

## ***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<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>) 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<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> 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<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> 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<sup>-6</sup> 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<sup>G</sup>-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  $\geq 90\%$  purity as determined by peroxidase staining (10).

**Cell Culture Technique.** Conditioned KC supernatant was generated by plating  $6 \times 10^6$  KC in 3 ml in 25-cm<sup>2</sup> flasks (Costar, Cambridge, MA) with recombinant rat IFN- $\gamma$  (100 units/ml; Amgen Biologicals, Thousand Oaks, CA). After 24 h, the medium was changed and LPS (10  $\mu\text{g/ml}$ ) was added (from *Escherichia coli*, 0111:B<sub>4</sub>; Difco Laboratories, Detroit, MI). The culture supernatants were collected 8 h later, filtered through 0.2  $\mu\text{m}$  pore filters, and stored at  $-70^\circ\text{C}$  until use.

HC were plated at  $2 \times 10^5$  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  $\text{NO}_2^-/\text{NO}_3^-$  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 [<sup>3</sup>H]leucine (5.0 Ci/mmol, 1.0  $\mu\text{Ci/well}$ ; 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 [<sup>3</sup>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  $\text{NO}_2^-/\text{NO}_3^-$  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  $\text{NO}^-$  release from cultured HC as described (5).

## Results and Discussion

Supernatants from KC exposed to IFN- $\gamma$  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  $\text{NO}_2^-/\text{NO}_3^-$  Production and the Suppression in HC Protein Synthesis*

HC culture conditions					
KC supernatant	NMA (0.1 mM)	L-Arg concentration	Protein synthesis ([ <sup>3</sup> H]leucine incorporation)	$\text{NO}_2^-/\text{NO}_3^-$	Citrulline
		mM	cpm	$\mu\text{M}$	$\mu\text{M}$
-	-	0.25	58,807 $\pm$ 3,977	59 $\pm$ 5	5 $\pm$ 2
+	-		34,753 $\pm$ 6,045 (41) <sup>†</sup>	227 $\pm$ 11	21 $\pm$ 1
-	+	0.25	59,830 $\pm$ 6,368	57 $\pm$ 11	6 $\pm$ 2
+	+		56,936 $\pm$ 4,700 (5)	93 $\pm$ 4	17 $\pm$ 1
-	+	2.0	60,970 $\pm$ 3,083	39 $\pm$ 10	14 $\pm$ 1
+	+		53,735 $\pm$ 2,230 (12)	132 $\pm$ 7	32 $\pm$ 4
-	+	5.0	56,984 $\pm$ 4,076	31 $\pm$ 7	22 $\pm$ 5
+	+		38,387 $\pm$ 2,709 (33)	174 $\pm$ 1	47 $\pm$ 6
Supernatant Agents					
IFN- $\gamma$ alone	-	0.25	47,938 $\pm$ 3,103	59 $\pm$ 4	-
LPS alone	-	0.25	48,410 $\pm$ 3,273	58 $\pm$ 4	-
IFN- $\gamma$ + LPS	-	0.25	48,128 $\pm$ 2,050	73 $\pm$ 7	-

\*  $\text{NO}_2^-/\text{NO}_3^-$  and citrulline concentrations in the KC supernatant were  $41 \pm 6$  and  $10 \pm 1$  respectively.

<sup>†</sup> The percent suppression compared with HC cultured without KC supernatant shown in parentheses.

tein synthesis, KC supernatant induced a threefold increase in  $\text{NO}_2^-/\text{NO}_3^-$  production by the HC. Both the increase in  $\text{NO}_2^-/\text{NO}_3^-$  production and the suppression in HC protein synthesis were blocked if NMA, an inhibitor of  $\text{NO}\cdot$  production from L-arg (8), was added to the HC cultures with the conditioned KC supernatant. As seen in M $\phi$  (8), the inhibitory effect of NMA on HC  $\text{NO}_2^-/\text{NO}_3^-$  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 M $\phi$ /KC, HC did not produce increases in citrulline that were equimolar in concentration to  $\text{NO}_2^-/\text{NO}_3^-$ . 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- $\gamma$  (100 U/ml) or LPS (10  $\mu\text{g}/\text{ml}$ ) to HC cultures, either alone or in combination, had no effect on HC protein synthesis or  $\text{NO}_2^-/\text{NO}_3^-$  production (Table I). Similarly, the known endothelial cell stimulants, acetylcholine (10  $\mu\text{M}$ ) (1, 2), bradykinin (100 nM), and histamine (30  $\mu\text{g}/\text{ml}$ ), did not induce L-arg metabolism within the HC cultures (data not shown). These findings rule out the possibility that the increase in  $\text{NO}_2^-/\text{NO}_3^-$  observed in KC supernatant-stimulated HC cultures was due to small numbers of contaminating M $\phi$  or endothelial cells. Furthermore, the findings show that these M $\phi$  and endothelial cell stimulants were not direct inducers of HC L-arg metabolism to  $\text{NO}\cdot$ .

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  $\text{NO}_2^-/\text{NO}_3^-$  Production and  
Suppression of HC Protein Synthesis are Dependent on L-Arg

Arginine concentration in HC cultures	L-Arg-free KC supernatant	Protein synthesis ( $^3\text{H}$ )leucine incorporation)	$\text{NO}_2^-/\text{NO}_3^-$	Citrulline
<i>mM</i>		<i>cpm</i>	$\mu\text{M}^*$	$\mu\text{M}^\dagger$
0	-	44,726 $\pm$ 1,675	27 $\pm$ 8	2 $\pm$ 1
	+	42,020 $\pm$ 2,563 (6) <sup>§</sup>	116 $\pm$ 12	6 $\pm$ 2
0.05	-	59,691 $\pm$ 3,154	23 $\pm$ 3	3 $\pm$ 1
	+	44,744 $\pm$ 3,153 (25)	335 $\pm$ 1	5 $\pm$ 1
0.5	-	59,061 $\pm$ 4,734	26 $\pm$ 8	4 $\pm$ 2
	+	31,876 $\pm$ 1,308 (46)	666 $\pm$ 13	10 $\pm$ 1
2.0	-	56,161 $\pm$ 5,239	21 $\pm$ 7	10 $\pm$ 2
	+	34,758 $\pm$ 2,383 (38)	749 $\pm$ 9	17 $\pm$ 1
4.0	-	58,842 $\pm$ 5,100	25 $\pm$ 8	16 $\pm$ 3
	+	34,820 $\pm$ 2,481 (41)	740 $\pm$ 16	25 $\pm$ 2

\*  $\text{NO}_2^-/\text{NO}_3^-$  concentrations of the L-arg-free KC supernatant exposed to 0, 0.5, and 4.0 mM L-arg for 18 h were 32  $\pm$  1, 31  $\pm$  2, and 27  $\pm$  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  $\pm$  1, 2  $\pm$  1, and 8  $\pm$  1, respectively.

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

requirement for L-arg concentrations  $\geq 0.5$  mM for both the suppression of protein synthesis and the maximal production of  $\text{NO}_2^-/\text{NO}_3^-$ . 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  $\text{NO}\cdot$ . In addition, no increase in  $\text{NO}_2^-/\text{NO}_3^-$  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  $\text{NO}\cdot$  synthetase enzyme was transferred in the supernatant. The minor changes in  $\text{NO}_2^-/\text{NO}_3^-$  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  $\text{NO}_2^-/\text{NO}_3^-$  production began after 8 h of KC supernatant exposure and increased in a linear manner from 8 to 24 h. Beyond 24 h, the  $\text{NO}_2^-/\text{NO}_3^-$  production appeared to plateau. The 8-h delay in  $\text{NO}_2^-/\text{NO}_3^-$  production is similar to that seen in  $\text{M}\phi$  in which the  $\text{NO}\cdot$  synthetase enzyme requires an induction period (13). The suppression of HC protein synthesis followed a similar pattern. However, the suppression lagged behind the  $\text{NO}_2^-/\text{NO}_3^-$  release with an initial delay of 12 h after exposure to KC supernatant.

Direct measurements of  $\text{NO}\cdot$  production by cells have been difficult because  $\text{NO}\cdot$  rapidly reacts with oxygen and water to form  $\text{NO}_2^-/\text{NO}_3^-$ . Recently, Stuehr et al. (5) have described an assay for  $\text{NO}\cdot$  production that can be performed under aerobic conditions using *Clostridium pasteurianum* ferredoxin (Fd). Fd exhibits a decrease in absorbance at 410 nm when  $\text{NO}\cdot$  (but not  $\text{NO}_2^-/\text{NO}_3^-$ ) 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  $\text{NO}\cdot$ .

These findings represent the first demonstration that parenchymal cells from a solid organ are capable of metabolizing large quantities of L-arg to  $\text{NO}\cdot$ . Previously, only endothelial cells (1, 2) and activated immune cells, such as  $\text{M}\phi$  (3-5) and neutrophils (7), have been shown to produce  $\text{NO}\cdot$  from L-arg. Based on the quantity of  $\text{NO}_2^-/\text{NO}_3^-$  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

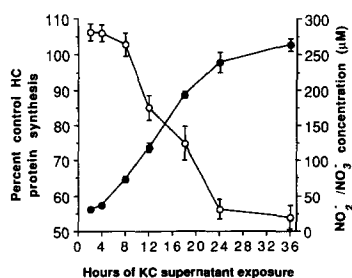


FIGURE 1. Kinetics of HC  $\text{NO}_2^-/\text{NO}_3^-$  (●) production and suppression of HC protein synthesis (○) after exposure to KC supernatant. At time intervals from 2-36 h after the addition of KC supernatant, HC production of  $\text{NO}_2^-/\text{NO}_3^-$  and protein synthesis were determined. 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  $\text{NO}_2^-/\text{NO}_3^-$  in the control HC cultures ranged from  $22 \pm 3 \mu\text{M}$  at 2 h to  $30 \pm 5 \mu\text{M}$  at 36 h. The concentration of  $\text{NO}_2^-/\text{NO}_3^-$  in the KC supernatant was  $30 \pm 4 \mu\text{M}$ . Results are the mean  $\pm$  SD of quadruplicate cultures from one of five similar experiments.

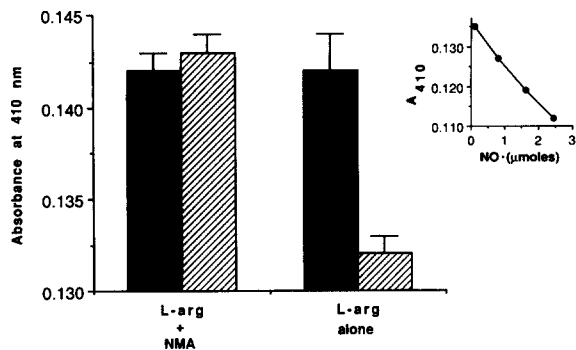


FIGURE 2. HC production of  $\text{NO}\cdot$  as determined by the loss of ferredoxin (Fd) absorbance at 410 nm. HC were exposed to L-arg-free KC supernatant (▨) or L-arg-free medium (■) for 18 h. The culture medium was aspirated and 0.7 ml of 4.1  $\mu\text{M}$  Fd in Eagle's medium without phenol red 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  $\pm$  SD of triplicate cultures from one of four

similar experiments. (Inset) Bleaching of Fd chromophore as a function of added  $\text{NO}\cdot$ . Authentic  $\text{NO}\cdot$  was transferred in a gas-tight syringe in 20- $\mu\text{l}$  aliquots to 1.5 ml of deoxygenated 4.1  $\mu\text{M}$  Fd in an anaerobic cuvette, and the absorbance at 410 nm determined.

$\text{NO}\cdot$  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  $\text{M}\phi$  in this manner is the murine adenocarcinoma cell line EMT-6 (14). The net observed effect of HC L-arg metabolism to  $\text{NO}\cdot$  is a decrease in protein synthesis. How  $\text{NO}\cdot$  production induces this change is unknown, but it may be that HC-derived  $\text{NO}\cdot$  impairs the mitochondrial respiration and ATP production needed for protein synthesis similar to the effects of  $\text{NO}\cdot$  on tumor cell mitochondrial function (4, 15). Also possible is that the HC-derived  $\text{NO}\cdot$  mediates changes in cell function through other mechanisms or enzyme systems. For example,  $\text{NO}\cdot$  appears to directly activate ADP-ribosyl transferase (16) and it is known that  $\text{NO}\cdot$  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  $\text{NO}\cdot$  within the HC. How this HC-derived  $\text{NO}\cdot$  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  $\text{NO}\cdot$  has recently been described in macrophages ( $\text{M}\phi$ ) 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  $\text{M}\phi$  and endothelial cells, citrulline and  $\text{NO}_2^-/\text{NO}_3^-$  are the stable endproducts of this metabolic pathway. In contrast, HC L-arg metabolism resulted in significantly greater production of  $\text{NO}_2^-/\text{NO}_3^-$  than citrulline. The generation of  $\text{NO}\cdot$  within HC was associated with a concurrent decrease in total protein synthesis.

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