Brief Definitive Report

Stimulation of the Nitric Oxide Synthase Pathway in Human Hepatocytes by Cytokines and Endotoxin

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Summary

Nitric oxide (NO) is a short-lived biologic mediator that is shown to be induced in various cell types and to cause many metabolic changes in target cells. Inhibition of tumor cell growth and antimicrobial activity has been attributed to the stimulation of the inducible type of the NO synthase (NOS). However, there is limited evidence for the existence of such inducible NOS in a human cell type. We show here the induction of NO biosynthesis in freshly isolated human hepatocytes (HC) after stimulation with interleukin 1, tumor necrosis factor (TNF), IFN-γ, and endotoxin. Increased levels of nitrite (NO_2^-) and nitrate (NO_3^-) in culture supernatants were associated with NADPH-dependent NOS activity in the cell lysates. The production of NO_2^- and NO_3^- was inhibited by N^G-monomethyl L-arginine and was associated with an increase in cyclic guanylate monophosphate release. The data presented here provide evidence for the existence of typical inducible NO biosynthesis in a human cell type.

A variety of cell types have been shown to produce nitric oxide (NO) from L-arginine by either a constitutive and/or an inducible enzyme (1). Recent reports provide strong evidence that vasorelaxation, induced through the constitutive and inducible NO pathway, might play an important role in the regulation of vascular tone in humans (2, 3). Little evidence for the existence of inducible NO enzyme activity in specific human cell types has been documented, despite its clear potential role in the elimination of tumor cells (4) and intra- and extracellular pathogens (recently reviewed in reference 5), as well as in the induction of sustained hypotension (6), as shown in animal models.

We have shown that rat hepatocytes (HC) cocultured with Kupffer cells and stimulated with LPS can produce large amounts of nitrite (NO_2^-) and nitrate (NO_3^-), the stable end products of NO (7). Furthermore, we have shown that HC alone are capable of biosynthesis of NO when exposed to various immunostimuli (8–10). The expression of the inducible nitric oxide synthase (NOS) alters various HC functions in vitro. These include a substantial decrease in total protein synthesis (7, 11), the inhibition of mitochondrial aconitase (12), and the stimulation of cyclic guanylate monophosphate (cGMP) synthesis and release (13). In vivo studies suggest that hepatic NO can protect the liver from damage in sepsis (14). In this communication, we report that nitrogen oxides are also produced in large amounts by human HC in a reproducible manner, demonstrating that a human cell type can, indeed, express an inducible NOS.

Materials and Methods

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

Hepatocyte Isolation. In accordance with institutional review guidelines, human HC were isolated from histologically normal wedge sections of liver resection specimens by collagenase (0.5%, type A; Boehringer Ingelheim, Germany) perfusion. Immediately after isolation, HC were purified over a Percoll gradient to obtain a highly purified cell population (16). After this procedure, purity exceeded 97% (assessed by microscopy) and viability ranged from 89 to 99% by trypan blue exclusion. HC (2×10^5) were cultured in 24-well tissue culture trays and incubated with 5% CO_2 at 37°C for 24 h before use in experiments.

Cell Culture Technique. HC were incubated with 0.5 ml of either a mixture of 10 μg/ml LPS (*Escherichia coli*, 011:B_4; Sigma Chemical Co., St. Louis, MO), 5 U/ml human recombinant (rh) IL-1β (Cistron), 500 U/ml rhTNF-α (Genzyme, Boston, MA), and 100 U/ml rhIFN-γ (Amgen Biologicals, Thousand Oaks, CA), or various combinations of these signals for 24 and 48 h. At the

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indicated time points, supernatants were collected and NO_2^-/NO_3^- and extracellular cGMP levels determined. When indicated, 0.1 mM NMA with or without 2.0 mM L-arginine was added to the cultures with the signals. The presence of calmodulin inhibitors (10 μM trifluoperazine, 10 μM W5 [*N*-(6-aminohexyl)-5-chloro-2-naphthalenesulfonamide]) on HC NO_2^- and NO_3^- formation was also tested.

Preparation of Cytosol. Either control HC in culture or HC stimulated with cytokines and LPS were scraped up and pelleted at 250 *g* for 5 min (24 h after the addition of the signals). H_2O with protease inhibitors (5 μg/ml aprotinin, 0.1 mM PMSF, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin) was added to 10^8 HC/ml, and the cells were lysed by three rapid freeze/thaw cycles. A 100,000 *g*, supernatant was used for the enzyme assay as described (10).

Determination of NO_2^-/NO_3^- and cGMP. cGMP levels were measured using a commercially available radioimmunoassay (Amersham Corp., Arlington Heights, IL) that had a sensitivity range of 2–128 fmol/assay sample. To assess the amount of NO produced, the culture supernatants were assayed for the stable end products of NO, NO_2^-, and NO_3^- with an automated procedure based on the Griess reaction, as previously described (7).

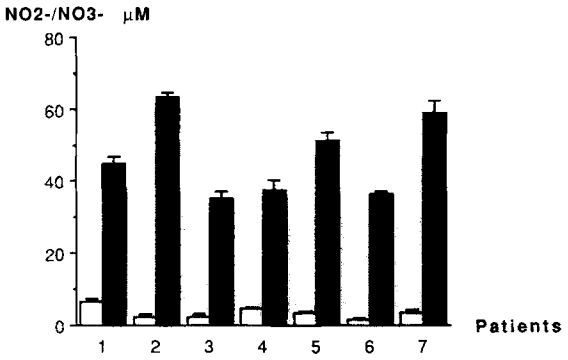

Figure 1. NO_2^- and NO_3^- levels in human HC supernatant 24 h after exposure to a mixture of cytokines and LPS. Markedly elevated levels of NO_2^- and NO_3^- were found in all stimulated cultures (■) when compared with nonstimulated (□). Results show the mean ± SEM for triplicate cultures from seven different individuals.

Results and Discussion

The stimulation of human HC with a combination of rhTNF-α, rhIL-1-β, rhIFN-γ, and LPS resulted in a marked increase in NO_2^- and NO_3^- in supernatants of human HC isolated from liver specimens from seven separate individuals (Fig. 1). The NO_2^-/NO_3^- levels were over three times higher at 48 h than 24 h (Fig. 2). The addition of NMA, a competitive inhibitor of NO synthesis (4), almost completely suppressed NO_2^-/NO_3^- production at both time points, an effect that could be partially overcome by additional L-arginine (2.0 mM). The addition of calmodulin inhibitors, trifluoperazine, and W5 (10 μM) had no significant effect on HC NO synthesis in response to cytokines plus LPS (data not shown). This result is in keeping with earlier observations, showing that the inducible NOS does not depend on the presence of Ca^{2+} or calmodulin (1).

The cytokines IL-1β, IFN-γ, and TNF-α have been shown to increase the expression of inducible NOS in several rodent cell types (1). These single cytokines or LPS did not increase NO_2^-/NO_3^- formation in human HC over control levels after either 24 or 48 h of incubation (Table 1). Combinations of two of these signals (six combinations) induced only minor (<5%) increases in NO_2^-/NO_3^- synthesis. Of the four possible combinations of three signals, the combination of TNF/IL-1/IFN-γ was most effective with NO_2^-/NO_3^- levels reaching 67% of the maximum seen at 24 h with all four signals (three = 25.4 ± 2.9, four = 37.8 ± 2.0). NO is a potent inducer of soluble guanylate cyclase resulting in increased cGMP production. Through this action, NO plays a role in smooth muscle relaxation (17), signaling in the central nerve system (18), and reduced platelet aggregation and adherence (19). We have recently shown that rat HC release large amounts of cGMP coincident with the synthesis of NO_2^-/NO_3^- after cytokine and LPS stimulation (13). After 24 h in culture, human HC were found to have released small amounts of cGMP if unstimulated or exposed to single cytokines or LPS (Table 1). NMA reduced both the cGMP and NO_2^-/NO_3^- generation, suggesting that low levels of NO may be produced under these conditions. When exposed to the combination of four signals, a marked increase in cGMP release was associated with high NO_2^-/NO_3^- synthesis. This cGMP release was inhibited by NMA, suggesting that the increased cGMP release was due to NO synthesis. cGMP levels were actually lower by the 48-h time point. This reduction in supernatant cGMP might be explained by the presence of phosphodiesterases (20).

Reconstitution of enzyme activity for NO synthesis in cytosolic preparations from both rat HC (10) and other cell types (1) requires the addition of NADPH. Cytosol was obtained from cultured human HC 24 h after stimulation with cytokines and LPS. The addition of L-arginine (8 mM), or NADPH (0.1 mM) alone, resulted in 0.84 nmol/mg protein/3 h and 0.05 nmol NO_2^-/mg protein/3 h, respectively.

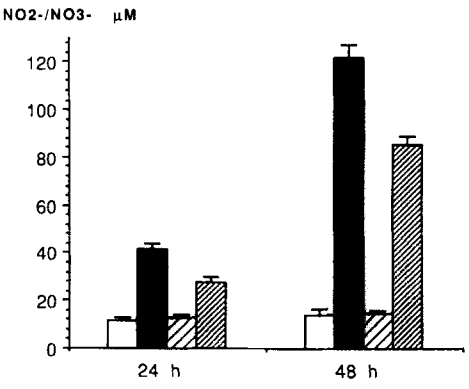

Figure 2. Human HC were stimulated as described above. Supernatants from control (□) and stimulated cultures (■) were tested for NO_2^-/NO_3^- 24 and 48 h later. To determine if the source of NO_2^-/NO_3^- was via the induction of NOS, cells were incubated in the presence of NMA (0.1 mM) (▨) or with NMA plus the simultaneous addition of 2.0 mM L-arginine (▨). Results are expressed as the mean of four different experiments ± SEM.

Table 1. *NO_2^-/NO_3^- and cGMP Levels in Supernatants of Human Hepatocyte Stimulated with Various Cytokines and LPS*

Signal	Treatment (0.1 mM NMA)	24 h NO_2^-/NO_3^-	24 h cGMP (per 2 × 10^5 hepatocytes)	48 h NO_2^-/NO_3^-	48 h cGMP (per 2 × 10^5 hepatocytes)
		μM	*fmol*	*μM*	*fmol*
Medium	–	8.3 ± 2.2‡	92.0 ± 5.8	14.1 ± 0.8	79.5 ± 4.8
	+	2.6 ± 0.1	31.0 ± 7.0	2.1 ± 0.02	<2.0
rhTNF-α	–	4.8 ± 0.2	51.5 ± 4.5	5.2 ± 0.9	<2.0
	+	1.8 ± 0.1	<2.0	1.6 ± 0.3	<2.0
rhIL-1	–	4.7 ± 0.3	42.5 ± 4.5	6.9 ± 0.5	<2.0
	+	2.0 ± 0.4	<2.0	3.1 ± 0.6	<2.0
rhIFN-γ	–	5.1 ± 0.3	<2.0	6.9 ± 0.3	<2.0
	+	1.4 ± 0.5	<2.0	1.7 ± 0.2	<2.0
LPS	–	4.7 ± 0.2	<2.0	7.1 ± 0.4	<2.0
	+	1.1 ± 0.2	<2.0	2.4 ± 0.5	<2.0
CM	–	47.8 ± 2.1	420.5 ± 23.2	125.2 ± 7.3	321.5 ± 7.3
	+	12.8 ± 0.5	21.0 ± 3.4	14.8 ± 0.8	63.5 ± 3.2

* rhTNF-α, 500 U/ml; rhIL-1, 5 U/ml; rhIFN-γ, 100 U/ml; LPS, 10 μg/ml; CM, all four signals combined.
‡ Values represent the mean ± SEM for triplicate cultures for one of three similar experiments.

The combination of L-arginine with NADPH resulted in 3.50 nmol/mg protein/3 h, while the addition of FAD (4 μM) and tetrahydrobiopterin (5 μM) increased NO production to 24.10 nmol/mg protein/3 h. The addition of EDTA had no effect on HC cytosol NOS activity and the addition of NADPH and L-arginine to cytosol obtained from unstimulated HC showed no NO generation (data not shown). These data show that human HC cytosolic enzyme activity does require NADPH and that cytosol from human HC in culture may be more dependent on added FAD and tetrahydrobiopterin than reported for freshly isolated rat HC (10).

The data presented here provide evidence for the existence of inducible NOS in a human cell type. A recent publication showed NO_2^- production in human monocytes (21). Unlike our findings in which human HC respond in a similar manner to rodent HC in terms of signals and time course, human monocytes exhibited marked differences compared with murine macrophages. Instead of responding to LPS and IFN-γ within hours, as seen in rodent cells, human monocytes required up to 5 d of stimulation with colony-stimulating factor and TNF for induction of detectable NO_2^- levels (22). The finding of the more typical type of inducible NOS in human HC obtained from histologically normal liver provides support for an inducible source of NO to explain the elevated nitrogen oxide levels in septic and cytokine-treated patients (22, 23). Recent identification of a constitutively expressed NOS that is Ca^{2+} independent from human colon adenocarcinoma cell lines suggests that certain human tumor cells may contain a unique NOS or may continuously express an NOS with inducible characteristics (24). Even though our data suggest that inducible HC NO may serve as a signaling molecule for cGMP production, the actual physiologic and pathologic significance of NO production in human HC is unknown and its role in the liver remains to be determined.

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