Nitric Oxide Derived from L-Arginine Impairs Cytoplasmic pH Regulation by Vacuolar-type H+ ATPases in Peritoneal Macrophages

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Summary

The ability of macrophages (Møs) to function within an acidic environment has been shown to depend on cytoplasmic pH (pH_i) regulation by vacuolar-type H^+ ATPases. Møs metabolize L-arginine via an oxidative pathway that generates nitric oxide, nitrate, and nitrite. Since each of these products could potentially inhibit vacuolar-type H^+ ATPases, we investigated the effect of L-arginine metabolism on Mø pH_i regulation in thioglycolate-elicited murine peritoneal Møs. H^+ ATPase-mediated pH_i recovery from an imposed cytoplasmic acid load was measured fluorometrically. When Møs were incubated with L-arginine (0.25–2.0 mM), their rate of pH_i recovery declined progressively from 2 to 6 h of incubation. By contrast, the recovery rate of cells incubated in arginine-free medium remained stable over the same period. The impairment of pH_i recovery was specific for L-arginine, and was blocked competitively by N^G-monomethyl-L-arginine, demonstrating its dependence on L-arginine metabolism. In addition, the inhibition of pH_i recovery was enhanced by lipopolysaccharide, an agent known to stimulate L-arginine metabolism by Møs. Scavenging the L-arginine metabolite nitric oxide with either ferrous sulphate or ferrous myoglobin prevented the inhibition of pH_i recovery, implying that L-arginine-derived nitric oxide was the species responsible for the inhibition. This concept was supported by the finding of elevated nitrite levels in the supernatant of cells incubated in L-arginine. Furthermore, incubation of Møs with sodium nitroprusside mimicked the L-arginine-dependent inhibition of H^+ ATPase activity. Treatment with the cyclic GMP analogue, 8-bromoguanosine 3':5'-cyclic monophosphate, similarly impaired Mø pH_i recovery, suggesting that a nitric oxide–stimulated elevation of cyclic GMP may contribute to the L-arginine-dependent inhibition of pH_i regulation.

Maintenance of cytoplasmic pH (pH_i)[1] within a narrow physiological range is crucial to the optimal function of mammalian cells. In macrophages (Møs), the cytoplasmic compartment is particularly at risk for acid loading, due to both increased metabolic acid generation during cell activation, and exposure to low extracellular pH (pH_o) within the microenvironment of abscesses, tumors, or wounds (1–4). To offset these challenges to their internal milieu, Møs have evolved several mechanisms of pH_i regulation. The best described of these are: (*a*) the Na^+/H^+ antiport, which exchanges extracellular Na^+ for intracellular H^+ (5–8); and (*b*) the Na^+-dependent HCO_3^-/Cl^- exchanger, which exchanges extracellular Na^+ and HCO_3^- for intracellular Cl^- and possibly H^+ (9, 10). Both of these mechanisms serve to prevent cytoplasmic acidification when pH_o is in the physiological range; however, both Na^+/H^+ exchange and Na^+-dependent HCO_3^-/Cl^- exchange become compromised in an acidic milieu, due to low pH_o and extracellular HCO_3^- depletion, respectively (11, 12).

We have previously demonstrated that Møs can recover from cytoplasmic acid loading by an additional, efficient mechanism that maintains pH_i in the physiological range even under acidic extracellular conditions (13): this mechanism extrudes protons from the cytoplasmic space into the extracellular medium in an ATP-dependent fashion (7, 14). The inhibitor sensitivity profile, ionic dependence, and electrogenic properties of this mechanism are consistent with the concept that proton extrusion is mediated by plasmalemmal vacuolar-type H^+ ATPases (15, 16). This Na^+- and HCO_3^--indepen-

[1] *Abbreviations used in this paper:* BCECF, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein; IBMX, 3-isobutyl-1-methyl-xanthine; Mø, macrophage; N-MMA, N^G-monomethyl-L-arginine; pH_i, cytoplasmic pH; pH_o, extracellular pH.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/11/1009/13 $2.00
Volume 174 November 1991 1009–1021

dent mechanism is inhibited by exposure of Møs to nitrate or to sulfhydryl reagents such as *N*-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (16). In addition, proton extrusion by this mechanism is exquisitely sensitive to the macrolide antibiotic bafilomycin A_1, which has been shown to be a potent and specific inhibitor of vacuolar-type H^+ ATPases (17).

Several recent studies have defined the ability of Møs to metabolize L-arginine via an oxidative pathway that yields L-citrulline as an end product (18, 19). A by-product of this metabolic pathway is the highly reactive species nitric oxide, which undergoes decomposition in the presence of O_2 and H_2O to produce nitrite and nitrate (20–22). Nitric oxide is thought to be an effector molecule of several important Mø functions, including tumor cytotoxicity and antimicrobial activity (23–29). These toxic effects appear to be mediated through inhibition of a variety of iron sulfur–dependent enzymes by nitric oxide. In tumor cells, this leads to an impairment of mitochondrial respiration, with resultant depletion of intracellular energy stores. The stable end product nitrite also exerts antimicrobial activity, through inhibition of enzymes containing essential sulfhydryl groups (30, 31).

Møs themselves have been found to be susceptible to the toxic effects of L-arginine metabolism. Incubation with L-arginine at concentrations typically present in commercial media (0.4–1.14 mM) results in inhibition of Mø phagocytosis, superoxide production, and cytotoxicity (32). In addition, inhibition of mitochondrial respiration occurs in Møs incubated with L-arginine (33). This could potentially lead to depletion of energy stores in the Møs themselves, as it does in target tumor cells. Because vacuolar-type H^+ ATPases can be inhibited by ATP depletion, by exposure to nitrate, or by a variety of sulfhydryl reagents, it is conceivable that, through one or more of these mechanisms, L-arginine metabolism might modulate pH_i regulation by vacuolar-type H^+ ATPases in Møs. To test this possibility, we investigated the effect of L-arginine on H^+ ATPase-mediated pH_i regulation in thioglycolate-elicited peritoneal Møs.

Materials and Methods

Materials and Solutions. Calcium- and magnesium-free HBSS and RPMI 1640 select-amine kit medium were obtained from Gibco Laboratories (Grand Island, NY). Heparin sodium) (1,000 USP U/ml) was from Organon Canada. Powdered brewer's thioglycolate medium and LPS B (*Escherichia coli* O.111:B4) were obtained from Difco Laboratories (Detroit, MI), and ferrous sulphate was from Fisher Scientific Co. (Pittsburgh, PA). Hepes, L-arginine, D-arginine, L-homoarginine, L-citrulline, L-ornithine, L-arginase (from bovine liver), L-ascorbic acid, myoglobin (from horse skeletal muscle), sodium nitroprusside, sodium nitrite, sulfanilamide, naphthylethylene diamine dihydrochloride, 3-isobutyl-1-methylxanthine (IBMX), and 8-bromoguanosine 3′:5′-cyclic monophosphate (sodium salt) were from Sigma Chemical Co. (St. Louis, MO). Nigericin, N^G-monomethyl-L-arginine (N-MMA), and luciferin/luciferase ATP assay kits were purchased from Calbiochem Behring Corp. (La Jolla, CA). The cGMP assay kits were from Amersham Corp. (Arlington Heights, IL). The acetoxymethyl ester of 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR). Bafilomycin A_1 was the kind gift of Prof. K. Altendorf (Universtät Osnabrück; see reference 17).

RPMI select-amine kit medium was normally reconstituted without adding bicarbonate, although 25 mM sodium bicarbonate was included in medium used to enrich Møs by adherence. L-arginine was omitted or added at the concentration indicated. All other components provided were included in the reconstituted medium. Hepes-RPMI was prepared by titrating HCO_3^--free RPMI with 20 mM Na-Hepes to pH 7.35 at 37°C. KCl medium contained 145 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.35, at 37°C. The osmolarity of KCl medium was adjusted to 290 ± 5 mosM with concentrated KCl. Thioglycolate medium was solubilized in H_2O and stored in the dark at 22°C until uniformly green.

Cell Isolation and Characterization. Peritoneal cells were harvested from 6–8-wk-old female Swiss Webster mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) by lavage with 10 ml HBSS containing 10 U/ml heparin sodium, then washed twice with cold HBSS (5°C), and counted using a cell counter (model Z_F; Coulter Immunology, Hialeah, FL). Resident Møs were obtained from untreated mice, while thioglycolate-elicited Møs were obtained by lavage 4 d after intraperitoneal injection with 2 ml of thioglycolate medium. The proportion of peritoneal cells identified as Møs by Wright's staining was consistently 80–85% for thioglycolate-elicited cells, and 20–25% for resident cells. After washing, thioglycolate-elicited cells were resuspended in Hepes-RPMI at 10^7 cells/ml for cell suspension experiments.

For some experiments, Møs populations were enriched by adherence. Peritoneal cells in arginine-free Hepes-RPMI including 25 mM sodium bicarbonate were loaded onto glass coverslips (Bellco Glass, Inc., Vineland, NJ) to achieve a final number of ~2×10^6/coverslip. The coverslips were incubated for 2 h at 37°C in 5% CO_2, then washed twice with warm HBSS (37°C). Mø enrichment to >93% was confirmed by Wright's stain for both resident and thioglycolate-elicited cells. The viability of both adherent cells and cells in suspension was >95% as assessed by trypan blue exclusion.

Incubation Conditions. For cell suspension experiments, 1-ml aliquots of thioglycolate-elicited peritoneal cells (10^7 cells/ml Hepes-RPMI with or without L-arginine, as indicated) were incubated in a water bath at 37°C in room air. Cells were gently agitated every 15 min throughout the incubation period. After the indicated incubation period (2–6 h), a sample of the cell suspension was obtained for cytoplasmic acid loading and subsequent measurement of pH_i or acid extrusion, or for measurement of intracellular ATP or cGMP. For some experiments, resident or thioglycolate-elicited Møs that had been enriched by adherence were incubated for a further 4 h in the presence of 10 μg/ml LPS at 37°C in room air in bicarbonate-free Hepes-RPMI, which was either arginine-free or contained 1.14 mM L-arginine. pH_i was then measured at the 4-h time point, as described below.

pH_i Measurement and Manipulation. pH_i was measured using the pH-sensitive fluorescent probe, BCECF. For cell suspension experiments, 2×10^6 thioglycolate-elicited cells were loaded with dye at the indicated time point by a 20-min incubation in the same medium with 2 μg/ml of the precursor acetoxymethyl ester at 37°C. Cytoplasmic acid loading was accomplished by simultaneous incubation with 40 mM NH_4Cl during this 20-min period, followed by sedimentation and resuspension in 2 ml NH_4^+-free KCl medium in a fluorometer cuvette. The principles of the NH_4^+ "prepulse" technique of cytoplasmic acid leading are described elsewhere (8, 11). The regimen used in these studies consistently yielded

acid loading to a pH_i of ~6.4. Coverslip-adherent cells were loaded with dye by a 20-min incubation in the same medium to which 2 μg/ml of the precursor acetoxymethyl ester was added. Cytoplasmic acid loading was achieved by simultaneous incubation with 40 mM NH_4Cl during the whole or latter part of this 20-min period, followed by placement of the coverslip in 2 ml of NH_4^+-free KCl medium in a fluorometer cuvette, so that the surface of the coverslip was oriented at 45° to the incident excitation beam (8). A 20-min incubation with NH_4Cl resulted in acid loading to a pH_i of ~6.10 in adherent resident Møs, whether they had been incubated with or without L-arginine. In the case of thioglycolate-elicited adherent Møs, it was necessary to adjust the length of the incubation with NH_4Cl to obtain equivalent acid loading in cells that had been incubated with and without L-arginine. Specifically, to achieve acid loading to a pH_i of ~6.80, Møs that had been incubated in arginine-free medium were treated with NH_4Cl for 20 min, whereas Møs that had been incubated in L-arginine-containing medium required only 9.5 min of exposure to NH_4Cl. Fluorescence was determined using a fluorescence spectrometer (650-40, LS-5, or LS-50; Perkin-Elmer Corp., Norwalk, CT) with excitation at 495 nm and emission at 525 nm using 5- and 10-nm slits, respectively. Calibration was done after addition of the K^+/H^+ ionophore nigericin (1 μM), using aliquots of concentrated 2-[morpholino] ethanesulfonic acid or Trizma base, as described (12). Recording of fluorescence commenced immediately upon transfer of cells to KCl medium, and the initial rate of pH_i recovery was measured.

Measurement of Acid Extrusion. Acid efflux was detected by measuring pH_o with a conventional combination pH electrode in a water-jacket chamber containing 2 ml of KCl medium prepared as above but without Hepes, to minimize the buffering power. After incubation in Hepes-RPMI with or without L-arginine, 2×10^6 cells were sedimented, resuspended in ~20 μl of the same medium, and added to the unbuffered KCl medium, under continuous magnetic stirring. pH_o was adjusted to 7.35 at 37°C by addition of KOH, and the change of pH_o was then monitored. Calibration was done by addition of known amounts of KOH and HCl, as described (14).

Nitrite Analysis. Thioglycolate-elicited Møs were incubated for 4 h at 37°C in Hepes-RPMI (10^7 cells/ml) in the absence or presence of 1.14 mM L-arginine. Cells were pelleted and the nitrite content of the supernatants determined in duplicate by reacting samples (100 μl) with the Griess reagent (50 μl of 0.1% naphthylethylene diamine dihydrochloride in H_2O plus 50 μl 1% sulfanilamide in 5% H_3PO_4) for 10 min in a microplate well (note reference 34). Absorbance at 540 nm was then measured, and nitrite concentrations were determined from a linear standard curve between 0 and 64 μM sodium nitrite.

Measurement of Intracellular ATP. Extraction was performed by addition of 0.8 ml ice cold perchloric acid (8%) to 5×10^6 pelleted cells. This extract was neutralized with 0.45 ml of ice-cold K_2CO_3 (1 M), and clarified by centrifugation. The ATP content of the cellular extracts was determined using an ATP assay kit (Calbiochem-Behring Corp.). The assay is based on the firefly luciferase-catalyzed oxidation of D-luciferin in the presence of ATP, the concentration of which is quantitated by the amount of light produced. The results were expressed as nanomoles of ATP per 10^7 cells.

Measurement of Intracellular cGMP. Thioglycolate-elicited Møs were incubated for 4 h at 37°C in Hepes-RPMI (10^7 cells/ml) in the absence or presence of 1.14 mM L-arginine. For the final 20 min of the incubation period, IBMX (100 μM) was added. Extraction was performed by addition of ice-cold ethanol to cell suspensions to give a final suspension volume of 65% ethanol. Supernatants were clarified by centrifugation at 4°C and evaporated in a vacuum oven. The cGMP content of the samples was then determined in duplicate, by RIA (Amersham Corp.).

Statistics. Results are presented as means ± 1 SE of *n* experiments. Statistical significance was established by one-way ANOVA followed by Newman-Keuls multiple intergroup comparisons or by *t* test where indicated.

Results

Inhibitory Effect of L-Arginine on H^+ ATPase-mediated pH_i Recovery. To examine pH_i regulation by H^+ ATPases, recovery from cytoplasmic acid loading was studied in Na^+- and HCO_3^--free KCl medium, where neither Na^+/H^+ exchange nor HCO_3^-/Cl^- exchange could restore pH_i. Under these conditions, the pH_i of thioglycolate-elicited Møs in suspension nevertheless recovers rapidly, as illustrated in Fig. 1 *A*. Between 85 and 90% of this pH_i recovery is mediated

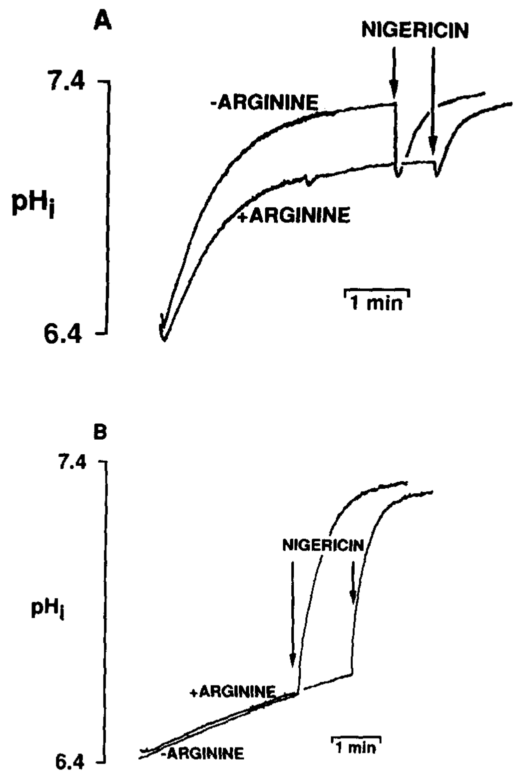

Figure 1. Effect of L-arginine on H^+ ATPase-mediated pH_i recovery of acid-loaded macrophages. pH_i was measured fluorometrically using the probe BCECF. Freshly harvested thioglycolate-elicited peritoneal cells were incubated for 4 h at 37°C in L-arginine–containing (*lower trace* in *A*, *upper trace* in *B*) or arginine-free (*upper trace* in *A*, *lower trace* in *B*) bicarbonate-free Hepes-RPMI (10^7 cells/ml). Cells were then acid loaded by the NH_4^+ prepulse method by preincubating 2×10^6 cells for 20 min at 37°C in the same medium, to which NH_4Cl was added (40 mM, final concentration); this was done in the absence (*A*) or presence (*B*) of bafilomycin A_1 (100 nM). Traces begin immediately upon resuspension of cells in 2 ml of NH_4^+-free KCl medium in a fluorometer cuvette. In each trace, nigericin (1 μM) was added where indicated by the arrows, for purposes of calibration. Traces are representative of four experiments. Temperature was 37°C.

by vacuolar-type H^+ ATPases, as indicated by its sensitivity to nanomolar concentrations of bafilomycin A_1, the potent, specific inhibitor of vacuolar-type H^+ ATPases (compare Fig. 1 *B* *A*; see references 15 and 17).

The NH_4^+ prepulse technique achieved a comparable degree of acid loading in Møs incubated with and without L-arginine (Fig. 1 *A*). Incubation for 4 h in L-arginine-containing medium reduced the initial rate of Na^+- and HCO_3^--independent pH_i recovery compared to incubation in arginine-free medium (0.67 ± 0.04 and 0.84 ± 0.02 pH/min, respectively; $n = 4$; $p < 0.05$; Fig. 1 *A*). This suggested that incubation with L-arginine was capable of inhibiting H^+ ATPase-mediated pH_i recovery. The inhibitory effect of L-arginine on pH_i recovery of thioglycolate-elicited cells was also observed in adherence-enriched Mø cell populations, where pH_i recovery rates were 0.20 ± 0.02 vs. 0.13 ± 0.02 pH/min in cells incubated in arginine-free vs. arginine-containing media, respectively ($p < 0.05$; $n = 6$). It should be noted that in adherent cells, the initial pH_i recovery rate appears slower than in suspended cells. This apparent discrepancy is due to the fact that the adherent cells were acidified to pH 6.80 vs. pH 6.40 for suspended cells (see Materials and Methods). Previous studies have shown that the initial rate of pH_i recovery mediated by H^+ ATPases is directly proportional to the magnitude of the imposed acid load (C. J. Swallow et al., unpublished observations).

In both suspended and plated Møs, a small portion (10–15%) of total Na^+- and HCO_3^--independent pH_i recovery was not mediated by vacuolar-type H^+ ATPases, as determined by its insensitivity to bafilomycin A_1 (15). It was therefore conceivable that incubation with L-arginine inhibited total pH_i recovery by reducing the bafilomycin-insensitive component of this recovery. To rule out this possibility, the effect of L-arginine on Na^+- and HCO_3^--independent pH_i recovery was studied in suspended cells treated with bafilomycin A_1. As shown in Fig. 1 *B*, L-arginine had no effect on the bafilomycin-insensitive component of the pH_i recovery (the recovery rates in bafilomycin-treated cells were 0.09 ± 0.02 and 0.10 ± 0.02 pH/min after incubation without and with L-arginine, respectively; $n = 4$); this result is consistent with the conclusion that L-arginine impairs H^+ ATPase-mediated pH_i regulation.

The time course of the inhibition of pH_i recovery by L-arginine is shown in Table 1. The initial rate of pH_i recovery from acid loading declined progressively from 2 to 6 h in Møs incubated in the presence of 1.14 mM L-arginine. By contrast, when Møs were incubated in arginine-free medium, the rate of pH_i recovery from acid loading remained stable. Mø viability was >90% over the 6-h incubation period, both with and without L-arginine, with no difference between the two incubation conditions. Thus, the presence of L-arginine in the incubation medium appeared to be associated with a progressive inhibition of H^+ ATPase-mediated pH_i recovery.

Two alternative explanations for this apparent inhibition of H^+ pump activity were considered. First, incubation with L-arginine might increase Mø buffering capacity. If this were the case, for the same amount of acid extruded by H^+ ATPases, the rate of pH_i recovery would appear reduced. This possibility seemed unlikely, since the same NH_4^+ prepulse conditions resulted in comparable acid loading in suspended cells incubated with and without L-arginine. Second, incubation with L-arginine might conceivably increase the rate of continuous metabolic acid generation sufficiently to slow the rate of pH_i recovery from a superimposed acute

Table 1. *Effect of L-Arginine on Macrophage Cytoplasmic pH Recovery*

Medium	LPS	Initial rate of cytoplasmic pH recovery* 2 h	4 h	6 h
			pH/min	
+ L-Arginine	−	0.78 ± 0.07	0.67 ± 0.04‡	0.51 ± 0.03‡§
Arginine-free	−	0.82 ± 0.04	0.84 ± 0.02‖	0.84 ± 0.02‖
+ L-Arginine	+	0.45 ± 0.04‖	0.46 ± 0.03‖	0.47 ± 0.05
Arginine-free	+	0.76 ± 0.02¶	0.72 ± 0.02¶	0.77 ± 0.03¶

* Freshly harvested thioglycolate-elicited peritoneal cells were incubated at 37°C for the indicated time period in bicarbonate-free Hepes-RPMI (10^7 cells/ml) with or without 1.14 mM L-arginine, in the presence or absence of LPS (10 μg/ml). Cells were then acid loaded by the NH_4^+ prepulse method by incubating 2 × 10^6 cells for 20 min at 37°C in the same medium, to which NH_4Cl (40 mM final concentration) was added. The initial rate of cytoplasmic pH recovery after resuspension in NH_4^+-free KCl medium was then measured fluorometrically. Results are expressed as means ± 1 SE of three to four experiments.
‡ $p < 0.05$ vs. t = 2 h.
§ $p < 0.05$ vs. t = 4 h.
‖ $p < 0.05$ vs. + L-arginine without LPS.
¶ $p < 0.02$ vs. + L-arginine with LPS.

Table 2. *Effect of L-Arginine on the Rate of Acid Extrusion by Acid-loaded Macrophages*

Medium	Rate of Acid Extrusion* 2 h	4 h	6 h
		nmol/min/10⁶ cells	
+ L-Arginine	9.27 ± 1.41	6.42 ± 1.04	5.33 ± 0.33‡
Arginine-free	9.18 ± 0.87	8.14 ± 0.75	8.46 ± 0.77

* Freshly harvested thioglycolate-elicited peritoneal cells were incubated at 37°C for the indicated time period in bicarbonate-free Hepes-RPMI (10^7 cells/ml) with or without 1.14 mM L-arginine. Cells were then acid loaded by the NH_4^+ prepulse method by incubating 2 × 10^6 cells for 20 min at 37°C in the same medium, to which NH_4Cl (40 mM final concentration) was added. The initial rate of acid extrusion after resuspension in NH_4^+-free KCl medium was then measured using a conventional pH electrode. Results are means ± 1 SE of four experiments.
‡ $p < 0.05$ vs. t = 2 h, and vs. arginine-free.

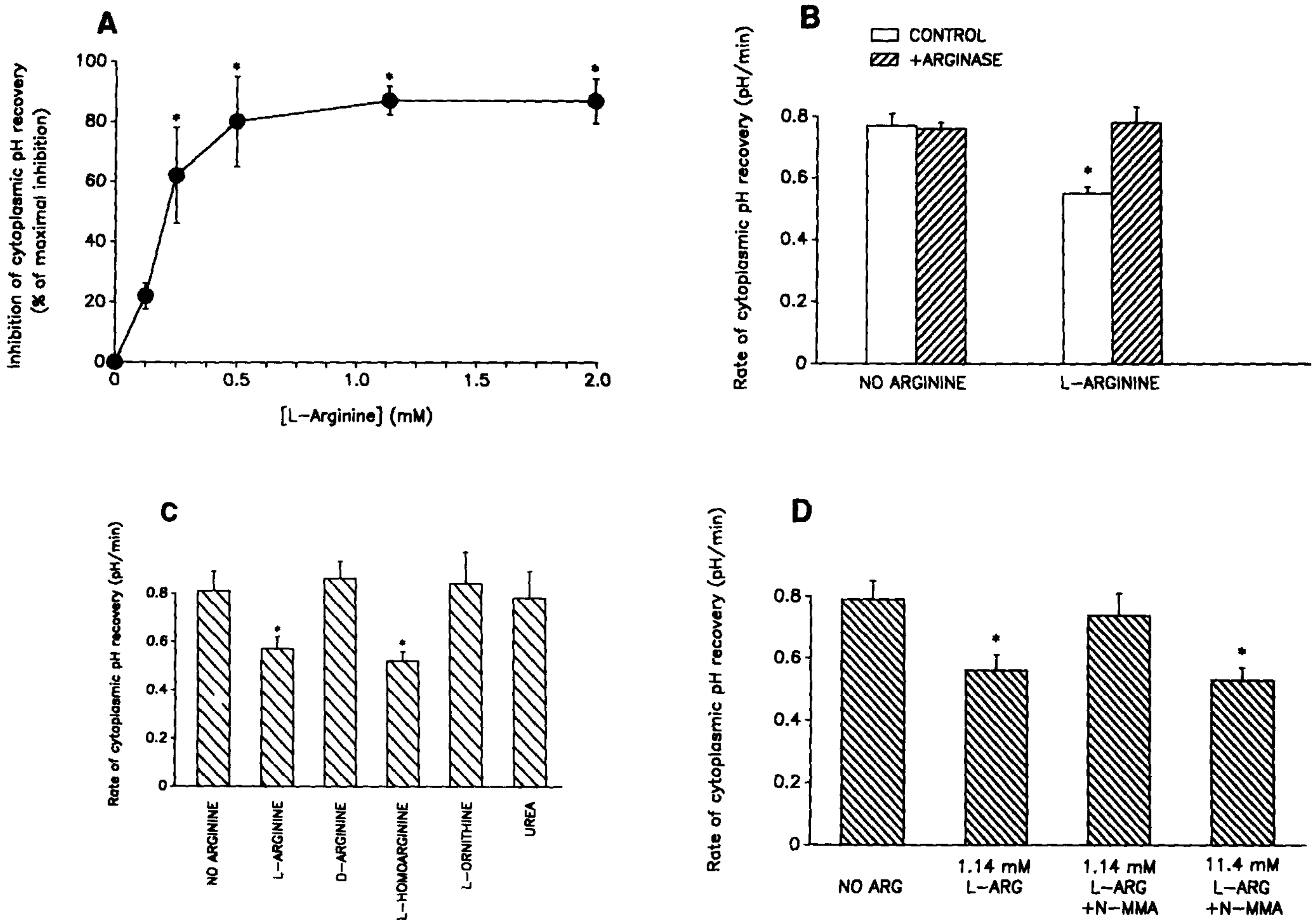

Figure 2. Effect of L-arginine, D-arginine, L-homoarginine, L-ornithine, urea, L-arginase, or N-MMA on pH_i recovery of acid-loaded macrophages. Thioglycolate-elicited peritoneal cells were incubated for 4 h at 37°C in bicarbonate-free Hepes-RPMI (10^7 cells/ml) in the presence or absence of L-arginine, with or without the indicated compounds. After this incubation, 2×10^6 cells were incubated for a further 20 min in the same medium, in the presence of 40 mM NH_4Cl. Cells were then resuspended in NH_4^+-free KCl medium, resulting in acid loading to pH_i ~6.4, as illustrated in Fig. 1. After calibration, the initial rate of pH_i recovery was calculated. (*A*) Cells were incubated for 4 h with various concentrations of L-arginine. The inhibition of pH_i recovery associated with each concentration of L-arginine is expressed as a percentage of the maximal inhibition observed on each given day. The mean maximal inhibition was 0.33 ± 0.06 pH/min; $n = 5$. $^*p < 0.05$ vs. no L-arginine. (*B*) Cells were incubated for 4 h with or without 1.14 mM L-arginine, in the absence or presence of L-arginase (10 U/ml) plus $MnCl_2 \cdot 4H_2O$ (10 μg/liter). $^*p < 0.01$ vs. no L-arginase, and vs. 1.14 mM L-arginine plus arginase; $n = 3$. (*C*) Cells were incubated for 4 h in medium containing no L-arginine or 1.14 mM of either L-arginine, D-arginine, L-homoarginine, L-ornithine, or urea. $^*p < 0.05$ vs. no L-arginine, vs. D-arginine, vs. L-ornithine, and vs. urea; $n = 5$. (*D*) Cells were incubated for 4 h in medium containing no L-arginine or L-arginine at a concentration of 1.14 or 11.4 mM, with or without 0.1 mM N-MMA, as indicated. $^*p < 0.05$ vs. no L-arginine and vs. 1.14 mM L-arginine plus 0.1 mM N-MMA; $n = 4$.

acid load. To determine whether either of these possibilities were responsible for the apparent inhibition of H^+ ATPase activity, the rate of acid extrusion into the extracellular medium by acid-loaded Møs was measured directly. Table 2 shows the rate of acid extrusion into unbuffered KCl medium by acid-loaded Møs, which had been incubated in either L-arginine-containing or arginine-free medium. Incubation with L-arginine resulted in a progressive reduction in the rate of acid extrusion by acid-loaded Møs over the experimental period, while this rate remained relatively stable in cells incubated in arginine-free medium. The parallel reduction in the rates of acid extrusion and pH_i recovery implies that the effect of L-arginine is due to inhibition of H^+ pump activity, rather than increased cytoplasmic buffering capacity or enhanced metabolic acid production.

To better define the role of L-arginine in modulating Mø pH_i recovery, the effect of incubation with varying doses of L-arginine was examined (Fig. 2 *A*). The inhibitory effect of each concentration of L-arginine on the initial rate of Na^+- and HCO_3^--independent pH_i recovery is expressed as a percentage of the maximal inhibition observed on each given day (maximal inhibition was defined as the difference between the rates of recovery in Møs incubated without L-arginine and Møs incubated with the most inhibitory concentration of L-arginine). The rate of pH_i recovery varied inversely with the concentration of L-arginine in the incubation medium.

pH_i recovery was significantly impaired by incubation with concentrations of L-arginine as low as 0.25 mM (0.61 ± 0.06 pH/min vs. 0.83 ± 0.09 pH/min in arginine-free medium; $n = 5$; $p < 0.05$). Incubation with 2 mM L-arginine did not result in any further reduction of the pH_i recovery rate at 4 h compared to incubation with 1.14 mM L-arginine (0.53 ± 0.05 vs. 0.55 ± 0.05 pH/min, respectively).

Inhibition of pH_i Recovery Is Specific for L-Arginine. To confirm that the inhibition of pH_i recovery was due specifically to the presence of L-arginine in the incubation medium, rather than to a contaminant, the effect of exogenous L-arginase on pH_i recovery was studied. This enzyme depletes L-arginine by converting it to L-ornithine plus urea. As shown in Fig. 2 *B*, 10 U/ml L-arginase plus $MnCl_2 \cdot 4H_2O$ (an arginase activator, 10 μg/liter) reversed the inhibitory effect of L-arginine on pH_i recovery ($n = 3$; $p < 0.01$). By contrast, addition of 10 U/ml L-arginase to arginine-free medium had no effect on the pH_i recovery rate, thereby ruling out a nonspecific stimulatory effect of arginase on pH_i recovery.

Møs themselves can release significant amounts of endogenous L-arginase (35). This suggested the possibility that the inhibition of pH_i recovery might be mediated by L-ornithine or urea derived from L-arginine through the action of endogenous L-arginase. The ability of exogenous L-arginase to reverse the inhibition, rather than accentuate it, made it unlikely that the generation of L-ornithine or urea was responsible for the inhibition. To directly rule out this possibility, Møs were incubated in arginine-free medium supplemented with 1.14 mM of either L-ornithine or urea. As illustrated in Fig. 2 *C*, neither compound had any effect on pH_i recovery compared to arginine-free medium alone. When considered together, these data provide strong evidence that the inhibitory effect was specifically related to the presence of L-arginine in the incubation medium.

To investigate the substrate specificity of the inhibition of H^+ ATPase-mediated pH_i recovery, the effect of incubation with the D-stereoisomer of arginine was compared to that of incubation with L-stereoisomer of arginine was compared to that of incubation with L-arginine itself (Fig. 2 *C*). The rate of pH_i recovery after a 4-h incubation with D-arginine did not differ from that observed in Møs incubated in the absence of arginine (0.86 ± 0.07 vs. 0.81 ± 0.08 pH/min, respectively; $n = 5$). By contrast, incubation with L-arginine reduced the pH_i recovery rate to 0.57 ± 0.05 pH/min ($n = 5$; $p < 0.05$ vs. D-arginine). These results indicated that the inhibition of H^+ ATPase activity had a specific requirement for the L-stereoisomer of arginine, likely through its oxidative metabolism.

Inhibition of pH_i Recovery Requires Metabolism of L-Arginine. A variety of cell types, including Møs, have been shown to metabolize L-arginine through a series of oxidative reactions that yield L-citrulline, nitrite, and nitrate as stable end products (18, 19, 22, 36). It was hypothesized that the L-arginine-dependent inhibition of pH_i recovery required metabolism of L-arginine via this pathway. This was suggested by the fact that the nonmetabolizable D-stereoisomer of arginine had no such inhibitory effect. However, it was still possible that the inhibition of H^+ ATPase-mediated pH_i recovery was due to the presence of extracellular L-arginine, rather than to its metabolism by Møs. To further investigate the requirement for metabolism, the effect of L-homoarginine (32, 36) on pH_i recovery was examined. As illustrated in Fig. 2 *C*, this metabolizable analogue mimicked the inhibitory effect of L-arginine on pH_i recovery.

Further evidence that metabolism of L-arginine was required for the inhibition of pH_i recovery was obtained from experiments using N-MMA, a potent, specific, competitive inhibitor of L-arginine metabolism. The effect of coincubation with N-MMA in the presence of L-arginine was studied (Fig. 2 *D*). N-MMA prevented the inhibition of pH_i recovery induced by L-arginine: the rate of pH_i recovery after incubation in the presence of 1.14 mM L-arginine with 0.1 mM N-MMA was comparable to that seen in cells incubated in arginine-free medium (0.74 ± 0.07 and 0.79 ± 0.06 pH/min, respectively; $n = 4$), while the pH_i recovery rate in Møs incubated with 1.14 mM L-arginine alone was 0.56 ± 0.05 pH/min ($n = 4$; $p < 0.05$ vs. 1.14 mM L-arginine plus N-MMA). The ability of N-MMA to prevent the L-arginine-dependent inhibition of pH_i recovery was overcome by incubation with a higher concentration of L-arginine: the rate of pH_i recovery of Møs incubated for 4 h in the presence of 0.1 mM N-MMA was 0.53 ± 0.04 pH/min when 11.4 rather than 1.14 mM L-arginine was included in the incubation medium (Fig. 2 *D*). This indicated that N-MMA prevented the inhibition of pH_i recovery by competing with L-arginine. The demonstration of competitive inhibition by N-MMA confirmed that the inhibitory effect of incubation with L-arginine on pH_i recovery was mediated through metabolism of L-arginine.

Enhancement of L-arginine-dependent Inhibition of pH_i Recovery by LPS. Treatment of Møs with LPS is known to stimulate metabolism of L-arginine to L-citrulline, and thus to enhance release of the highly reactive metabolite, nitric oxide, as well as of nitrite/nitrate (19, 22, 37). We therefore hypothesized that the L-arginine-dependent inhibition of H^+ ATPase-mediated pH_i recovery might be enhanced by LPS. To test this possibility, the effect of LPS (10 μg/ml) on pH_i recovery was examined (Table 1). Mø viability remained >88% over the course of 6 h of incubation with 10 μg/ml LPS, both in the presence and absence of L-arginine, and did not differ between groups. pH_i recovery after incubation for 2 h in the presence of 1.14 mM L-arginine was markedly reduced in LPS-treated cells compared to control cells (0.45 ± 0.04 vs. 0.78 ± 0.07 pH/min, respectively; $n = 3$; $p < 0.001$). By contrast, when Møs were incubated for 2 h in arginine-free medium, the rate of pH_i recovery of LPS-treated cells approximated that seen in control cells. This demonstrated the L-arginine dependence of the LPS-mediated inhibition of pH_i recovery. The difference in pH_i recovery rates between LPS-treated Møs incubated with and without L-arginine persisted after 4 h (Table 1), demonstrating the ongoing L-arginine dependence of the LPS-induced inhibition of pH_i recovery. By 6 h, the mean pH_i recovery rate in

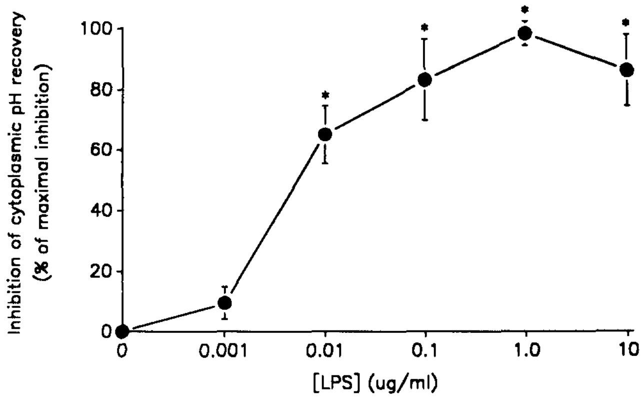

Figure 3. Effect of LPS on L-arginine-dependent inhibition of H^+ ATPase-mediated pH_i recovery of acid-loaded macrophages. Thioglycolate-elicited peritoneal cells were incubated for 2 h at 37°C in Hepes-RPMI (10^7 cell/ml) with 1.14 mM L-arginine, without or with various concentrations of LPS. 2×10^6 cells were then incubated for a further 20 min in the same medium, in the presence of 40 mM NH_4Cl. The initial rate of pH_i recovery after resuspension in NH_4^+-free KCl medium was then measured fluorometrically. The inhibition of pH_i recovery associated with each incubation condition is expressed as a percentage of the maximal inhibition observed on each given day. The mean maximal inhibition was 0.31 ± 0.04 pH/min; $n = 4$. $^*p < 0.05$ vs. no LPS.

Møs incubated with L-arginine but without LPS approximated that seen in cells incubated with both (0.51 ± 0.03 and 0.47 ± 0.05 pH/min, respectively; $n = 4$).

The ability of various concentrations of LPS to promote L-arginine-dependent inhibition of pH_i recovery was studied in Møs incubated in the presence of 1.14 mM L-arginine for 2 h (Fig. 3). Incubation with as little as 10 ng/ml LPS resulted in a significant inhibition of pH_i recovery compared to control cells incubated with L-arginine but without LPS (0.53 ± 0.06 vs. 0.75 ± 0.06 pH/min, respectively; $n = 4$; $p < 0.05$). There was no further enhancement of the inhibition when the concentration of LPS was increased beyond 0.1 μg/ml.

Nitric Oxide Mediates the L-Arginine-dependent Inhibition of pH_i Recovery. Since Mø metabolism of L-arginine leads to the generation of L-citrulline, nitrite, and nitrate as stable end products (18–20, 22), the possibility that one of these metabolites was responsible for the inhibition of H^+ ATPase-mediated pH_i regulation was examined. Neither nitrate nor L-citrulline (up to 10 mM) was able to inhibit pH_i recovery (data not shown).

The short-lived intermediate nitric oxide reacts with and inhibits a variety of enzymes, including several in the citric acid cycle and the mitochondrial electron transport chain (18, 25–27). We hypothesized that nitric oxide was the L-arginine metabolite responsible for the observed inhibition of H^+ ATPase-mediated pH_i recovery. Several approaches were taken to test this hypothesis. First, the effect of scavenging nitric oxide on the L-arginine-dependent inhibition of pH_i recovery was examined. A combination of ferrous sulphate (100 μM) and ascorbate (1 mM) was used to generate the nitric oxide scavenger, superoxide (23). Superoxide anions avidly react with nitric oxide, markedly shortening its pharmacologic half-life (38, 39).

As shown in Fig. 4 *A*, simultaneous incubation with this scavenging system reversed the inhibitory effect of L-arginine plus LPS on pH_i recovery. Ascorbate alone had no such effect. In addition, ferrous sulphate plus ascorbate had no effect on the pH_i recovery of cells incubated in arginine-free medium, arguing against a nonspecific stimulatory effect of ferrous sulphate and ascorbate on pH_i recovery. A second nitric oxide scavenging system, with a different mode of action, was also tested. When kept in the ferrous state, heme proteins such as hemoglobin and myoglobin avidly bind and inactivate nitric oxide (23, 40, 41). Fig. 4 *B* illustrates the effect of coincubation with myoglobin and ascorbate on the pH_i recovery of Møs incubated with L-arginine and LPS (10 μg/ml). This scavenging system was similarly effective in

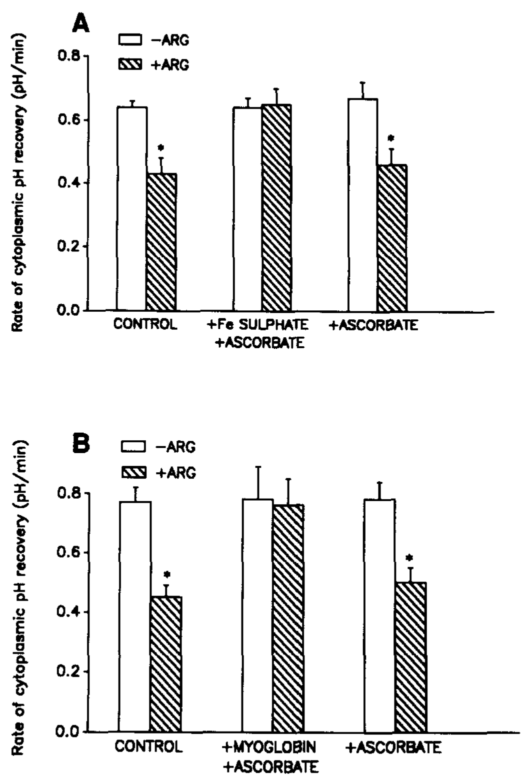

Figure 4. Effect of scavenging nitric oxide on the L-arginine-dependent inhibition of pH_i recovery in acid-loaded macrophages. Thioglycolate-elicited peritoneal cells were incubated for 2 h at 37°C in Hepes-RPMI (10^7 cells/ml) with or without 1.14 mM L-arginine, in the presence of 10 μg/ml LPS. 2×10^6 cells were incubated for a further 20 min in the same medium, in the presence of 40 mM NH_4Cl. The initial rate of pH_i recovery after resuspension in NH_4^+-free KCl medium was then measured fluorometrically. (*A*) Cells were incubated without or with either ferrous sulphate (Fe sulphate, 100 μM) plus ascorbate (1 mM) or ascorbate alone (1 mM) throughout the entire 140-min incubation period. $^*p < 0.05$ vs. no L-arginine and vs. L-arginine and ferrous sulphate plus ascorbate; $n = 4$. (*B*) Cells were incubated without or with either myoglobin (3 mg/ml) plus ascorbate (1 mM) or ascorbate alone (1 mM) throughout the entire 140-min incubation period. $^*p < 0.05$ vs. no L-arginine and vs. L-arginine and myoglobin plus ascorbate; $n = 4$.

abrogating the inhibition of pH_i recovery normally observed in cells incubated with L-arginine. Prevention of the L-arginine-dependent inhibition of pH_i recovery by two distinct nitric oxide scavenging systems suggested that the L-arginine metabolite responsible for the inhibition is nitric oxide.

This concept was further supported by examining the effect of nitroprusside on pH_i recovery. This vasodilator has been shown to spontaneously generate nitric oxide. If nitric oxide were the species responsible for inhibition of H^+ ATPase-mediated pH_i recovery, nitroprusside would be expected to mimic this inhibition by acting as an exogenous source of nitric oxide. To determine its effect on pH_i recovery, sodium nitroprusside (1 mM) was added to Møs after a 2-h preincubation in arginine-free medium. Incubation with nitroprusside for 20 min significantly reduced the rate of pH_i recovery from acid loading (0.37 ± 0.08 vs. 0.70 ± 0.04 pH/min in nitroprusside-treated vs. control cells, respectively; n = 4; $p < 0.05$). Representative analogue tracings that demonstrate this inhibition are shown in Fig. 5 *A*. These data suggested that nitroprusside was able to inhibit H^+ ATPase-mediated pH_i recovery. To confirm that the observed impairment of pH_i recovery was due to inhibition of the H^+ ATPase-mediated component of Na^+- and HCO_3^--independent recovery, the effect of nitroprusside on bafilomycin A_1-insensitive pH_i recovery was examined. As shown in Fig. 5 *B*, incubation for 20 min with 1 mM nitroprusside had no effect on the pH_i recovery rate of bafilomycin A_1-treated cells, indicating that the component of the Na^+- and

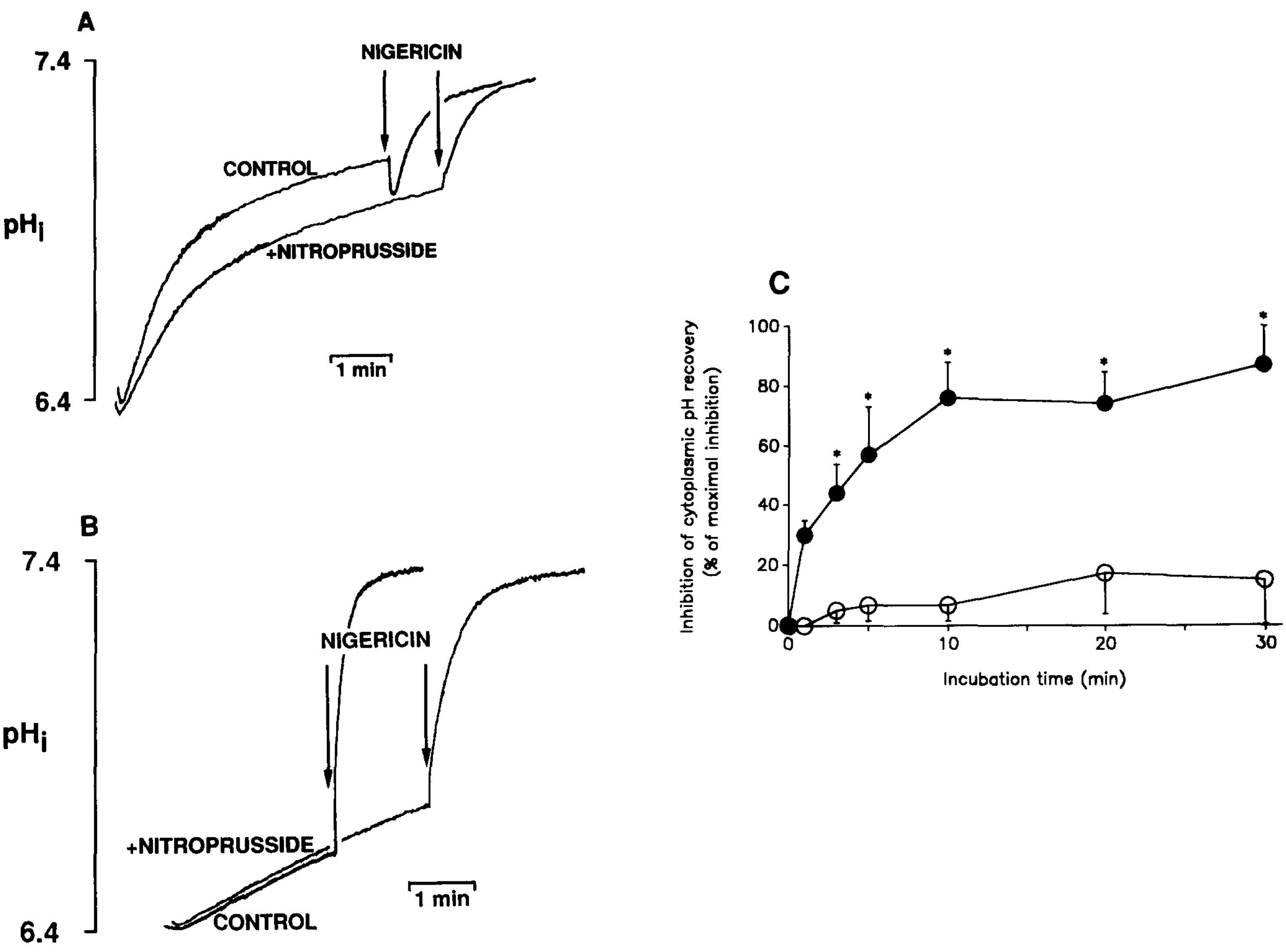

Figure 5. Effect of nitroprusside on H^+ ATPase-mediated pH_i recovery of acid-loaded macrophages. Thioglycolate-elicited peritoneal cells were incubated for 2 h at 37°C in arginine-free Hepes-RPMI (10^7 cells/ml). Cells were then acid loaded by the NH_4^+ prepulse method by incubating 2 × 10^6 cells for a further 20 min at 37°C in the same medium, to which NH_4Cl was added (40 mM, final concentration); this 20-min incubation was done in the presence (*B*) or absence (*A* and *C*) of bafilomycin A_1. pH_i recovery after resuspension in NH_4^+-free KCl medium was then measured fluorometrically. Where indicated (*lower trace* in *A*, *upper trace* in *B*, *filled circles* in *C*) sodium nitroprusside (1 mM) was added for the final 20 min, synchronous with the incubation with NH_4Cl (*A* and *B*), or for the time period indicated, synchronous with the incubation with NH_4Cl ± the final 10 min of the 2-h preincubation (*C*). Control cells were incubated in parallel in arginine-free medium without nitroprusside (*upper trace* in *A*, *lower trace* in *B*, *open circles* in *C*). Traces begin immediately upon resuspension of cells in 2 ml of NH_4^+-free KCl medium in a fluorometer cuvette. In each trace, nigericin (1 μM) was added where indicated by the arrows, for purposes of calibration. (*A* and *B*) Traces are presentative of four experiments. Temperature was 37°C. (*C*) The inhibition of initial pH_i recovery at each time point is expressed as a percentage of the maximal inhibition observed on each given day. The mean maximal inhibition was 0.42 ± 0.05 pH/min; n = 4. $^*p < 0.05$ vs. time = 0 vs. without nitroprusside.

HCO_3^--independent pH_i recovery inhibited by nitroprusside in Fig. 5 *A* was the H^+ ATPase-mediated recovery. Nitric oxide–induced relaxation of smooth muscle occurs within 1 min of exposure to nitroprusside (42). The effect of nitroprusside on Mø pH_i recovery was similarly rapid in onset: inhibition was apparent within the first few minutes of incubation with nitroprusside (Fig. 5 *C*).

Finally, solution decomposition under standard experimental conditions yields nitrite and nitrate as stable end products. Accumulation of nitrite/nitrate thus reflects cellular nitric oxide production. Incubation of cells in arginine-containing medium for 4 h resulted in a significant increase in nitrite levels in the supernatant compared to cells incubated in arginine-free medium (2.33 ± 0.26 μM vs. 1.69 ± 0.29 μM; $n = 13$; $p < 0.05$). When considered together with the studies presented above using nitric oxide scavengers and sodium nitroprusside, these data further support the concept that the L-arginine-dependent impairment of pH_i regulation is mediated through the action of nitric oxide.

Mechanism of Nitric Oxide–induced Inhibition of pH_i Recovery. Nitric oxide has been shown to impair mitochondrial respiration in both target cells and in Møs themselves, by inhibiting the aconitase of the citric acid cycle and the NADH/ubiquinone and succinate/ubiquinone oxidoreductase of the mitochondrial electron transport chain (26, 27, 33, 36). Such inhibition of mitochondrial respiration could potentially lead to ATP depletion. Since bafilomycin-sensitive proton pumping is ATP dependent, nitric oxide–induced ATP depletion represented one possible mechanism by which L-arginine metabolism could inhibit the regulation of Mø pH_i by H^+ ATPases. To investigate this possibility, the ATP content of cells incubated in the presence or absence of L-arginine, with or without LPS, was measured (Table 3). Over the 6-h incubation period, there was a gradual ATP depletion observed in all groups. The reason for the gradual decline in cellular ATP content is not clear. It is unlikely to result from progressive adherence of the Møs to the incubation tubes, since extraction was performed by adding perchloric acid directly to the tubes. A more likely alternative explanation was that, although viability remained high, the general condition of the cells slowly deteriorated over the course of the 6-h incubation ex vivo. However, there were no significant differences in ATP levels among the various groups at any time point. These results indicate that inhibition of mitochondrial respiration with resultant ATP depletion is not the mechanism whereby nitric oxide impairs H^+ ATPase-mediated pH_i recovery.

One further possibility is that L-arginine-derived nitric oxide might activate guanylate cyclase, resulting in an increase in the second messenger cyclic GMP (43–45). The vasorelaxant effect of nitric oxide is mediated through elevation of cGMP in vascular smooth muscle cells (18, 43, 44). To test the hypothesis that an elevation in cGMP might be responsible for the observed inhibition of Mø pH_i regulation, Møs were treated with a membrane-permeant form of this second messenger, 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo cGMP), after a 2-h preincubation in arginine-free medium. As shown in Fig. 6, exposure to 8-bromo cGMP (100 μM to 10 mM) significantly reduced the pH_i recovery rate of acid-loaded Møs (the rate of recovery was 0.52 ± 0.04 pH/min after incubation with 1 mM 8-bromo cGMP vs. 0.76 ± 0.06 pH/min in control cells; $n = 4$; $p < 0.05$). In fur-

Table 3. *Effect of L-Arginine on Cellular ATP Content*

Medium	LPS	Cellular ATP 2 h	4 h	6 h
			nmol/10⁷ cells	
+ L-Arginine	–	17.4 ± 2.3	16.7 ± 3.0	11.3 ± 2.9‡
Arginine-free	–	20.0 ± 2.9	16.3 ± 3.2	11.2 ± 3.1‡
+ L-Arginine	+	17.6 ± 3.1	16.6 ± 3.2	11.2 ± 2.8‡
Arginine-free	+	19.5 ± 2.4	17.2 ± 3.0	11.3 ± 3.1‡

* Freshly harvested thioglycolate-elicited peritoneal cells were incubated at 37°C for the indicated time period in bicarbonate-free Hepes-RPMI (10^7 cells/ml) with or without 1.14 mM L-arginine, in the presence or absence of LPS (10 μg/ml), as indicated. 5 × 10^6 cells were then pelleted, extracted with perchloric acid, and ATP content was determined using the luciferin/luciferase assay system. The ATP content of freshly harvested cells was 22.1 ± 2.6 nmol/10^7 cells. Results are expressed as means ± 1 SE of four to six experiments.
‡ $p < 0.05$ vs. $t = 0$.

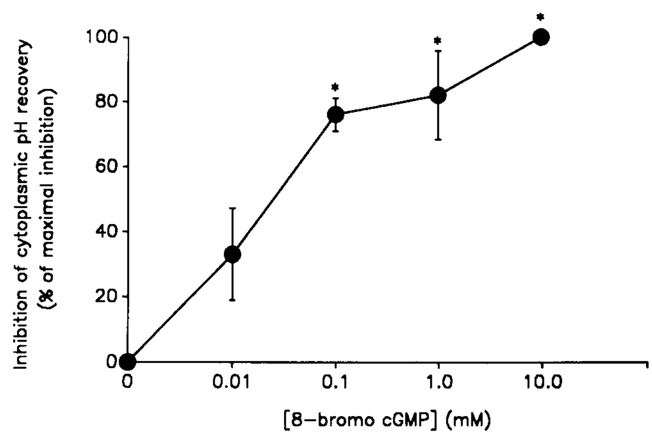

Figure 6. Effect of 8-bromo cGMP on the H^+ ATPase-mediated pH_i recovery of acid-loaded macrophages. Freshly harvested thioglycolate-elicited peritoneal cells were incubated for 2 h at 37°C in arginine-free Hepes-RPMI (10^7 cells/ml). 2 × 10^6 cells were incubated for a further 20 min in the same medium, in the presence of 40 mM NH_4Cl. The initial rate of pH_i recovery after resuspension in NH_4^+-free KC1 medium was then measured fluorometrically. The indicated concentration of 8-bromo cGMP was present during the final 10 min of the 2-h of preincubation and throughout the 20-min incubation with NH_4Cl. The inhibition of pH_i recovery associated with each concentration of L-arginine is expressed as a percentage of the maximal inhibition observed on each given day. The mean maximal inhibition was 0.30 ± 0.01 pH/min; $n = 4$. Where absent, the error bar was smaller than the symbol. *$p < 0.05$ vs. no 8-bromo cGMP.

ther support of this concept, nitroprusside (1 mM), shown above to inhibit pH_i recovery caused a significant rise in intracellular cGMP within 30 min after addition to cells (0.187 ± 0.023 pmol/mg protein; $n = 3$ vs. 0.037 ± 0.011 pmol/mg protein; $n = 3$; $p < 0.001$). However, total intracellular cGMP was not measurably different in cells incubated for 4 h in L-arginine-containing vs. arginine-free medium (0.084 ± 0.018 pmol/mg protein; $n = 7$ vs. 0.070 ± 0.019 pmol/mg protein; $n = 7$, respectively).

Discussion

Our previous studies defined the presence of H^+ extrusion mediated by vacuolar-type H^+ ATPases in murine peritoneal Møs. These proton pumps constituted a prominent pH_i regulatory mechanism, and were found to be critical to the preservation of normal Mø function within an acidic milieu (13). The present studies demonstrate that H^+ pump-mediated pH_i regulation may be modulated by an L-arginine-dependent mechanism. Incubation of Møs in the presence of L-arginine caused a dose-dependent inhibition of Mø pH_i recovery from an imposed acid load. The impairment of pH_i recovery required the presence of L-arginine, was reversed by the addition of L-arginase, and was blocked competitively by N-MMA, a competitive inhibitor of L-arginine metabolism. Furthermore, LPS, which is a potent stimulator of Mø L-arginine metabolism, enhanced the inhibitory effect of L-arginine on pH_i recovery. Finally, it was the bafilomycin A_1–sensitive component of the pH_i recovery, which was inhibited by L-arginine, implicating an effect on H^+ pump activity. When considered together, these data indicate that a product of L-arginine metabolism by Møs is able to inhibit the vacuolar-type H^+ ATPases involved in Mø pH_i regulation.

The metabolite responsible for this inhibition was investigated. Among the stable end products of L-arginine metabolism, nitrates are known to inhibit vacuolar-type H^+ ATPases. The reported K_i values for this inhibition are in the range of 40–100 mM (46–48). The total amount of nitrate released by activated Møs after 48 h in vitro has been estimated to reach ~400 nmol/10^6 cells (43). If this amount of nitrate remained within the cells, the intracellular concentration of nitrate would be predicted to reach well over 100 mM. However, since nitrate is a permeant anion, it is unlikely that it would remain trapped in the intracellular compartment, and thus the total amount of nitrate generated by L-arginine metabolism would likely be distributed throughout the extra- and intracellular compartments. If 10^7 cells suspended in 1 ml of medium produced a total of 4 μmol of nitrate, the predicted final concentration of nitrate would reach only 4 mM, well below the K_i for H^+ ATPase inhibition. Thus, it was unlikely that nitrate was the L-arginine metabolite responsible for the observed inhibitory effect. Accordingly, nitrate in concentrations up to 10 mM failed to inhibit pH_i recovery.

Instead, the present studies indicate that the molecular species responsible for L-arginine-dependent inhibition of H^+ pump activity is nitric oxide. Several lines of evidence support this concept: (*a*) two distinct nitric oxide scavenging systems reversed the effect of L-arginine metabolism on pH_i recovery; (*b*) the exogenous nitric oxide–generating agent, nitroprusside, was found to mimic the inhibitory effect of L-arginine; (*c*) nitrite levels were higher in the supernatants of cells incubated in L-arginine-containing medium than in arginine-free medium; (*d*) the stable end products of various pathways of L-arginine metabolism had no effect on pH_i recovery. Two potential mechanisms by which nitric oxide might impair H^+ pump activity were investigated. The ATP content of Møs incubated with or without L-arginine was equivalent, making it unlikely that the inhibition resulted from a nitric oxide–induced depletion of ATP. By contrast, the possibility that cGMP mediated the inhibition is supported by two findings. First, nitroprusside caused a rapid inhibition of pH_i recovery in association with a marked rise in cGMP levels. Second, incubation of Møs with the membrane-permeant form of cGMP, 8-bromo cGMP, impaired H^+ ATPase-mediated pH_i regulation. We were unable to demonstrate elevated cGMP levels in cells incubated in L-arginine-containing medium for 4 h, a time point where inhibition of pH_i recovery was clearly evident. This finding suggests that L-arginine-derived nitric oxide may exert its effect on pH_i recovery via a cGMP-independent mechanism, or alternatively that the measurement of total intracellular cGMP is not sufficiently sensitive to detect small or focal increases in cGMP within the cytoplasmic compartment, which might be responsible for the inhibition of pH_i recovery.

There are several potential mechanisms through which an elevation in intracellular cGMP might potentially modulate H^+ ATPase activity. One possible mechanism is through activation of a cGMP-dependent kinase (49). This appears to be the signal transduction pathway through which nitric oxide induces relaxation of vascular smooth muscle (43, 50–53), inhibits aggregation of platelets (54), and transmits signals in cerebellar cells (55). In addition, Semrad et al. (56) have recently shown that 8-bromo cGMP inhibits Na^+/H^+ exchange in chicken enterocytes, resulting in cytoplasmic acidification; this effect appeared to be mediated through an increase in $[Ca^{2+}]_i$, which was in turn secondary to cGMP-dependent phosphorylation. The H^+ ATPases that regulate Mø pH_i might similarly be modulated by cGMP-dependent phosphorylation.

An additional possibility is that cGMP could indirectly affect H^+ ATPases by altering the intracellular level of cAMP. A family of cAMP phosphodiesterases exists that is inhibited by physiological levels of cGMP (49). Increasing cGMP could thus potentially lead to decreased cAMP hydrolysis and thereby elevated cAMP levels, which could in turn stimulate cAMP-dependent kinase activity. The effect of cAMP on the pH_i regulating H^+ ATPases of Møs has not been examined. Recent studies by Gurich and Dubose (57) have demonstrated that 8-bromo cAMP inhibits H^+ ATPase-mediated acidification of endosomal vesicles prepared from rabbit renal cortex. Since the H^+ ATPases that regulate Mø pH_i are of the vacuolar-type, like those located in endosomal membranes, one could speculate that they might be similarly susceptible to inhibition by cAMP. ATPase-mediated pumping of protons into acidic organelles is facilitated by the comigration of chloride anions (16), which offsets the electrogenicity of H^+ pumping. Accordingly, H^+ ATPase-mediated pH_i recovery in Mφs is partially dependent on the presence of intracellular chloride (15). Thus, cAMP could theoretically inhibit H^+ pumping by decreasing the conductance for the counterion, chloride. However, Bae and Verkman (58) have recently reported that, in rabbit proximal tubule endosomes, counterion conductance is increased in response to phosphorylation through a cAMP-dependent protein kinase. This suggests that cAMP would be more likely to inhibit H^+ pumping through an effect on H^+ ATPases themselves, rather than through an effect on chloride conductance.

The present studies have focused on the role of vacuolar-type H^+ ATPases as regulators of Mφ pH_i. However, vacuolar-type H^+ ATPases are also responsible for acidification of a variety of intracellular compartments, including secretory granules, endosomes and lysosomes, and the Golgi apparatus (16), as well as phagosomes (59). The ability to acidify these organelles is central to many important cellular functions, including receptor-ligand dissociation, protein sorting and degradation, and microbicidal activity. For example, phagosomal acidification after microbial ingestion is crucial to the maintenance of normal cellular microbicidal function. Acidification enhances the activity of acid hydrolases within the phagolysosome, promotes the formation of the toxic oxygen metabolite, hydrogen peroxide, and may be directly microbicidal. Impairment of vacuolar-type H^+ ATPases by L-arginine metabolism could potentially alter the pH homeostasis of these organelles and reduce normal microbicidal activity. It is also interesting to note that incubation with concentrations of L-arginine typically present in commercially available prepared media (0.4 mM in MEM, 1.14 mM in RPMI 1640) has been shown to inhibit a variety of activation-associated functions in resident and *Corynebacterium parvum*–elicited peritoneal Mφs, including phagocytosis and protein synthesis (32). Inhibition of vacuolar-type H^+ ATPase activity constitutes one mechanism by which L-arginine metabolism could interfere with effective Mφ function.

Whether L-arginine metabolism by Mφs can modulate H^+ ATPase function in vivo is not clear. Estimates of extracellular L-arginine concentrations range from ∼100 μM in plasma (32) to <50 μM in healing wounds and inflammatory sites (60, 61). Incubation of thioglycolate-elicited Mφs with L-arginine in this concentration range did not result in significant impairment of pH_i regulation. However, endotoxin present in the inflammatory milieu could potentially enhance metabolism of low concentrations of L-arginine to produce significant amounts of nitric oxide. Preliminary studies indicate that incubation of Mφs with LPS causes inhibition of pH_i recovery at L-arginine concentrations as low as 12.5 μM. This suggests that the low levels of L-arginine present in vivo could modulate pH_i regulation of Mφs in which the nitric oxide synthase pathway had been activated by stimuli present in the septic milieu. On the other hand, activated Mφs release increased amounts of arginase (60–62); reduction of pericellular L-arginine concentrations by this mechanism may prevent the undesirable sequelae of L-arginine metabolism to nitric oxide. Enhanced release of arginase by activated Mφs can thus be viewed as a self-protective mechanism. This might be of particular importance to Mφs attempting to function in the acidic milieu of an abscess, where H^+ ATPase activity is essential to maintenance of pH_i (13).

The present studies investigated proton pump activity in moderately activated cells, i.e., those elicited by thioglycolate. Adherence-enriched populations of resident peritoneal macrophages did not demonstrate an L-arginine-dependent inhibition of H^+ ATPase-mediated proton extrusion (pH_i recovery rate: without L-arginine, 0.11 ± 0.03 pH/min vs. with L-arginine, 0.12 ± 0.03 pH/min $n = 5$). The finding that pH_i recovery in adherent thioglycolate-elicited cells incubated in arginine-free medium was significantly higher than in resident cells (0.20 ± 0.02 pH/min vs. 0.11 ± 0.03 pH/min; $p < 0.05$) suggests that the component of H^+ ATPase-mediated pH_i recovery inhibitable by L-arginine metabolism may be induced by Mφ activation. Whether this enhanced H^+ ATPase activity occurs via synthesis of new pumps or upregulation of existing quiescent pumps after exposure to inflammatory stimuli requires further investigation.

In summary, metabolism of L-arginine by Mφs causes inhibition of H^+ ATPase-mediated pH_i regulation in vitro. The evidence that this inhibition may be mediated through a nitric oxide–induced elevation of cGMP suggests a novel mechanism by which the activity of vacuolar-type H^+ ATPases may be modulated. Since H^+ ATPase-mediated pH_i regulation is critical for effective Mφ function within an acidic milieu, metabolism of L-arginine to nitric oxide could thus lead to impaired Mφ performance within the inflammatory microenvironment.

This work was supported by the Medical Research Council of Canada. C. J. Swallow is the recipient of an Ethicon-Society of University Surgeons Surgical Research Fellowship and a Medical Research Council of Canada Fellowship.

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Received for publication 6 March 1991 and in revised form 23 July 1991.

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