AN L-ARGININE-DEPENDENT MECHANISM MEDIATES KUPFFER CELL INHIBITION OF HEPATOCYTE PROTEIN SYNTHESIS IN VITRO

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The hepatic failure associated with severe sepsis is characterized by specific, progressive, and often irreversible defects in hepatocellular metabolism (1). Although the etiologic microbe can often be identified, the direct causes and mechanisms of the hepatocellular dysfunction are poorly understood. We have hypothesized that Kupffer cells (KC), which interact with ambient septic stimuli, respond by providing signals to adjacent hepatocytes (HC) in sepsis. Furthermore, we have provided evidence (2, 3) that KC activated by LPS from Gram-negative bacteria can induce profound changes in the function of neighboring HC in coculture. In our model, coculture of either KC (2) or peritoneal macrophages (Mφ) (3) with HC normally promotes HC protein synthesis ([^{3}H]leucine incorporation). The addition of LPS or killed *Escherichia coli* to such cocultures induces a profound decrease in HC protein synthesis, as well as qualitative changes ([^{35}S]methionine, SDS-gel electrophoresis) in protein synthesis without inducing HC death (2, 3). In this report we show that the inhibition in protein synthesis is mediated via an L-arginine-dependent mechanism.

The metabolism of L-arginine by activated Mφ to substances with cytostatic and even lethal effects on target cells is a relatively recent discovery. After the description by Stuehr and Marletta (4, 5) that LPS-triggered Mφ produced nitrite/nitrate (NO_2^-/NO_3^-), Hibbs et al. (6, 7) and Iyengar et al. (8) demonstrated that L-arginine was the substrate for the formation of both these nitrogen end products and citrulline. A role for the arginine-dependent mechanism in Mφ tumor cytotoxicity (6, 7) and microbiostatic activity (9) has been suggested. However, the in vivo functions of this novel Mφ mechanism have not yet been defined, but it is possible that there are both physiologic as well as pathologic roles. Our in vitro results raise the possibility that some metabolic responses to microbial invasion may be partially mediated by the L-arginine-dependent mechanism. What other metabolic responses are affected and the possible pathologic consequences remain to be studied.

Materials and Methods

Animals. Liver cells were obtained from unfasted male Sprague Dawley rats weighing 200–300 g (Harlan Sprague Dawley, Inc., Indianapolis, IN).

Cell Isolation.   HC were obtained using an in situ collagenase (Sigma Chemical Co., St. Louis, MO) perfusion technique as described (3). The HC were purified by three differential sedimentations at 50 g for 2 min. Viability consistently exceeded 85% by trypan blue exclusion.

Liver nonparenchymal cells were obtained using a pronase E (Sigma) digestion of the parenchyma as described (2). The KC were separated from the other nonparenchymal cells using centrifugal elutriation and found to be 90–95% pure KC by peroxidase staining.

Cell Culture Technique.   On day 1, HC were plated at 2 × 10⁵ cells/ml onto either 96-well microtiter plastic culture plates in a 0.1- or 1.2-ml vol in 35-mm wells (Costar, Cambridge, MA) coated with gelatin. Culture medium consisted of Williams medium E (Gibco Laboratories, Grand Island, NY) supplemented with 10^{-6} M insulin, 15 mM Hepes, L-glutamine, penicillin, streptomycin, and 10% calf serum (CS) (HyClone Laboratories, Logan, UT). On day 2, KC (10^6 cells/ml) were added to wells with HC to establish a 5:1 KC/HC ratio, or cultured alone. The KC were added in fresh MEM (including 0.1 mM L-arginine) prepared in our laboratory and supplemented as above, except 20 mM Hepes and 5% dialyzed CS (Gibco) were used. L-arginine was excluded from the medium added to cultures that did not receive L-arginine in the subsequent incubation with LPS. On the third day, the old medium was removed and LPS (from *E. coli* 0111:B₄; Difco Laboratories, Detroit, MI) was added in fresh MEM plus 1–2.5% dialyzed CS and various L-arginine concentrations. Total protein synthesis or citrulline and NO_2^-/NO_3^- production were measured 24 h after the addition of LPS.

Determination of Protein Synthesis.   Total protein synthesis was determined using a 4-h labeling interval with [³H]leucine (5.0 Ci/mmol, 1.0 μCi/well; New England Nuclear, Cambridge, MA) in fresh MEM without L-arginine or unlabeled leucine. The labeling medium was the same for all of the experiments. L-arginine was excluded to avoid promoting the cytostatic effect during the 4-h label and the lack of L-arginine did not significantly change the total [³H]leucine incorporation. The incorporation of [³H]leucine into protein was determined using scintillation counting (2, 3).

Determination of Citrulline and NO_2^-/NO_3^-.   Supernatant (35-mm wells) citrulline concentrations were determined using a colorimetric assay based on the reaction of diacetyl monoxime with citrulline after the removal of protein and urea (10). The NO_2^-/NO_3^- concentrations were determined using an automated procedure based on the Griess reaction (4).

Results

The end products of L-arginine metabolism by activated Mφ are citrulline and NO_2^-/NO_3^- (6–8). In Fig. 1, the total protein synthesis is compared with the production of L-arginine metabolites for HC, HC cultured with KC, and KC cultured alone in response to LPS. Most of the protein synthesis takes place in the HC. LPS had no effect on the amount of protein synthesized by HC or KC in the absence of the other cell type. In the absence of LPS, KC/HC coculture protein synthesis was significantly enhanced over HC or KC alone. When exposed to critical concentrations of LPS (≥100 ng/ml), protein synthesis was inhibited and decreased to levels below control HC protein synthesis at the highest concentrations of LPS (2, 3). Identical changes in uptake of label was seen if [³⁵S]methionine was used (data not shown). The production of L-arginine metabolites paralleled the decrease in protein synthesis. HC release of citrulline and NO_2^-/NO_3^- did not increase and KC release of these metabolites increased only slightly in response to LPS. In contrast, KC/HC cocultures produced much greater quantities of L-arginine metabolites when exposed to LPS, with the highest production corresponding to the greatest inhibition of protein synthesis. Fig. 2 shows that inhibition of protein synthesis in KC/HC coculture was dependent on the L-arginine concentration as well as the LPS concentration. A notable decrease in protein synthesis first occurred at L-arginine concentration of 0.05 mM, whereas maximal inhibition occurred at L-arginine concentrations of ≥0.5 mM. Nei-
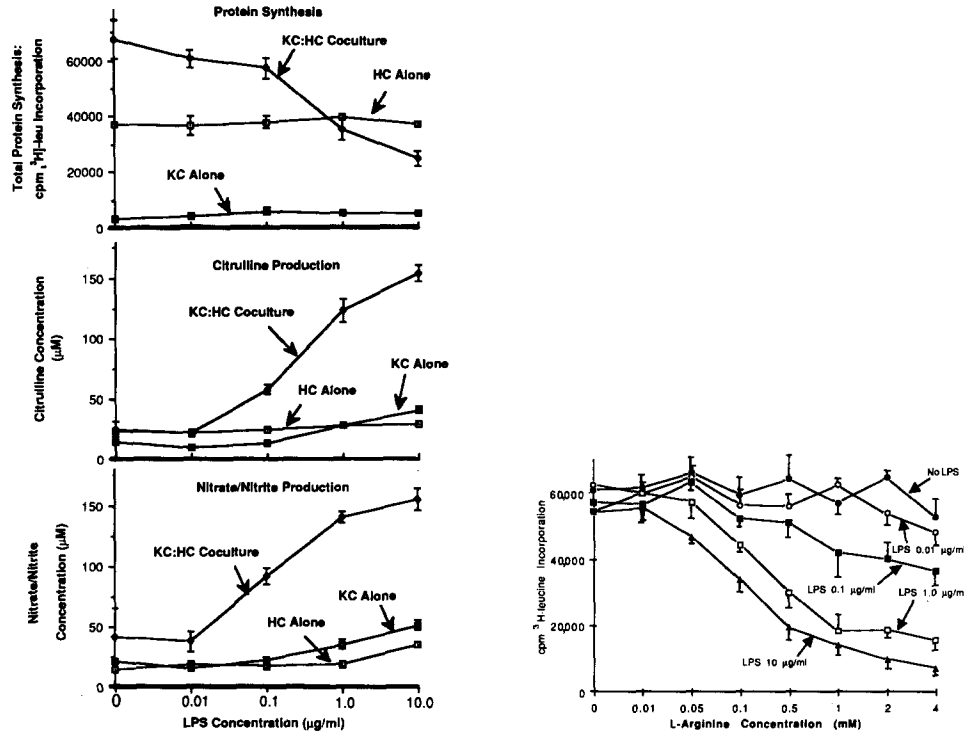
FIGURES 1 AND 2.   (*Fig. 1, left*) Total protein synthesis ([³H]leucine incorporation), citrulline production, and total nitrate/nitrite production is shown for HC, KC, and KC cocultured with HC (5:1 KC/HC ratio) 24 h after the addition of LPS. The LPS was added in complete MEM (including 0.5 mM L-arginine) plus 2.5% dialyzed CS. Values represent the mean of three to four cultures ± SD. Where error bars are not shown, they fall within the symbols. (*Fig. 2, right*) KC/HC (5:1 ratio) coculture protein synthesis 24 h after the addition of LPS is shown as a function of the L-arginine concentration in the culture medium. LPS was added in complete MEM plus 1% dialyzed CS with L-arginine concentrations ranging from 0 to 4 mM. The values represent the means ± SD of quadruplicate cultures.

ther D-arginine nor L-homoarginine could replace L-arginine in our model (percent inhibition using 1 mM amino acid and 10 μg/ml LPS: no arginine, 9.7%; L-arginine, 41.3%; D-arginine, 0%; L-homoarginine, 11.4%).

The presence or absence of MEM amino acids other than L-arginine did not contribute to the inhibition of protein synthesis or to the production of L-arginine metabolites (Table I). Significant inhibition in KC/HC protein synthesis was seen only if L-arginine was present in the medium in which LPS was added and occurred even if L-arginine was the only amino acid added with the LPS. Similarly, coculture protein synthesis was significantly diminished only when both citrulline and NO_2^-/NO_3^- were produced. Again, the production of L-arginine metabolites was greater by coculture than by KC alone. There was some small production of citrulline and NO_2^-/NO_3^- in the absence of added L-arginine, which might be due to L-arginine derived from Mφ catabolism of serum proteins present in the culture system (6). This may explain the slight inhibition in protein synthesis in the absence of L-arginine. We have previously shown that HC viability is not significantly decreased in LPS-trig-

TABLE I

L-arginine Is the only MEM Amino Acid Required for the LPS-induced Inhibition of Protein Synthesis and Production of Citrulline and Nitrate/Nitrite

Media amino acid content	LPS added*	KC/HC protein synthesis ([³H]leu incorporation)	Citrulline		Nitrate/nitrite	
		cpm	KC/HC	KC	KC/HC	KC
			μM		μM	
MEM amino acids‡	−	56,919 ± 5,745§	7.9 ± 0.4	4.4 ± 3.2	13.8 ± 0.6	15.5 ± 0.9
	+	28,651 ± 2,888 (49.7%)‖	133.7 ± 2.5	33.6 ± 2.5	131.6 ± 2.0	43.3 ± 2.1
MEM amino acids except L-Arg	−	49,411 ± 2,450	8.7 ± 3.6	3.2 ± 0.7	12.4 ± 0.6	10.9 ± 1.7
	+	44,691 ± 2,951 (9.6%)	13.0 ± 0.8	11.0 ± 1.4	44.4 ± 2.2	25.8 ± 3.3
L-Arg only	−	81,768 ± 6,128	3.8 ± 0.8	4.7 ± 1.1	12.3 ± 0.7	16.0 ± 0.5
	+	41,857 ± 2,372 (48.8%)	150.5 ± 2.1	52.3 ± 4.6	108.7 ± 7.2	34.1 ± 1.4
No amino acids	−	75,688 ± 5,362	2.1 ± 0.4	1.1 ± 0.4	11.0 ± 2.7	12.3 ± 0.6
	+	67,207 ± 1,650 (11.2%)	11.6 ± 2.5	8.7 ± 2.5	40.3 ± 1.4	23.0 ± 0.3

* LPS (10 μg/ml) added in MEM with the amino acid content indicated and 2.5% dialysed calf serum.
‡ MEM amino acids include L-Arg (0.5 mM) and Cys, Gln, His, Ile, Leu, Lys, Phe, Met, Thr, Trp, Try, and Val.
§ Results represent the mean ± SD of at least three cultures.
‖ Shows percent inhibition of protein synthesis.

Table II

Effect on NMA on KC/HC Protein Synthesis and Citrulline Production in the Presence of LPS and Increasing Concentrations of L-arginine

L-Arginine*	LPS added[‡]	NMA added[§]	Protein synthesis ([³H]leu)	Citrulline
mM			*cpm*	*μM*
0.5	−	−	75,512 ± 4,043[‖]	0
0.5	+	−	17,618 ± 1,172 (76.7%)[¶]	148.1 ± 8.5
0.5	−	+	71,008 ± 3,283	0.1
0.5	+	+	55,959 ± 900 (21.2%)	18.5 ± 3.5
1.0	+	+	45,831 ± 3,466 (35.5%)	43.5 ± 4.8
2.0	+	+	38,845 ± 3,745 (45.3%)	76.0 ± 0.9
4.0	+	+	31,414 ± 3,194 (55.8%)	116.7 ± 6.5

* L-Arginine added in MEM with all other MEM amino acids and 2.5% dialysed calf serum.
‡ LPS 10 μg/ml added as indicated.
§ NMA 0.05 mM added at the same time as the LPS, as indicated.
‖ Values represent the mean ± SD of three to four cultures.
¶ Shows percent inhibition of protein synthesis.

gered coculture (3). In this study, the minimal release of the HC enzyme, aspartate aminotransferase, by KC/HC coculture was not altered by the presence or absence of L-arginine or LPS (data not shown).

N^Gmonomethyl-L-arginine (NMA) (Calbiochem-Behring Corp., La Jolla, CA) is a specific and reversible inhibitor of the Mφ L-arginine-dependent mechanism of tumor cell cytotoxicity (7, 8). NMA (0.05 mM) added with LPS to KC/HC coculture prevented most of the inhibition of protein synthesis (Table II) and the production of citrulline. The inhibitory effect of NMA was overcome with higher concentrations of L-arginine, confirming the reversible nature of this inhibitor in our system.

Discussion

Metabolism of L-arginine to citrulline and NO_2^-/NO_3^- by activated murine peritoneal Mφ is well established (4–8). Cellular injuries associated with induction of this pathway include inhibition of the Krebs cycle enzyme aconitase, inhibition of complex I and complex II components of the electron transport chain, and inhibition of DNA synthesis as studied in tumor cell targets (11) and fungus (9). It has been suggested that this represents a general mechanism whereby Mφ inhibit tumor cell (6, 7) and microbial growth (9). However, a role for this Mφ mechanism in in vivo physiologic or pathologic intercellular interactions has not been established. It has been shown that another cell type, endothelial cells, utilize L-arginine to produce nitric oxide, which acts as a smooth muscle relaxant (12).

Our results show that LPS-activated rat KC inhibit HC protein synthesis by an L-arginine-dependent mechanism. Besides the strict requirement for L-arginine, other characteristics of the arginine-dependent mechanism true in the KC/HC system include the requirement for proximity between the Mφ and target cell for the cytostatic effect, an inability to transfer the cytostatic effect in conditioned media from LPS-triggered KC or KC/HC coculture, and an inability of citrulline or NO_2^-/NO_3^- to inhibit HC protein synthesis (data not shown) (6, 7, 9). It has been suggested that unstable intermediate metabolites of this pathway, termed reactive nitrogen intermediates (13), mediate the target cell injuries. Unlike murine peritoneal Mφ (7), rat KC could not use L-homoarginine as substrate for this pathway.

The combination of KC with HC resulted in a dramatic increase in the production of L-arginine metabolites in response to LPS. These experiments show that this is not a result of the conversion of other amino acids by the HC to substrate for citrulline or NO_2^-/NO_3^- production by the KC. Also excluded is a difference in viability of the KC cultured alone or cocultured with HC (trypan blue exclusion, data not shown). KC/HC cocultures do, however, produce more TNF and IL-1 than KC cultured alone in response to LPS (manuscript in preparation), and it may be that these monokines potentiate the metabolism of L-arginine by the KC (5, 13). Other explanations include that HC are induced by KC products to metabolize L-arginine as seen in the Mϕ EMT-6 adenocarcinoma cell model (14) or that HC produce cytokines that promote KC metabolism of L-arginine. These possibilities are currently under investigation.

References

1. Cerra, F. B., J. H. Siegel, J. R. Border, J. Wiles, and R. R. McMenamy. 1979. The hepatic failure of sepsis: cellular versus substrate. *Surgery (St. Louis)*. 86:409.
2. West, M. A., T. R. Billiar, J. E. Mazuski, R. D. Curran, F. B. Cerra, and R. L. Simmons. 1988. Endotoxin modulation of hepatocyte secretory and cellular protein synthesis is mediated by Kupffer cells. *Arch. Surg.* 123:1400.
3. West, M. A., G. A. Keller, F. B. Cerra, and R. L. Simmons. 1985. Killed *Escherichia coli* stimulates macrophage-mediated alterations in hepatocellular function during *in vitro* coculture: a mechanism of altered liver function in sepsis. *Infect. Immun.* 49:563.
4. Stuehr, D. J., and M. A. Marletta. 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. USA*. 82:7738.
5. Stuehr, D. J., and M. A. Marletta. 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. *J. Immunol.* 139:518.
6. Hibbs, J. B., R. R. Taintor, and Z. Vavrin. 1987. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science (Wash. DC)*. 240:473.
7. Hibbs, J. B., Z. Vavrin, and R. R. Taintor. 1987. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* 138:550.
8. Iyengar, R., D. J. Stuehr, and M. A. Marletta. 1987. Macrophage synthesis of nitrite, nitrate, and N-nitrosamine: precursors and role of respiratory burst. *Proc. Natl. Acad. Sci. USA*. 84:6369.
9. Granger, D. L., J. B. Hibbs, J. R. Perfect, and D. T. Durack. 1988. Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. *J. Clin. Invest.* 81:1129.
10. Boyde, T. R. C., and M. Rahmatullah. 1980. Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. *Anal. Biochem.* 107:424.
11. Drapier, J. C., and J. B. Hibbs. 1986. Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. *J. Clin. Invest.* 78:790.
12. Palmer, R. M. J., D. S. Ashton, and S. Moncada. 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature (Lond.)*. 333:664.
13. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J. Immunol.* 141:2407.
14. Amber, I. J., J. B. Hibbs, R. R. Taintor, and A. Varrin. 1988. Cytokines induce an L-arginine-dependent effector system in nonmacrophage cells. *J. Leukocyte Biol.* 44:58.