Brief Definitive Report

HEPATOCYTES PRODUCE NITROGEN OXIDES FROM L-ARGININE IN RESPONSE TO INFLAMMATORY PRODUCTS OF KUPFFER CELLS

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Recent findings suggest that nitric oxide (NO·) is an important biologic mediator produced by a number of cell types including endothelial cells (1, 2), macrophages (Mϕ (3–5), cerebellar neurons (6), and neutrophils (7). L-Arginine (L-arg) serves as the substrate for NO· production (1–4), while L-citrulline and nitrite/nitrate (NO_2^-/NO_3^-) are the stable endproducts of this metabolic pathway (8, 9). We have previously shown that cocultures of rat Kupffer cells (KC) and hepatocytes (HC) also metabolize L-arg to citrulline and NO_2^-/NO_3^- in response to lipopolysaccharide (LPS) (10). This L-arg metabolism was associated with a profound suppression of coculture total protein synthesis. When KC and HC were cultured alone, only KC metabolized L-arg in response to LPS. Therefore, we originally hypothesized that KC, like other Mϕ, were the sole site of L-arg metabolism. However, KC:HC cocultures exposed to LPS produced three to five times more NO_2^-/NO_3^- and citrulline than LPS-activated KC cultured alone. Investigation of this discrepancy led to the subject of this report, that transferable KC products induce the conversion of significant quantities of L-arg to NO· in HC and that this metabolism of L-arg is associated with a concurrent reduction in HC protein synthesis.

Materials and Methods

Culture Medium. HC and KC cultures were performed in standard Williams medium E (0.24 mM L-arg) or L-arg-free Williams medium E (Gibco Laboratories, Grand Island, NY) supplemented with 10^{-6} M insulin, 15 mM Hepes, L-glutamine, penicillin, streptomycin, and 5% dialyzed calf serum. Additional culture reagents included L-arg HCl (Gibco Laboratories) and N^G-monomethyl-L-arginine acetate (NMA), prepared by a modification of the method described by Corbin and Reporter (11).

Cell Isolation. Liver cells were obtained from unfasted male Sprague-Dawley rats weighing 200–300 g (Harlan Sprague Dawley, Inc., Indianapolis, IN). HC were harvested by an in situ collagenase (Type IV; Sigma Chemical Co., St. Louis, MO) perfusion and separated from nonparenchymal cells by differential sedimentation to >95% purity as previously described (12). Liver nonparenchymal cells were harvested by a pronase E (Sigma Chemical

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Co.) digestion and KC separated from endothelial cells by centrifugal elutriation to ≥90% purity as determined by peroxidase staining (10).

Cell Culture Technique. Conditioned KC supernatant was generated by plating 6×10^6 KC in 3 ml in 25-cm^2 flasks (Costar, Cambridge, MA) with recombinant rat IFN-γ (100 units/ml; Amgen Biologicals, Thousand Oaks, CA). After 24 h, the medium was changed and LPS (10 μg/ml) was added (from *Escherichia coli*, 0111:B_4; Difco Laboratories, Detroit, MI). The culture supernatants were collected 8 h later, filtered through 0.2 μm pore filters, and stored at −70°C until use.

HC were plated at 2×10^5 cells/ml on 96-well microtiter plates (0.1 ml/well) for protein synthesis determination and 12-well tissue culture trays (0.8 ml/well) for measurement of L-arg metabolite production. 24 h later, the medium on the HC cultures was removed and KC supernatant (50% dilution) or fresh medium added. HC total protein synthesis or citrulline and NO_2^-/NO_3^- production were assessed after 18 h of KC supernatant exposure unless otherwise specified.

Determination of Protein Synthesis. HC total protein synthesis was determined using a 4-h labeling interval with [^{3}H]leucine (5.0 Ci/mmol, 1.0 μCi/well; New England Nuclear, Cambridge, MA) in fresh MEM without unlabeled leucine. Experiments performed in L-arg-free medium were also labeled in MEM without L-arg. The incorporation of [^{3}H]leucine into protein was determined using scintillation counting.

Determination of L-Arg Metabolites. The citrulline concentrations of the HC culture supernatants 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 (9). The chromophore, ferredoxin (from *Clostridium pasteurianum*; Sigma Chemical Co.) was used to detect NO· release from cultured HC as described (5).

Results and Discussion

Supernatants from KC exposed to IFN-γ and LPS induced a 35–50% suppression in HC total protein synthesis (Table I). Associated with the suppression in pro-

TABLE I

NMA Inhibits KC Supernatant Induction of HC NO_2^-/NO_3^- Production and the Suppression in HC Protein Synthesis

HC culture conditions					
KC supernatant	NMA (0.1 mM)	L-Arg concentration	Protein synthesis ([^{3}H]leucine incorporation)	NO_2^-/NO_3^-	Citrulline
		mM	*cpm*	*μM*	*μM*
−	−	0.25	58,807 ± 3,977	59 ± 5	5 ± 2
+	−		34,753 ± 6,045 (41)‡	227 ± 11	21 ± 1
−	+	0.25	59,830 ± 6,368	57 ± 11	6 ± 2
+	+		56,936 ± 4,700 (5)	93 ± 4	17 ± 1
−	+	2.0	60,970 ± 3,083	39 ± 10	14 ± 1
+	+		53,735 ± 2,230 (12)	132 ± 7	32 ± 4
−	+	5.0	56,984 ± 4,076	31 ± 7	22 ± 5
+	+		38,387 ± 2,709 (33)	174 ± 1	47 ± 6
Supernatant Agents					
IFN-γ alone	−	0.25	47,938 ± 3,103	59 ± 4	—
LPS alone	−	0.25	48,410 ± 3,273	58 ± 4	—
IFN-γ + LPS	−	0.25	48,128 ± 2,050	73 ± 7	—

* NO_2^-/NO_3^- and citrulline concentrations in the KC supernatant were 41 ± 6 and 10 ± 1 respectively.
‡ The percent suppression compared with HC cultured without KC supernatant shown in parentheses.

tein synthesis, KC supernatant induced a threefold increase in NO_2^-/NO_3^- production by the HC. Both the increase in NO_2^-/NO_3^- production and the suppression in HC protein synthesis were blocked if NMA, an inhibitor of NO· production from L-arg (8), was added to the HC cultures with the conditioned KC supernatant. As seen in Mϕ (8), the inhibitory effect of NMA on HC NO_2^-/NO_3^- production could be overcome with higher L-arg concentrations. These results strongly support the hypothesis that conditioned KC supernatant induced L-arg metabolism within the HC. However, in contrast to Mϕ/KC, HC did not produce increases in citrulline that were equimolar in concentration to NO_2^-/NO_3^-. Furthermore, no citrulline could be measured in lysates of HC exposed to KC supernatant, indicating that the citrulline was not retained intracellularly (data not shown). It is possible that the citrulline was further metabolized, such as in the urea cycle.

Addition of IFN-γ (100 U/ml) or LPS (10 μg/ml) to HC cultures, either alone or in combination, had no effect on HC protein synthesis or NO_2^-/NO_3^- production (Table I). Similarly, the known endothelial cell stimulants, acetylcholine (10 μM) (1, 2), bradykinin (100 nM), and histamine (30 μg/ml), did not induce L-arg metabolism within the HC cultures (data not shown). These findings rule out the possibility that the increase in NO_2^-/NO_3^- observed in KC supernatant-stimulated HC cultures was due to small numbers of contaminating Mϕ or endothelial cells. Furthermore, the findings show that these Mϕ and endothelial cell stimulants were not direct inducers of HC L-arg metabolism to NO·.

An active KC supernatant could be generated in the absence of L-arg (Table II). However, when this L-arg-free KC supernatant was added to HC, there was a strict

TABLE II

KC Supernatant Induction of HC NO_2^-/NO_3^- Production and Suppression of HC Protein Synthesis are Dependent on L-Arg

Arginine concentration in HC cultures	L-Arg-free KC supernatant	Protein synthesis ([^{3}H]leucine incorporation)	NO_2^-/NO_3^-	Citrulline
mM		*cpm*	*μM**	*μM*‡
0	–	44,726 ± 1,675	27 ± 8	2 ± 1
	+	42,020 ± 2,563 (6)§	116 ± 12	6 ± 2
0.05	–	59,691 ± 3,154	23 ± 3	3 ± 1
	+	44,744 ± 3,153 (25)	335 ± 1	5 ± 1
0.5	–	59,061 ± 4,734	26 ± 8	4 ± 2
	+	31,876 ± 1,308 (46)	666 ± 13	10 ± 1
2.0	–	56,161 ± 5,239	21 ± 7	10 ± 2
	+	34,758 ± 2,383 (38)	749 ± 9	17 ± 1
4.0	–	58,842 ± 5,100	25 ± 8	16 ± 3
	+	34,820 ± 2,481 (41)	740 ± 16	25 ± 2

* NO_2^-/NO_3^- concentrations of the L-arg-free KC supernatant exposed to 0, 0.5, and 4.0 mM L-arg for 18 h were 32 ± 1, 31 ± 2, and 27 ± 2, respectively.

‡ Citrulline concentrations of the L-arg-free KC supernatant exposed to 0, 0.5, and 4.0 mM L-arg for 18 h were 2 ± 1, 2 ± 1, and 8 ± 1, respectively.

§ The percent suppression compared with HC cultured without KC supernatant shown in parentheses.

requirement for L-arg concentrations ≥0.5 mM for both the suppression of protein synthesis and the maximal production of NO_2^-/NO_3^-. This finding excludes the possibility that a stable nitrosyl compound derived from L-arg was transferred in the KC supernatant, which then degraded in the HC cultures, providing a source of NO·. In addition, no increase in NO_2^-/NO_3^- levels was seen if L-arg was added to the L-arg–free KC supernatant in the absence of cells, excluding the possibility that the NO· synthetase enzyme was transferred in the supernatant. The minor changes in NO_2^-/NO_3^- concentration and protein synthesis that occurred in the absence of exogenous L-arg may reflect another L-arg source in the culture system, such as from the catabolism of serum proteins or intracellular L-arg stores.

The time interval required for the induction of HC L-arg metabolism and the inhibition of protein synthesis is illustrated in Fig. 1. HC NO_2^-/NO_3^- production began after 8 h of KC supernatant exposure and increased in a linear manner from 8 to 24 h. Beyond 24 h, the NO_2^-/NO_3^- production appeared to plateau. The 8-h delay in NO_2^-/NO_3^- production is similar to that seen in Mϕ in which the NO· synthetase enzyme requires an induction period (13). The suppression of HC protein synthesis followed a similar pattern. However, the suppression lagged behind the NO_2^-/NO_3^- release with an initial delay of 12 h after exposure to KC supernatant.

Direct measurements of NO· production by cells have been difficult because NO· rapidly reacts with oxygen and water to form NO_2^-/NO_3^-. Recently, Stuehr et al. (5) have described an assay for NO· production that can be performed under aerobic conditions using *Clostridium pasteurianum* ferredoxin (Fd). Fd exhibits a decrease in absorbance at 410 nm when NO· (but not NO_2^-/NO_3^-) reacts with its intrinsic iron-sulfur center. HC that had been exposed to KC supernatant induced a significant decrease in the absorbance of Fd compared to unstimulated HC (Fig. 2). This HC-mediated bleaching of Fd absorbance was blocked by NMA, confirming that the HC used L-arg to produce NO·.

These findings represent the first demonstration that parenchymal cells from a solid organ are capable of metabolizing large quantities of L-arg to NO·. Previously, only endothelial cells (1, 2) and activated immune cells, such as Mϕ (3–5) and neutrophils (7), have been shown to produce NO· from L-arg. Based on the quantity of NO_2^-/NO_3^- produced per cell, HC appear to generate far greater quantities of nitrogen oxides than these other cell types. In addition, HC L-arg metabolism to

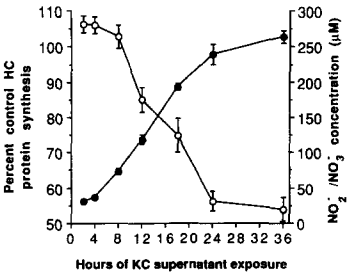

FIGURE 1. Kinetics of HC NO_2^-/NO_3^- (●) production and suppression of HC protein synthesis (○) after exposure to KC supernatant. At time intervals from 2–36 h after the addition of KC supernatant, HC production of NO_2^-/NO_3^- and protein synthesis were determined. The protein synthesis of HC exposed to KC supernatant is expressed as a percent of the protein synthesis of control (unstimulated) HC cultures from the corresponding time interval. The concentration of NO_2^-/NO_3^- in the control HC cultures ranged from 22 ± 3 μM at 2 h to 30 ± 5 μM at 36 h. The concentration of NO_2^-/NO_3^- in the KC supernatant was 30 ± 4 μM. Results are the mean ± SD of quadruplicate cultures from one of five similar experiments.

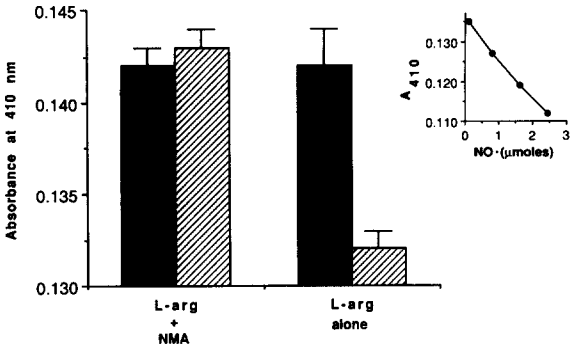

FIGURE 2. HC production of NO· as determined by the loss of ferredoxin (Fd) absorbance at 410 nm. HC were exposed to L-arg-free KC supernatant (▨) or L-arg-free medium (■) for 18 h. The culture medium was aspirated and 0.7 ml of 4.1 μM Fd in Eagle's medium without phenol red added. At this time, the cultures were also supplemented with 2 mM L-arg either alone or with 0.5 mM NMA. The incubation with Fd continued to 18 h after which the culture supernatants were collected and the absorbance at 410 nm was determined. Results are the mean ± SD of triplicate cultures from one of four similar experiments. (*Inset*) Bleaching of Fd chromophore as a function of added NO·. Authentic NO· was transferred in a gas-tight syringe in 20-μl aliquots to 1.5 ml of deoxygenated 4.1 μM Fd in an anaerobic cuvette, and the absorbance at 410 nm determined.

NO· was induced by the inflammatory products of activated KC, its neighboring cell type in vivo. The only other cell known to respond to products of activated Mϕ in this manner is the murine adenocarcinoma cell line EMT-6 (14). The net observed effect of HC L-arg metabolism to NO· is a decrease in protein synthesis. How NO· production induces this change is unknown, but it may be that HC-derived NO· impairs the mitochondrial respiration and ATP production needed for protein synthesis similar to the effects of NO· on tumor cell mitochondrial function (4, 15). Also possible is that the HC-derived NO· mediates changes in cell function through other mechanisms or enzyme systems. For example, NO· appears to directly activate ADP-ribosyl transferase (16) and it is known that NO· induces increases in intracellular cGMP by activating guanylate cyclase (1).

We originally established the coculture model as a method to study the role of KC and HC interactions in the changes in liver function associated with sepsis and organ failure states. These data provide evidence that KC modulation of HC function may include the induction of L-arg metabolism to NO· within the HC. How this HC-derived NO· may contribute to the physiologic and pathologic responses of the liver remains to be determined.

Summary

A metabolic pathway by which L-arginine (L-arg) is converted to the biologically active compound NO· has recently been described in macrophages (Mϕ) and endothelial cells. This report demonstrates that transferable products from activated Kupffer cells (KC) induce the conversion of large quantities of L-arg to nitrogen oxides within hepatocytes (HC). In Mϕ and endothelial cells, citrulline and NO_2^-/NO_3^- are the stable endproducts of this metabolic pathway. In contrast, HC L-arg metabolism resulted in significantly greater production of NO_2^-/NO_3^- than citrulline. The generation of NO· within HC was associated with a concurrent decrease in total protein synthesis.

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