions indicated in the table. When so shown, IFN-γ was added at 10 U/ml together with LPS at 1 μg/ml, and/or N-MMA at 0.5 mM. Culture media contained 1 mM L-arginine and 0.1 μCi/ml [*guanido*-^{14}C]-L-arginine. The partition of label between urea and citrulline was determined after overnight culture. For each metabolite, values followed by different letters are different.

Table III. *Effect of overnight culture in different O_2 tensions on arginase and NOS activity during re-oxygenation*[a]

Cultured Overnight in	Urea	Citrulline
21% O_2	1.6 ± 0.5 a	3.3 ± 1 a
0% O_2	28.7 ± 1.2 b	13.3 ± 0.6 b

[a] Wound-derived macrophages were cultured in air in the presence of 1 mM L-arginine and 0.1 μCi/ml [*guanido*-^{14}C]-L-arginine for 2 h after overnight culture in air or in an anoxic environment. The partition of label between urea and citrulline was then determined in culture supernatants and is here shown as nmol product/h/10^6 cells. For each product, values followed by different letters are different ($p < 0.05$).

conducted using resident and *C. parvum*-elicited peritoneal macrophages (Fig. 2 and Table II).

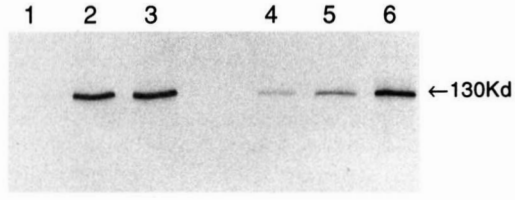
As predicted by previous observations (3), *C. parvum*-elicited macrophages processed more arginine through NOS in aerobic culture than resident cells. Neither cell type catabolized L-arginine through arginase when cultured in 5 to 21% O_2. Decreasing O_2 availability below 5% suppressed NOS activity and allowed for the increased expression of arginase.

In contrast to findings in wound cells, anoxic culture did not increase total L-arginine utilization through arginase plus NOS in resident or in *C. parvum*-elicited peritoneal macrophages (Figs. 1 and 2, Tables I and II). Arginine flux in these cells appeared to be simply redirected toward arginase by the anoxic suppression of NOS (Table II).

The metabolic response of resident and *C. parvum*-elicited peritoneal macrophages to IFN-γ and LPS is also shown in Table II. Stimulation increased L-arginine utilization through NOS in resident macrophages, but failed to do so in *C. parvum*-elicited cells. The addition of *N*-MMA to IFN-γ- and LPS-treated cultures revealed that arginase activity was enhanced somewhat by these signals, but that total L-arginine utilization was, once again, markedly reduced.

Anoxic cultured macrophages demonstrate increased NOS activity during re-oxygenation

To determine whether NOS activity was recoverable following anoxic culture, wound-derived macrophages were incubated overnight in room air or 0% O_2, and then exposed to room air for 2 h. Radiolabeled L-arginine was present in the cultures during these 2 h, and its metabolism through arginase or NOS was determined by product formation. Data from one of these experiments are shown in Table III and demonstrate that wound-derived macrophages cultured in an anoxic environment actually increased their ability to metabolize L-arginine through arginase and NOS upon re-oxygenation.



Resident Peritoneal Macrophages

	O.D. (mm²)	NO_2^- (μM)
Freshly Harvested	N.D.	-
21% O_2	2.1±0.3 [a]	18.5±3.2 [a]
0% O_2	1.9±0.3 [a]	0.2±0.4 [b]

Wound Derived Macrophages

	O.D. (mm²)	NO_2^- (μM)
Freshly Harvested	0.1±0.1 [a]	-
21% O_2	0.5±0.2 [b]	48.5±0.3 [a]
0% O_2	1.9±0.3 [c]	0.9±0.2 [b]

FIGURE 3. Immunoblot analysis of NOS in resident peritoneal or wound-derived macrophages, demonstrating the accumulation of NOS protein in cells cultured in an anoxic environment. Freshly harvested or 24-h cultured resident peritoneal or wound-derived macrophages were processed, as indicated in *Materials and Methods*, and immunoblotted using a mouse mAb against NOS. *Lane 1*, freshly harvested resident peritoneal macrophages; *lane 2*, resident peritoneal macrophages after aerobic overnight culture; and *lane 3*, after anoxic culture. *Lane 4*, freshly harvested wound-derived macrophages; *lane 5*, wound-derived macrophages after aerobic overnight culture; and *lane 6*, after anoxic culture. The table contains means ± SD from densitometric readings of immunoblots from five independent experiments for resident macrophages and eight for wound-derived cells, and the corresponding concentrations of NO_2^-, a degradative product of NO, in culture supernatants at the end of culture. Within each cell type, values followed by different superscripts are different ($p < 0.05$). N.D. = not detectable.

Enhanced immunoreactive NOS protein content of wound-derived and resident peritoneal macrophages following culture

Data shown in Figure 3 indicate that freshly harvested wound-derived and resident peritoneal macrophages contained little immunoreactive NOS protein, and that NOS protein content was increased markedly following overnight culture in both cell types. In the case of wound-derived cells, immunoreactive NOS content was higher in cells subjected to anoxic culture than in those cultured aerobically. Beyond indicating a previously unrecognized effect of

Table IV. *Effect of O_2 tension on spontaneous and stimulated TNF-α release*[a]

	Resident Peritoneal Macrophages		Wound-Derived Macrophages	
Oxygen:	21%	0%	21%	0%
No additives	2.55 ± 0.30 a	3.92 ± 0.28 b	0.52 ± 0.03 a	1.26 ± 0.09 b
IFN-γ + LPS	3.45 ± 0.24 b	4.48 ± 0.25 c	3.14 ± 0.12 c	4.65 ± 0.08 d

[a] Resident peritoneal or wound-derived macrophages were cultured overnight in room air or 0% O_2. Culture media contained IFN-γ (10 U/ml) and LPS (1 μg/ml) when so indicated in the table. Immunoreactive TNF-α contained in the supernatants at the end of culture was measured using ELISA as indicated in the *Materials and Methods*. Results are nanogram of TNF-α per milliliter. Within each cell type, values followed by different letters are different ($p < 0.05$).

Table V. *Effect of O_2 tension on spontaneous and stimulated IL-6 release*[a]

	Resident Peritoneal Macrophages		Wound-Derived Macrophages	
Oxygen:	21%	0%	21%	0%
No additives	1984 ± 277 a	6626 ± 144 b	39 ± 5 a	145 ± 14 b
IFN-γ + LPS	4281 ± 312 c	9195 ± 656 d	555 ± 30 c	6626 ± 144 d

[a] Resident peritoneal or wound-derived macrophages were cultured overnight in room air or 0% O_2. Culture media contained IFN-γ (10 U/ml) and LPS (1 μg/ml) when so indicated in the table. IL-6 activity in the supernatants at the end of culture was determined using the bioassay described in the *Materials and Methods*. Results are U IL-6/ml. Within each cell type, values followed by different letters are different ($p < 0.05$).

culture on NOS protein content, these observations may provide a mechanism for the increased capacity of wound-derived cells to metabolize arginine through NOS following anoxic culture that was described in the preceding section.

Anoxic culture enhances macrophage TNF-α and IL-6 production

The marked increase in NOS protein found in anoxic wound-derived and resident peritoneal macrophages and the increase in arginase activity most prominently displayed by the wound-derived cells raised the question as to whether these effects were isolated alterations or reflections of an enhanced activation state of the cells. To address this issue, the capacity of wound-derived or resident peritoneal macrophages cultured aerobically or anaerobically to release TNF-α and IL-6 was determined. Data in Tables IV and V show the impact of O_2 availability on these parameters, demonstrating that anoxia enhanced cytokine release by both macrophage types. The addition of IFN-γ and LPS to these cultures further increased cytokine release from the cells at either O_2 tension.

Discussion

Experiments reported in this work were conducted to investigate the potential role of O_2 availability in modulating NOS activity in wound-derived macrophages. Findings exceeded this original intent in that they revealed that anoxic culture results in the enhanced expression of activation-associated traits, including increases in total utilization of L-arginine and its metabolism through arginase, cellular NOS protein content, and the release of TNF-α and IL-6 by the cells. Peritoneal macrophages did not respond to anoxic culture with increases in total L-arginine metabolism, arginase flux, or immunoreactive NOS content, but shared with wound-derived cells the anoxia-dependent enhancement in cytokine release.

Taken in order, the finding that hypoxia suppressed NOS activity was fully predictable from the known requirement of this enzyme for molecular O_2 (4, 5). Indeed, the production of L-citrulline from L-arginine was reduced in all cell types by culture in media at a pO_2 <37 torr. It was, however, unexpected to observe that total arginine consumption by wound-derived macrophages cultured in anoxic conditions was markedly higher than that in hypoxic/normoxic cultures, and that this increase was due to a >15-fold elevation in L-arginine metabolism through arginase.

Arginase and NOS compete for their common substrate, L-arginine. Hibbs, Taintor, and Vavrin (16) demonstrated that the inhibition of arginase or NOS redirects L-arginine flux toward the complementary enzyme. The increase in arginase activity detected in wound macrophages during anoxia was not a result of such redirection of L-arginine due to the anoxic suppression of NOS. It represented an actual increase in enzyme activity. This is so because total L-arginine utilization was increased simultaneously, be cause L-arginine flux through arginase exceeded the sum of fluxes through arginase and NOS in aerobic cultures, and because neither *N*-MMA nor DPI, two NOS inhibitors with markedly different mechanisms of action (13, 14) that efficiently suppressed NOS activity, was able to increase arginase flux to the level found in anoxic cultures (Table I).

The effects of anoxia on L-arginine metabolism by wound-derived cells were different from those of the classical activators IFN-γ and LPS, in that these stimuli promoted L-arginine metabolism through NOS rather than arginase. The presence of NOS inhibitors was required to reveal that arginase activity was also enhanced by IFN-γ and LPS, and that the ability of the cells to metabolize L-arginine through this enzyme was cloaked by its preferential flux through NOS.

Resident and *C. parvum*-elicited peritoneal macrophages were included in these experiments to attempt to establish whether the alterations in L-arginine metabolism found in response to anoxia in wound-derived cells were peculiar to these inflammatory macrophages. Unlike wound-derived cells, neither resident nor *C. parvum*-elicited macrophages increased their total L-arginine metabolism during anoxic culture. There was, however, a detectable increase in the amount of L-arginine processed through arginase in these cells when they were cultured in anoxic conditions. These results appear similar to those with wound-derived cells cultured with NOS inhibitors, and can be interpreted as indicating that L-arginine metabolized through NOS under aerobic conditions was shunted toward arginase in anoxic cultures (Table II).

Immunoreactive NOS was not detectable in resident peritoneal macrophages and present at very low levels in wound-derived macrophages when cells were examined immediately after harvesting. Overnight culture led to a significant increase in the cellular content of this protein in both cell types (Fig. 3). In wound macrophages, but not in resident peritoneal macrophages, anoxic culture resulted in a greater accumulation of immunoreactive NOS than normoxic culture. It has been shown, in this regard, that NO can feedback inhibit NOS activity (17). Current data may indicate that, in addition, NO can down-regulate the amount of NOS protein contained in cells. This conclusion is strengthened by recent results indicating that macrophage culture in L-arginine-deficient media prevents NO production and results in increased immunoreactive NOS content, thus mimicking the effect of O_2 deprivation (J.E.A., unpublished observations). The smaller NO production by resident peritoneal macrophages in aerobic culture could, in contrast to wound-derived cells, be insufficient to suppress the accumulation of NOS protein.

In summary, then, anoxia appears to be selective for wound-derived macrophages in its ability to increase NOS protein content and arginase activity. In addition, anoxia can also act as a nonselective stimulator of macrophage function in that it promotes cytokine release from macrophages obtained from different sites.

To correlate these findings with those reported in wounds, it is relevant to remember that wounds provide hypoxic/anoxic environments comparable with those used in these experiments (6, 7). It appears, then, reasonable to propose that the hypoxic/anoxic environment of the wound should at least prevent or reduce the production of NO through NOS, if not actually increase the arginase activity of macrophages present in the wound. These effects of O_2 availability on the metabolism of L-arginine may help explain the pattern of L-arginine metabolites found in wounds during macrophage infiltration that was reported previously (1, 2) and is described in this work.

The physiology of macrophages involved in the healing of wounds has not been fully established. Because they are obviously involved in an inflammatory response, wound macrophages are likely to share at least some of the functional and metabolic characteristics of other inflammatory macrophages. It can, however, be hypothesized that wound-associated macrophages are functionally adapted to respond to conditions found in the wound environment and to perform the specific tasks demanded by the process of repair. It is their presence in a hypoxic environment that may, among other factors, modulate their functional profile and lead to the expression of a specific phenotype different from that of macrophages found in other anatomical locations or those associated with other immune events. It does not necessarily follow that all macrophage capacities are altered distinctly by their involvement in the healing event. Evidence presented in this work indicates that at least two macrophage traits, the expression of arginase and their NOS content, are enhanced predominantly by anoxia in wound-derived macrophages. In contrast, cytokine release was found to be modulated similarly by O_2 availability in macrophages obtained from wounds and in those resident in the peritoneal cavity. Ongoing work intended to fully characterize the wound macrophage phenotype will, hopefully, lead to a better understanding of the role of the wound environment in shaping the functional profile of these cells.

Interestingly, the enhanced release of TNF-α in anoxic conditions can provide a link between the finding that anoxia promotes the production of angiogenic factors by macrophages (18), and the proposal that TNF-α may mediate such angiogenic activity (19). Whether the accelerated release of TNF-α requires, like the promotion of arginase activity, a fully anoxic environment or responds in a dose-dependent manner to decreases in ambient O_2 tensions remains to be determined. Present observations and the known hypoxic nature of the microenvironment in tumors (20), parenthetically, allow the speculative conclusion that the use of NO by macrophages as a tumoricidal molecule in vivo may be limited by O_2 availability.

Finally, it does not escape the authors' attention that the expression of NOS by human macrophages remains controversial (21). The relevance of current findings to the healing of human wounds will have to await the clarification of the specifics of arginine metabolism in human cells.

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