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Enhancement of Macrophage Microbicidal Activity: Supplemental Arginine and Citrulline Augment Nitric Oxide Production in Murine Peritoneal Macrophages and Promote Intracellular Killing of *Trypanosoma cruzi*

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The generation of nitric oxide (NO) is largely responsible for the intracellular killing of *Trypanosoma cruzi* by activated macrophages. The present study was carried out to determine whether the production of NO by activated murine macrophages cultured in physiologic levels of arginine can be augmented by increasing the availability of arginine, the substrate for NO biosynthesis. Increased exogenous arginine or citrulline resulted in a significant increase in NO production and complete clearance of the parasites by activated macrophages. As citrulline fully substituted for arginine in supporting NO production and trypanocidal activity, these results demonstrate the expression of a highly active citrulline-NO cycle in activated macrophages and that levels of arginine in plasma are limiting with respect to both NO production and trypanocidal activity in these cells. The results indicate that increasing plasma substrate levels for both arginine and NO biosynthesis may represent a means of enhancing microbicidal activity in vivo.

Recent studies with a number of microbial pathogens, including *Leishmania major*, *Cryptococcus neoformans*, *Mycobacterium tuberculosis*, and *Toxoplasma gondii*, have established the critical role of nitric oxide (NO) and other reactive nitrogen intermediates in the microbicidal activity of cytokine-stimulated murine macrophages (reviewed in reference 8). Although increased production of NO is responsible for heightened microbicidal activity, in some cases a small number of microorganisms can persist, leading to a subpatent infection and possibly chronic sequelae. Increasing the level of NO production by macrophages may lead to enhanced microbial killing and even the complete elimination of intracellular microorganisms. Using the protozoan parasite *Trypanosoma cruzi* as a model, we tested the effect of providing increased levels of substrate for NO biosynthesis on the microbicidal activity of gamma interferon (IFN-γ)-activated murine macrophages.

T. cruzi is the causative agent of Chagas' disease, which is a major public health concern in rural areas of Central and South America, where it is estimated that 16 to 18 million people are currently infected. Infectious trypomastigotes are introduced into the vertebrate host by insect vectors and infect a number of cell types, including macrophages. Macrophage activation by IFN-γ is a critical factor in controlling the infection and in clearance of the organisms. IFN-γ treatment increases survival of acutely infected mice and enhances trypanocidal activity of activated macrophages. Although there is an increase in IFN-γ levels early in the host response to *T. cruzi* infections, it is apparently insufficient for complete clearance of the parasites (18). IFN-γ-treated murine peritoneal macrophages and human monocytes exhibit increased killing of *T. cruzi* amastigotes, attributable to NO production via an L-arginine dependent pathway (5, 11). In addition, increased NO levels correlated with reduced parasitemias in experimentally infected mice (13), and treatment of experimentally infected mice with inhibitors of nitric oxide sythase exacerbated the infection (19). These results suggest that augmentation of macrophage microbicidal activity, specifically through the increased generation of NO, represents a previously unexplored avenue through which early control of the infection may be achieved.

The production of NO by activated macrophages is the result of increased expression of the inducible isoform of nitric oxide synthase (iNOS), which produces NO and citrulline from arginine. Once iNOS is expressed, the level of NO production is dependent in part upon the concentration of arginine, which is the only physiologic nitrogen donor for NO production (reviewed in reference 10). Previous studies have shown that physiologic levels of arginine are rate limiting for NO production (9, 12) and that citrulline, the byproduct of NO production, can be recycled to arginine via argininosuccinate synthetase (AS) and argininosuccinate lyase, thus increasing the available arginine for NO production in activated iNOS-expressing cells (12, 20). The arginine biosynthetic enzyme AS and iNOS are coinduced upon stimulation of a macrophage cell line with IFN-γ or lipopolysaccharide (LPS), and coinduction correlates with increased enzymatic activity and NO production (12). Taken together, these results suggest that NO production and arginine biosynthesis are coupled and that increased extracellular arginine or citrulline could support increased NO production in an IFN-γ-activated macrophage cell line (10).

The present study addresses three major questions. (i) What is the extent of NO-dependent killing of *T. cruzi* by activated macrophages at physiologic levels of arginine? (ii) Do increases in extracellular arginine concentration above the physiologic level significantly increase killing of *T. cruzi* in activated macrophages? (iii) Is citrulline, via recycling to arginine, effec-

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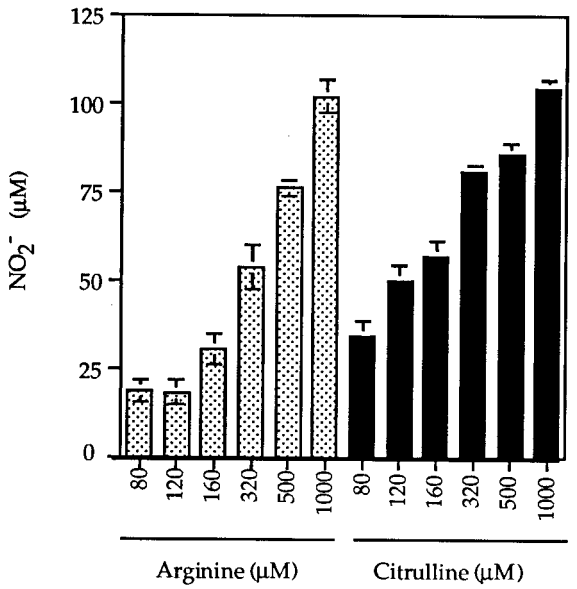

FIG. 1. Increased concentrations of extracellular arginine and citrulline augment NO_2^- production by murine peritoneal macrophages. Macrophages were primed with IFN-γ and LPS and then plated in complete RPMI Select-Amine medium supplemented with arginine or citrulline as indicated. All citrulline-supplemented media contained a baseline level of 80 μM arginine. Data are expressed as the mean NO_2^- concentration ± standard error of the mean of triplicate samples from six independent experiments.

tive in enhancing NO production and intracellular killing of *T. cruzi* by activated macrophages?

To determine if increasing extracellular arginine or citrulline levels represented an effective means of increasing macrophage NO production, thioglycolate-elicited peritoneal exudate cells were recovered from female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) and plated at 2.5×10^5 cells per well in 96-well microtiter plates (Corning, Corning, N.Y.) at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1% modified Eagle medium nonessential amino acids, 5% NCTC-109, and 50 μg of gentamicin per ml (all from GIBCO). After 4 h, nonadherent cells were removed by washing with supplemented RPMI Select-Amine (GIBCO) medium (with 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES and 50 μg of gentamicin per ml) containing 80 μM arginine. The medium was then replaced with RPMI Select-Amine medium containing various levels of arginine or citrulline as indicated in Fig. 1. All citrulline-supplemented media contained 80 μM arginine, which represents physiologic levels in plasma (3, 17). IFN-γ (Genentech, San Francisco, Calif.) was added to each well at 100 U/ml, and the plates were incubated for 18 h at 37°C in 5% CO_2. The plates were washed twice, and the medium was replaced with fresh arginine- or citrulline-supplemented complete RPMI Select-Amine medium. Tissue culture-derived trypomastigotes (strain Y) were harvested as previously described (16) and added to the wells at a multiplicity of infection of 5. After 6 h, extracellular trypomastigotes were removed by washing the macrophages twice. The medium was replaced with complete RPMI Select-Amine medium containing 10 ng of LPS (Sigma Chemical Co., St. Louis, Mo.) per ml and arginine or citrulline as indicated in Fig. 1. The cells were incubated at 37°C in 5% CO_2 for 72 h. Infection levels were assessed microscopically by staining the cells with Diff-quik (Baxter, McGaw Park, Ill.) and counting the number of infected cells in 10 random fields (×400 magnification) in triplicate sets for each experimental condition. Additionally, the number of amastigotes per cell was quantitated by counting the number of parasites in 30 random infected cells for each condition. For each culture condition NO_2^- was measured in the culture supernatants as an indicator of NO production by a modified Griess assay as described elsewhere (7).

Under the culture conditions described above, intracellular amastigotes were readily detectable 72 h after infection. Infection of peritoneal macrophages in the presence of 80 μM arginine without prior activation with IFN-γ and LPS resulted in a heavily infected culture (>50% infected cells) without detectable NO production (not shown). Thus, the parasites themselves do not appear to stimulate NO production in these cells. When the cells were activated and then cultured in media containing increasing levels of arginine, a dose-dependent increase in NO production was observed up to 1 mM arginine, indicating that under physiologic conditions, arginine is limiting for NO production by IFN-γ-activated peritoneal macrophages (Fig. 1). The results shown represent the mean NO_2^- levels at 72 h postinfection of triplicate samples from six independent experiments.

Because activated macrophages can also produce arginase which will suppress NO production by hydrolyzing extracellular arginine (1, 4), we investigated whether citrulline could replace arginine in augmenting NO production. Our results demonstrate that at levels as low as 80 μM, citrulline can augment NO production in activated peritoneal macrophages over the background level of 80 μM arginine (Fig. 1) and that cells in arginine- and citrulline-supplemented media produced comparable levels of NO over the range of concentrations tested.

The trypanocidal activity of activated macrophages in the presence of increased arginine and citrulline also increased dramatically. We consistently observed that once NO_2^- levels exceeded 75 μM, the infections were completely cleared or prevented. The NO produced under these conditions did not affect cell adherence in the cultures observed after 72 h, indicating that it is still below cytotoxic levels (not shown). These results were consistent in six independent experiments, with the results of a representative experiment shown in Table 1. At arginine and citrulline concentrations of 320 μM, there was a significant decrease in the number of infected cells, as well as the number of parasites per cell compared with numbers in controls (Table 1). These results suggest that there is a critical threshold level of NO which can completely eliminate the infection in activated macrophages in vitro but that physiologic levels of arginine are probably limiting for NO production and therefore this level may not be attained during the course of an infection. This may be a critical feature in the pathogenesis of *T. cruzi* infections, since parasites may persist at very low levels and contribute to the development of chronic disease.

To establish that the trypanocidal activity observed was attributable to arginine-dependent NO production, N^G-monomethyl-L-arginine (NMMA) (Calbiochem, La Jolla, Calif.), an inhibitor of iNOS, was added to the cultures at the initiation of infection. At all arginine and citrulline levels, NMMA (500 μM) completely inhibited NO production (not shown) and the trypanocidal activity was completely abrogated (Table 1). These results clearly show that the effect of supplemental arginine and citrulline on trypanocidal activity of activated macrophages is the result of increased NO production by these cells. The high efficiency of citrulline in supporting NO production is likely a consequence of the fact that mRNA encod-

TABLE 1. Effect of arginine and citrulline concentration on NO_2^- production and infectivity of *T. cruzi* in IFN-γ- and LPS-stimulated peritoneal macrophages[a]

Medium supplement and concn (μM)	NO_2^- concn (μM)	No. of infected cells/field[b]		No. of parasites/infected cell[c]	
		−NMMA (% reduction[d])	+NMMA	−NMMA (% reduction[e])	+NMMA
Arginine					
80	17.1 ± 1.1	35 ± 3 (37.5)	56 ± 4	15 ± 1 (6)	16 ± 1
160	29.7 ± 0.7	34 ± 3 (55.3)	76 ± 6	ND	ND
320	52.8 ± 0.5	19 ± 2 (75.0)	76 ± 4	6 ± 1 (62.5)[f]	16 ± 2
500	77.4 ± 2.7	0 (100)	56 ± 4	NA	NA
1,000	110.0 ± 2.6	0 (100)	82 ± 2	NA	NA
Citrulline					
80	33.1 ± 0.5	41 ± 3 (43.8)	73 ± 4	15 ± 2 (31.9)[f]	22 ± 3
160	45.9 ± 1.1	44 ± 3 (31.3)	64 ± 4	ND	ND
320	74.8 ± 1.4	11 ± 2 (80.4)	56 ± 4	4 ± 1 (80)[f]	20 ± 2
500	88.2 ± 1.4	0 (100)	44 ± 3	NA	NA
1,000	113.6 ± 1.5	0 (100)	65 ± 5	NA	NA

[a] Data are expressed as the mean ± standard error of the mean for triplicate samples of a representative experiment as described in the legend to Fig. 1. Data were analyzed for significant differences by Student's *t* test.
[b] Ten random fields in each well of triplicate sets were counted at a magnification of ×400.
[c] Data are expressed as the mean ± standard error of the mean parasites per cell from 30 infected cells per well of triplicate samples. ND, not done; NA, not applicable.
[d] 100 − [(infected cells per field without NMMA/infected cells per field with NMMA) (100)]. All values of infected cells per field were significantly reduced in the absence of NMMA ($P \leq 0.001$).
[e] 100 − [(parasites per infected cell without NMMA/parasites per infected cell with NMMA) (100)].
[f] Values are significantly reduced in the absence of NMMA ($P \leq 0.0001$).

ing AS, the rate-limiting enzyme in arginine biosynthesis, is coinduced with iNOS mRNA in these cells (Fig. 2). Together, these results support the conclusion that arginine biosynthesis and NO production are coordinately regulated in these cells as in other cell types (10), so that supplemental citrulline is efficiently recycled to arginine, thus boosting NO production and parasite killing.

The results presented here confirm the critical role of NO in mediating trypanocidal activity of activated macrophages and demonstrate that physiologic levels of arginine are limiting for NO production and parasite killing by IFN-γ-activated peritoneal macrophages. Modest increases in arginine concentration above physiologic levels blocked infection with *T. cruzi* in vitro. Furthermore, citrulline could effectively replace arginine as a means of boosting production of NO by activated macrophages, thus demonstrating an active arginine biosynthetic pathway in these cells.

Previous studies have shown that levels of arginine in plasma may be increased by dietary supplementation (2, 6, 14, 15), suggesting that, on the basis of our results, dietary supplementation with arginine or citrulline may be an effective way to boost NO production in vivo. Because citrulline was shown to be nearly as effective as arginine in supporting NO production and it is not a substrate for arginase, delivery of this amino acid may be particularly effective in enhancing microbicidal activity of activated macrophages in vivo. The present study indicates that modulation of macrophage function by increasing the availability of arginine for iNOS may represent a means of early and efficient control of infectious agents known to be susceptible to NO toxicity.

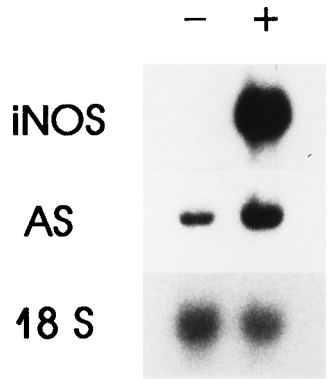

FIG. 2. Induction of iNOS and AS mRNA in peritoneal macrophages. Cells were cultured in the presence (+) or absence (−) of IFN-γ and LPS for 24 h prior to RNA preparation. Northern blot analysis of total RNA was performed as described previously (12). Equal loading of samples was confirmed by hybridization of the same membrane with a DNA probe for 18S rRNA.

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REFERENCES

1. **Albina, J., C. D. Mills, A. Barbul, C. Thirkill, W. Henry, B. Mastrofrancesco, and M. Caldwell.** 1988. Arginine metabolism in wounds. Am. J. Physiol. **254:**E459–E467.
2. **Chyun, J.-H., and P. Griminger.** 1984. Improvement of nitrogen retention by arginine and glycine supplementation and its relation to collagen synthesis in traumatized mature and aged rats. J. Nutr. **114:**1697–1704.
3. **Clowes, G., H. Randall, and C. Cha.** 1980. Amino acid and energy metabolism in septic and traumatized patients. J. Parenter. Enteral Nutr. **4:**195–205.
4. **Currie, G., L. Gyure, and L. Cifuentes.** 1979. Microenvironmental arginine depletion by macrophages *in vivo*. Br. J. Cancer **39:**613–620.
5. **Gazzinelli, R., I. Oswald, S. Hieny, S. James, and A. Sher.** 1992. The microbicidal activity of interferon-γ treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor β. Eur. J. Immunol. **22:**2501–2506.
6. **Gianotti, L., J. W. Alexander, T. Pyles, and R. Fukushima.** 1993. Arginine-supplemented diets improve survival in gut-derived sepsis and peritonitis by modulating bacterial clearance. The role of nitric oxide. Ann. Surg. **217:**644–654.
7. **Green, L. C., D. A. Wagner, J. A. Gogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum.** 1982. Analysis of nitrite, nitrate, and [^{15}N]-nitrite in biological fluids. Anal. Biochem. **126:**131–138.
8. **Green, S. J., and C. A. Nacy.** 1993. Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis. Curr. Opin. Infect. Dis. **6:**383–396.
9. **Iyengar, R., D. Stuehr, and M. Marletta.** 1987. Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. Proc. Natl. Acad. Sci. USA **84:**6369–6373.

10. **Morris, S., and T. Billiar.** 1994. New insights into the regulation of inducible nitric oxide synthesis. Am. J. Physiol. **266:**E829–E839.
11. **Muñoz-Fernandez, M. A., M. A. Fernandez, and M. Fresno.** 1992. Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-α and IFN-γ through a nitric oxide-dependent mechanism. Immunol. Lett. **33:**35–40.
12. **Nussler, A., T. Billiar, Z. Liu, and S. Morris.** 1994. Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line—implications for regulation of nitric oxide production. J. Biol. Chem. **269:**1257–1261.
13. **Petray, P., M. Rottenberg, S. Grinstein, and A. Orn.** 1994. Release of nitric oxide during the experimental infection with *Trypanosoma cruzi*. Parasite Immunol. **16:**193–199.
14. **Pui, U., and H. Fisher.** 1979. Factorial supplementation with arginine and glycine on nitrogen retention and body weight gain in the traumatized rat. J. Nutr. **109:**240–246.
15. **Reynolds, J., J. Daly, S. Zhang, E. Evantash, J. Shou, R. Sigal, and M. Ziegler.** 1988. Immunomodulatory mechanisms of arginine. Surgery **104:** 142–151.
16. **Sanderson, C., J. Thomas, and C. Twomey.** 1980. The growth of *Trypanosoma cruzi* in human diploid cells for the production of trypomastigotes. Parasitology **80:**153–162.
17. **Sax, H., P. Hasselgren, M. Talamini, L. Edwards, and J. Fischer.** 1988. Amino acid uptake in isolated, perfused liver: effect of trauma and sepsis. J. Surg. Res. **45:**50–55.
18. **Tarleton, R.** 1991. Regulation of immunity in *Trypanosoma cruzi* infection. Exp. Parasitol. **73:**106–109.
19. **Vespa, G., F. Cunha, and J. Silva.** 1994. Nitric oxide is involved in control of *Trypanosoma cruzi*-induced parasitemia and directly kills the parasites in vitro. Infect. Immun. **62:**5177–5182.
20. **Wu, G., and J. T. Brosnan.** 1992. Macrophages can convert citrulline into arginine. Biochem. J. **281:**45–48.