Multiple Cytokines Are Required to Induce Hepatocyte Nitric Oxide Production and Inhibit Total Protein Synthesis

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The etiology and mechanisms by which severe trauma or sepsis induce hepatic failure are unknown. Previously we showed that Kupffer cells (KC), the fixed macrophages of the liver, induce a profound decrease in hepatocyte (HC) total-protein synthesis when exposed to endotoxin. Furthermore we demonstrated that endotoxin-activated KCs induce these changes in HC protein synthesis through the induction of a novel L-arginine–dependent biochemical pathway within the HC. In this pathway, the guanido nitrogen of L-arginine is converted to the highly reactive molecule nitric oxide (NO·). To identify the KC factors that act as signals for induction of HC NO· biosynthesis, recombinant cytokines were added to HC cultures and HC nitrogen oxide production and protein synthesis levels were determined. We found that no single cytokine, but rather a specific combination of tumor necrosis factor, interleukin-1, interferon-gamma, and endotoxin, were required for maximal induction of HC nitrogen oxide production. This specific combination of cytokines induced a 248.8 ± 26.0 μmol/L (micromolar) increase in HC nitrogen oxide production and simultaneously inhibited HC total protein synthesis by 36.1% ± 3.1%. These data demonstrate that multiple cytokines, produced by endotoxin-activated KC, induce the production of NO· within HC, which in turn leads to the inhibition of HC total-protein synthesis.

SIGNIFICANT CHANGES IN hepatic function occur in surgical patients after severe trauma, extensive operations, and in sepsis. Some of these changes in metabolism, such as the hepatic acute-phase response, are clearly physiologic and adaptive, whereas other changes appear to be pathologic, most clearly the hepatocellular dysfunction accompanying multisystem organ failure. Many advances have been made toward the supportive management of the hepatic failure associated with sepsis, but very little is known of the etiology or mechanisms by which this hepatocellular dysfunction occurs. We have hypothesized that Kupffer cells (KC), the fixed macrophages of the liver, interact with septic stimuli within the portal circulation and respond by producing mediators that alter the function of contiguous hepatocytes (HC) in sepsis. In support of this hypothesis, we have demonstrated in a rat HC:KC coculture model that KC activated by endotoxin (lipopolysaccharide, LPS) or killed *Escherichia coli* induce a profound decrease in HC total-protein synthesis.[1,2] Furthermore we identified a novel L-arginine biochemical pathway as the mechanism by which these changes in HC protein synthesis occur.[3-5] In this pathway, a guanido nitrogen of L-arginine is oxidized to form the highly-reactive molecule nitric oxide (NO·), which, in the presence of oxygen and water, rapidly decomposes to the stable and inactive end products, nitrite and nitrate (NO_2^-/NO_3^-).[6-8] Recently we demonstrated that conditioned medium from interferon-gamma (IFNg)- and LPS-activated KC induce the conversion of large quantities of L-arginine to NO· within HC, which, in turn, leads to the inhibition of HC total-protein synthesis.[9] The studies reported here were undertaken to determine the KC factors that act as signals for induction of HC NO· biosynthesis.

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Materials and Methods

Reagents

Liver cell cultures were performed in Williams medium E (0.24 mmol/L [millimolar] L-arginine) supplemented

with 15 mmol/L HEPES, 2 mmol/L L-glutamine, 10^5 U/L penicillin, 100 mg/L streptomycin (Gibco, Grand Island, NY), 10^{-6} mol/L (molar) bovine insulin (Eli Lilly Co., Indianapolis, IN), and 10% heat-activated, low endotoxin calf serum (Hyclone Laboratories, Logan, UT). Hepatocyte culture medium was also supplemented with 10^{-6} mol/L dexamethasone (Sigma Chemical Co., St. Louis, MO). Recombinant rat IFNg (rrIFNg) was purchased from Amgen Biologicals (Thousand Oaks, CA) and recombinant human interleukin 1 beta (rhIL-1) from Cistron Technology (Pine Brook, NJ). Recombinant murine tumor necrosis factor-alpha (rmTNFα) and recombinant human interleukin 6 (rhIL-6) were obtained from Genzyme Corporation (Cambridge, MA). L-[4,5-^{3}H(N)] leucine (^{3}H-leu, specific activity 5.0 Ci/mmol/L) and [methyl-^{3}H] thymidine (^{3}H-thymidine, specific activity 6.7 Ci/mmol/L) were from New England Nuclear (Boston, MA). Lipopolysaccharide (from *E. coli*, 0111:B_4) was obtained from Difco Laboratories (Detroit, MI). N^G monomethyl-L-arginine (NMA) was prepared according to the method of Corbin and Reporter[10] modified as previously described.[11]

Cell Isolation

Liver cells were obtained from male Sprague-Dawley rats that weighed 200 to 300 g (Harlan Sprague-Dawley, Madison, WI). Hepatocytes were harvested using a modification of the Seglan perfusion technique,[12] as previously described.[1] The liver was perfused *in situ* with a 0.05% collagenase solution (Type IV, Sigma) for 20 minutes. After perfusion the capsule of the liver was dissected and the parenchyma gently combed to produce a cell suspension. Hepatocytes then were separated from nonparenchymal cells to more than 98% purity[13] by repeated differential sedimentation at 50*g* for 2 minutes and washing with medium. The HCs were enumerated and viability was consistently more than 85% by trypan blue exclusion.

Liver nonparenchymal cells, which by definition include KC, endothelial cells, Ito cells, and pit cells, were obtained using the pronase perfusion technique of Einess and Planque,[14] as previously described.[15] The liver was perfused *in situ* for 2 minutes with Gey's balanced salt solution (Gibco) containing 0.2% pronase E (Sigma). The liver was then minced into 2- to 3-mm cubes and incubated with continuous stirring for 60 minutes at 37 C. DNase (1 mg, Sigma) was added after 20 and 40 minutes to prevent cell clumping. The liver slurry was filtered through gauze mesh, washed in cold RPMI, and centrifuged at 250*g* for 5 minutes. Centrifugal elutriation was performed to separate the KCs from the other nonparenchymal cells. Viability exceeded 98% by trypan blue exclusion and peroxide staining of the cell suspension identified 85% to 95% of the cells as KCs.

Cell Culture Technique

Conditioned KC supernatants were generated by plating 6×10^6 KC in 3 mL in 25-cm^2 flasks (Costar Data Packaging Co, Cambridge, MA). During the initial incubation, the KCs were cultured in standard medium or medium supplemented with rrIFNg 100 units/mL. After 24 hours the medium was aspirated and replaced with fresh medium containing rrIFNg (100 U/mL) and LPS (10 μg/mL) or LPS alone. After the addition of LPS, the KC supernatants were collected at various time intervals, filtered through 0.2-μm pore-size filters, and stored at -70 C until use.

Hepatocytes were plated in supplemented Williams medium E at a concentration of 2×10^5 cells/mL on gelatin-coated 96-well tissue culture trays (0.1 mL/well) for determination of total protein synthesis, and in 35-mm wells (2 mL) for measurement of NO_2^-/NO_3^- production. Following an initial 18-hour incubation, the medium on the HC cultures was aspirated and KC supernatant (50% dilution), rrIFNg, rhIL-1, rmTNFa, rhIL-6, and/or LPS was added. Hepatocyte total-protein synthesis and NO_2^-/NO_3^- production were assessed after 24 hours of exposure to KC supernatant or recombinant cytokines.

Total-protein Synthesis and NO_2^-/NO_3^- Determination

Hepatocyte total-protein synthesis was measured using a 4-hour labeling interval with ^{3}H-leu (1.0 μCi/well) added in fresh Minimal Essential Medium without L-leucine (Gibco). The label was terminated by lysing the HC with 1 N NaOH and 0.025% triton X-100. The protein was precipitated with cold 40% trichloroacetic acid and collected on filter paper using a multichannel cell harvester. ^{3}H-leu incorporation into protein was measured by liquid scintillation counting. The NO_2^-/NO_3^- concentration of the HC culture supernatants was measured using an automated colorimetric procedure based on the Griess reaction.[16]

Cytokine Assays

TNF. Kupffer cell production of TNF *in vitro* was determined with the lytic assay of L929 fibroblasts, as described by Aggarwal.[17] Briefly serial dilutions of the KC supernatants were added to murine L929 cells cultured in the presence of actinomycin D. After an 18-hour incubation, the cultures were washed and the degree of cell lysis was determined by staining the plates with 0.5% crystal violet in methanol/water. The optical density of the L929 cultures was measured at 560 nm and the quantity of TNF in the conditioned KC supernatants determined from standard curves generated for each experiment using rmTNFα.

Interleukin 1. Interleukin 1 activity in the KC supernatants was determined in the D10.G4.1 cell proliferative assay.[18] Serial dilutions of KC supernatant and 2.5 μg/mL concanvalin A were added to 2 × 10^4 D10.G4.1 cells. After a 65-hour incubation, the cultures were labeled with 2 μCi/well of ^{3}H-thymidine for 6 hours. The cultures were harvested into filter paper and ^{3}H-thymidine incorporation onto DNA was measured by liquid scintillation counting. The concentration of IL-1 in the KC supernatants was extrapolated from standard curves generated for each experiment using rhIL-1.

Interleukin 6. The B-9 hybridoma, provided by Dr. R. Nordan (National Institutes of Health, Bethesda, MD), was used to measure IL-6 activity.[19] Briefly 2 × 10^3 cells were cultured in the presence of serial dilutions of the KC supernatant for 3.5 days. The cultures were labeled with ^{3}H-thymidine (2 μCi/well) for 4 hours and ^{3}H-thymidine incorporation into DNA was determined. The IL-6 activity in the KC supernatants was determined from standard curves constructed with rhIL-6.

Results

Previously we demonstrated that conditioned medium from activated KCs induces HC NO• production and suppresses HC total-protein synthesis.[9] To generate this active KC supernatant, it was necessary to expose the KC to rrIFNg for 24 hours before the addition of LPS. This rrIFN and LPS-activated KC supernatant harvested 8 hours after the addition of LPS induced a 284 ± 7.7 μmol/L (micromolar) increase in HC NO_2^-/NO_3^- production and simultaneously inhibited HC total-protein synthesis by 42.1% ± 3.3% (Fig. 1). If harvested later, 24 and 36 hours after the addition of LPS, the KC supernatant was less active, inducing lower amounts of NO_2^-/NO_3^- production by HC and inhibiting protein synthesis to a lesser degree. While LPS alone is sufficient stimulus to induce L-arginine metabolism to NO• in KC:HC cocultures,[3-5] KC supernatants generated with LPS alone stimulated only a modest increase (56 to 110 μmol/L) in HC NO_2^-/NO_3^- production and had no effect on HC protein synthesis. Therefore maximal induction of HC NO• production and inhibition of total-protein synthesis were dependent not only on the presence of both rrIFNg and LPS but also on a precise interval at which the KC supernatant was collected after stimulation.

There were two possible hypotheses to explain the need for IFNg in the generation of an active KC supernatant. The first possibility was that IFNg may have been acting on the KC to stimulate the synthesis of one or more KC factors, which were, in turn, needed to induce NO• biosynthesis by the HC. A second alternative hypothesis was that IFNg could act directly on the HC, functioning as one of the signals for induction of HC NO• production.

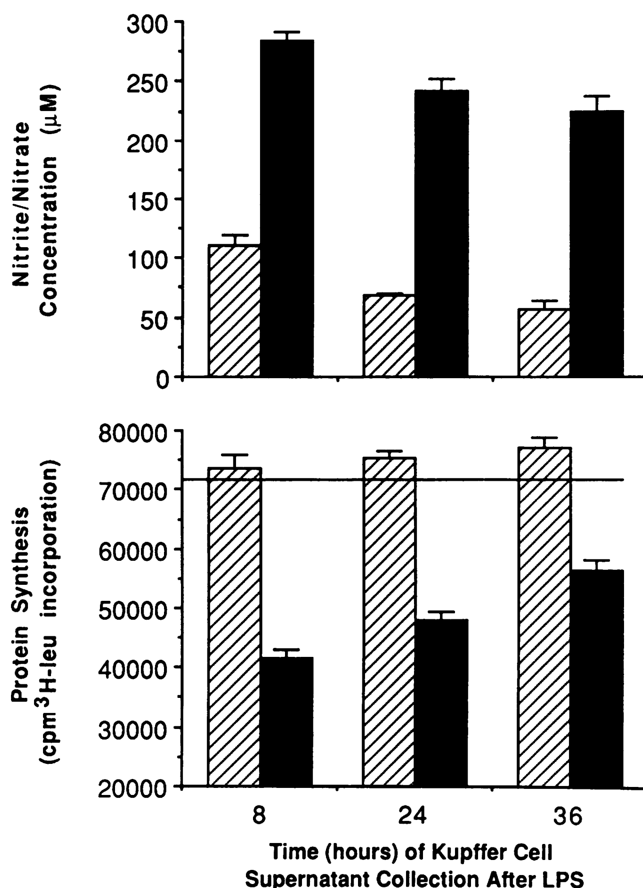


FIG. 1. HC NO_2^-/NO_3^- production and total-protein synthesis in response to KC supernatants generated under different conditions. KC supernatants were generated with the combination of rrIFNg and LPS (■) or LPS alone (▨) and then collected at various time intervals after the addition of LPS. Results are the mean ± SEM of one of three similar experiments in which the KC supernatants were generated in duplicate and then added to quadruplicate HC cultures. The total-protein synthesis of HC cultured in medium alone (—) is shown as a reference. The NO_2^-/NO_3^- concentration in control HC cultures was 2.2 ± 0.6 μmol/L. HC NO_2^-/NO_3^- production and protein synthesis in response to the KC supernatant generated with rrIFNg and LPS were significantly different ($p < 0.05$) from that of the medium control and HC cultures exposed to KC supernatant generated with LPS alone.

To test the first hypothesis, we examined the effects of rrIFNg on KC cytokine production. It is known that IFNg enhances macrophage production of certain cytokines, such as IL-1, while inhibiting the production of other macrophage products, such as PGE_2.[20] Consistent with the literature, higher levels of TNF, IL-1, and IL-6 were found in the KC supernatants activated with rrIFNg and LPS, as compared to the KC supernatants generated with LPS alone (Table 1). Exposure to rrIFNg significantly increased the IL-1 activity in the KC supernatant at all three time points tested. In addition rrIFNg induced a significant early increase in IL-6 levels, following which the levels remained elevated at the later time points. The effects of rrIFNg on TNF levels were less dramatic. While exposure to rrIFNg resulted in slightly greater TNF activity at all

TABLE 1. *Cytokine Levels in KC Supernatants Generated with rrIFNg and LPS or LPS Alone at Varying Time Intervals After the Addition of LPS*

Time (Hours) of KC Supernatant Collection After LPS Exposure	rrIFNg (100 μ/mL)	Cytokine Levels (units/mL)*		
		IL-1	IL-6	TNF
8	−	3.1 ± 0.2	163.2 ± 8.3	363.5 ± 35.8
	+	4.1 ± 0.3†	469.1 ± 21.3†	445.4 ± 36.6
24	−	4.1 ± 0.2	310.5 ± 24.0	286.7 ± 10.2
	+	6.0 ± 0.3†	444.0 ± 21.9	317.4 ± 30.7
36	−	4.7 ± 0.3	454.7 ± 8.9	111.1 ± 11.8
	+	6.4 ± 0.2†	525.7 ± 24.3	240.6 ± 26.1†

* Results are expressed as the mean ± SEM.
† $p \leq 0.05$ when compared with the paired value using the Student's t test.

times tested, only the differences observed at 36 hours were significant. More importantly TNF levels were highest at the 8-hour time and then declined, an activity pattern that is similar to that of the KC supernatant. In contrast IL-1 and IL-6 activity could be detected at 8 hours but peaked at later times. Thus rrIFNg enhanced KC cytokine production but qualitatively all of the cytokines tested were present in measurable quantities, whether the KCs were exposed to rrIFNg and LPS or to LPS alone.

To determine if IFNg might be required as one of the signals directly influencing the HC response, rrIFNg was added directly to the HC cultures with a KC supernatant prepared using only LPS as a stimulating agent. The LPS-activated KC supernatant had minimal activity alone, but when added to HC with rrIFNg it induced significant increases in HC NO_2^-/NO_3^- production and decreases in protein synthesis. These changes were dependent on the rrIFNg concentration (Fig. 2) so that at the highest rrIFNg concentration tested (100 U/mL) a 258.4 ± 4.2 μmol/L increase in the NO_2^-/NO_3^- concentration and 39.7% ± 3.4% decrease in the protein synthesis of the HC cultures were observed. The suppression of HC protein synthesis and increase in NO_2^-/NO_3^- production induced by adding rrIFNg (100 U/mL) directly to the HC cultures, together with the LPS-activated KC supernatant, were not significantly different from that observed with the originally described rrIFNg- and LPS-activated KC supernatant. Adding IFNg (100 U/mL) to the HC cultures alone had no effect on total-protein synthesis and induced only a 1.9 ± 0.4 μmol/L increase in NO_2^-/NO_3^- production, demonstrating that rrIFNg alone was not responsible for the changes. These data indicate that rrIFNg not only increases KC cytokine production but also functions directly on the HC as one of the signals inducing HC L-arginine metabolism to NO•. In addition the data also show clearly that maximal induction of HC L-arginine metabolism to NO• requires more than one factor because neither rrIFNg nor the LPS-activated KC supernatant alone were sufficient stimuli.

To identify the factors within the LPS-activated KC supernatant that were acting in concert with rrIFNg to stimulate HC L-arginine metabolism to NO•, recombinant cytokines were added to the HC cultures with rrIFNg

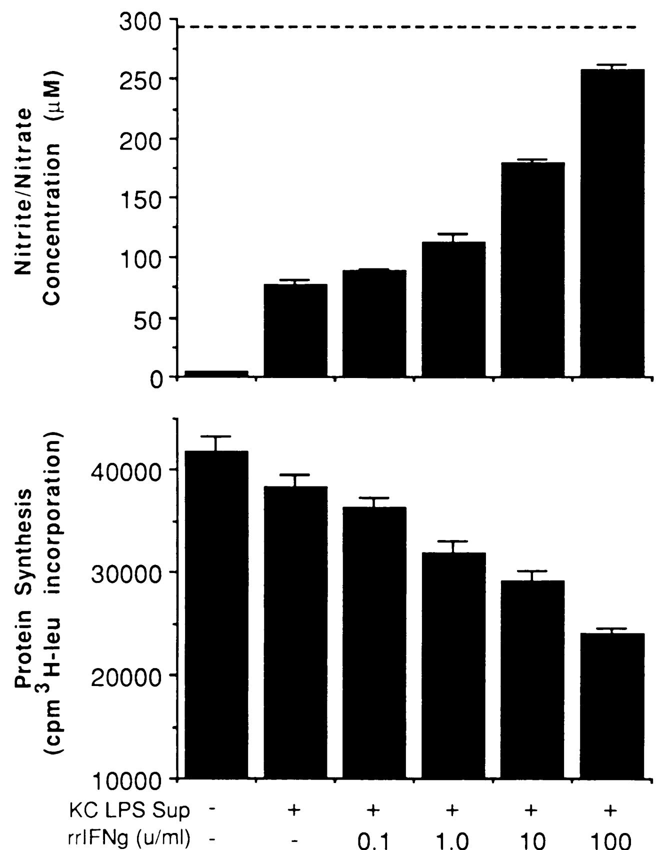


FIG. 2. Concentration-dependent effects of rrIFNg on HC NO_2^-/NO_3^- production and total-protein synthesis. rrIFNg was added directly to HC cultures in combination with LPS-activated KC supernatant and NO_2^-/NO_3^- production and protein synthesis assessed after 24 hours. Results are the mean ± SEM of quadruplicate cultures from one of four similar experiments. HC NO_2^-/NO_3^- production in response to the KC supernatant generated with both rrIFNg and LPS is shown as a reference (---). Significant differences ($p < 0.05$) in HC NO_2^-/NO_3^- production and total-protein synthesis were observed at rrIFNg concentrations ≥ 1.0 U/mL when compared to medium controls or HC cultures exposed to LPS-activated KC supernatant alone.

TABLE 2. *Effect of rrIFNg in Combination with Recombinant Cytokines or LPS on HC NO_2^-/NO_3^- Production and HC Total-protein Synthesis*

HC Culture Additive	Concentration	rrIFNg (100 U/mL)	Protein Synthesis* (cpm ^{3}H-leu Incorporation)	NO_2^-/NO_3^- (μmol/L)*
None	—	–	47,803 ± 2419	1.5 ± 0.1
		+	48,741 ± 1056	1.9 ± 0.4
rmTNF	100 U/mL	+	45,737 ± 1521	21.4 ± 1.2†
	500 U/mL	+	45,699 ± 1074	42.6 ± 2.4†
rhIL-1	1.0 U/mL	+	52,550 ± 2221	8.7 ± 1.7†
	5.0 U/mL	+	51,004 ± 1237	46.4 ± 2.0†
rhIL-6	100 U/mL	+	48,399 ± 796	11.7 ± 0.6†
	250 U/mL	+	54,309 ± 1027	38.1 ± 2.8†
LPS	1.0 U/mL	+	46,532 ± 1148	3.0 ± 0.4†
	10.0 U/mL	+	43,831 ± 817	30.2 ± 1.0†

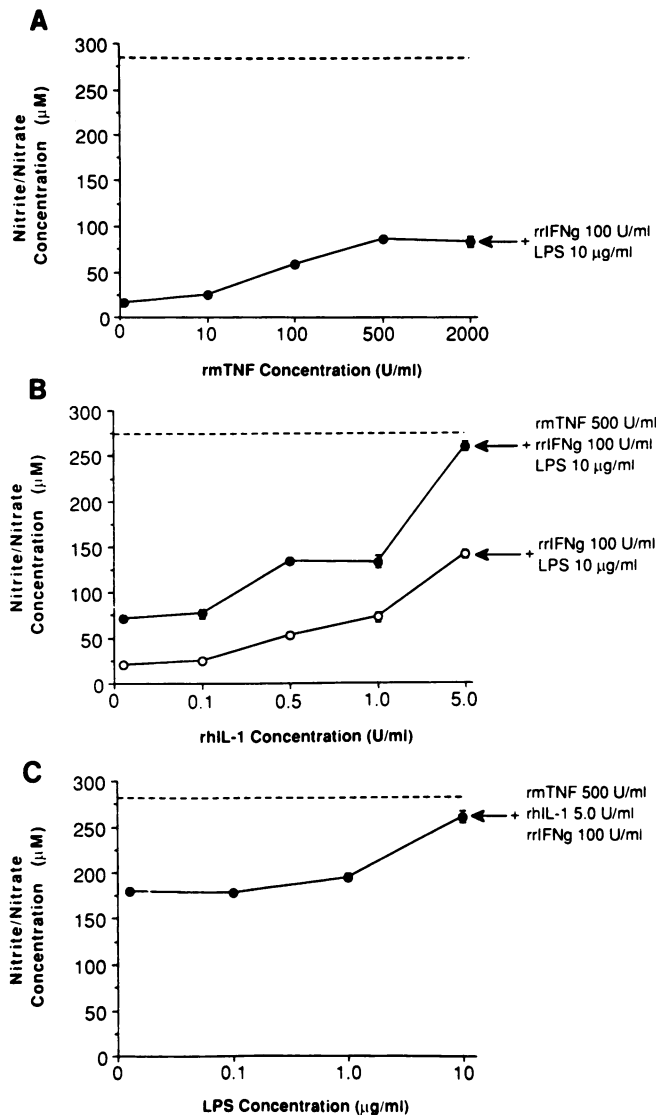
* Results are expressed as the mean ± SEM.
† $p \leq 0.05$ when compared to HC cultured in median alone using the Student's t test.

(Table 2). Addition of any single cytokine, *i.e.*, either rmTNF or rhIL-1 or rhIL-6 or LPS, at concentrations present within the LPS-activated KC supernatant to HC cultures with rrIFNg (100 U/mL) induced only minimal increases in NO_2^-/NO_3^- production. Furthermore these minor increases in HC NO_2^-/NO_3^- production were not associated with any significant suppression of HC protein synthesis. In fact, at the highest concentration of rhIL-6 tested (250 U/mL), the combination of rhIL-6 and rrIFNg actually increased HC total-protein synthesis by 13.6% ± 1.9%. Hepatocyte protein synthesis also was increased slightly in the presence of IL-1 and rrIFNg. Therefore neither LPS nor any single cytokine alone or in combination with rrIFNg could reproduce the increase in HC NO• synthesis and decrease in protein synthesis observed with rrIFNg- and the LPS-activated KC supernatant. However each of the factors functioned synergistically with rrIFNg to induce small but measurable increases in HC NO_2^-/NO_3^- production.

The maximal induction of HC NO_2^-/NO_3^- production was found to require the combination of several factors, including rrIFNg, rmTNF, rhIL-1, and LPS. As illustrated in Figure 3A, rmTNFa induced a concentration-dependent increase in HC NO_2^-/NO_3^- synthesis when added to HC cultures in combination with rrIFNg and LPS. At a rmTNF concentration of 500 U/mL, this combination of factors induced an 86.1 μmol/L ± 1.9 increase in HC NO_2^-/NO_3^- production, nearly two times higher than that observed with the combination of any two factors. Furthermore Figure 3B shows that addition of rhIL-1 to this combination resulted in even greater increases in HC NO_2^-/NO_3^- production to levels near that induced by rrIFNg and the LPS-activated KC supernatant. As observed with rmTNF, induction of HC NO_2^-/NO_3^- biosynthesis by rhIL-1 was concentration dependent, with maximal HC NO_2^-/NO_3^- production requiring 5 U/mL of IL-1. If rmTNF was omitted from this combination of cytokines plus LPS, HC NO_2^-/NO_3^- production decreased from 259.8 ± 5.2 μmol/L to 141.2 ± 4.8 μmol/L. Similarly omitting LPS from the combination of rmTNF, rhIL-1, and rrIFNg resulted in a 79.8 ± 2.1 μmol/L decrease in HC NO_2^-/NO_3^- production (Figure 3C). Unlike the cytokines that induced a concentration-dependent increase in HC NO_2^-/NO_3^- production, LPS stimulation of HC NO• synthesis occurred only at concentrations of 10 μg/mL or more.

The cytokine- and LPS-induced increase in HC NO_2^-/NO_3^- production was associated with a decrease in total-protein synthesis. This is demonstrated in Figure 4, in which the addition of rhIL-1 to the combination with rmTNF, rrIFNg, and LPS induced a concentration-dependent inhibition of HC total-protein synthesis. At the highest concentration tested (5 U/mL), rhIL-1, in combination with these other factors, suppressed HC total-protein synthesis by 36.1% ± 3.1%, which is similar to that observed with the combination of rrIFNg and the LPS-activated KC supernatant. It should be noted also that while the mixture of cytokines and LPS induced both HC NO_2^-/NO_3^- production and inhibited protein synthesis, these effects were not completely parallel. Maximal induction of HC nitrogen oxide synthesis required an rhIL-1 concentration of 5 U/mL, whereas the inhibition of protein synthesis induced by the combination of cytokines was near maximal on exposure to 0.5 U/mL of IL-1, suggesting that a threshold level of NO• production was required to induce the suppression in protein synthesis.

To confirm that the increase in HC NO_2^-/NO_3^- production and inhibition of total-protein synthesis induced by the combination of rmTNF, rhIL-1, rrIFNGg, and LPS were mediated *via* the production of NO•, we added these cytokines to HC cultures in the presence of NMA, a competitive inhibitor of L-arginine metabolism to NO•.[21] NMA (1.0 mmol/L) had no effect on the normal levels of protein synthesis and NO_2^-/NO_3^- production of unstimulated HC. However, in HC cultures exposed to the



FIGS. 3A–C. Concentration-dependent effects of (A) rmTNF, (B) rhIL-1, and (C) LPS on HC NO_2^-/NO_3^- production. The indicated cytokine and LPS combinations were added to HC cultures for 24 hours and the culture supernatants collected for determination of NO_2^-/NO_3^- concentration. The results for each combination are the mean ± SEM of three separate experiments each with triplicate cultures. Where error bars are not shown, they fall within the symbols. HC NO_2^-/NO_3^- production in response to the combination of rrIFNg (100 U/mL) and the LPS-activated KC supernatant is shown as a reference (---). Significant increase ($p < 0.05$) in HC NO_2^-/NO_3^- production occurred at (A) rmTNF concentrations > 100 U/mL, (B) rhIL-1 concentrations ≥ 0.5 U/mL, and (C) at 10 μg/ml of LPS.

combination of cytokines and LPS, or rrIFNg and the LPS-activated KC supernatant, NMA totally blocked the increase in HC NO_2^-/NO_3^- production and substantially reversed the suppression of HC protein synthesis (Table 3). These data indicate that similar to the KC supernatant, the combination of rmTNF, rhIL-1, rrIFNg, and LPS induced HC NO_2^-/NO_3^- production and inhibited total-protein synthesis *via* the conversion of L-arginine to NO•.

Discussion

In 1985 Stuehr and Marletta[22] demonstrated that mammalian cells, specifically macrophages, produce nitrogen oxides when stimulated with interferon gamma (IFNg) and endotoxin. Hibbs et al.[21,23] and Iyengar et al.[24] went on to show in 1987 that L-arginine is the substrate for macrophage nitrite and nitrate (NO_2^-/NO_3^-) production *via* a novel biochemical pathway in which L-arginine in converted to L-citrulline. It has been shown since then that the highly reactive molecule nitric oxide is formed as a short-lived intermediate in this pathway.[6-8] Since the original description in macrophages, a number of other cell types, including endothelial cells,[25-27] neutrophils,[28,29] cerebellar neurons,[30-32] murine EMT-6 adenocarcinoma cells,[33,34] KCs,[3-5] and HCs[9] have been shown to produce NO• from L-arginine. These cell types vary not only in timing and quantity of NO• production but also in the signals that stimulate the cells. Endothelial cells are triggered by vasoactive stimuli such as acetylcholine, bradykinin, and histamine. Neutrophils respond to leukotriene B_4, f-met-leu-phe, and platelet-activating factor, while brain NO• production occurs when L-glutamate binds to N-methyl-D-aspartate receptors on the cerebellar neurons. In these three cell types, expression of the NO• synthase enzyme appears to be constitutive, requiring no previous induction period, and stimulation results in the instantaneous production of small quantities of NO•. In contrast, macrophages, HCs, and murine EMT-6 adenocarcinoma cells synthesize NO• only in response to a specific combination of multiple immunostimulants, which seem not only to stimulate enzyme activity but also may be required to induce the enzyme. In these cells

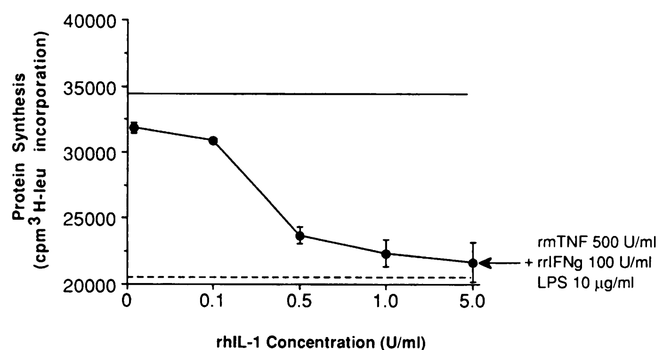


FIG. 4. Concentration-dependent effects of rhIL-1 in combination with rmTNF, rrIFNg, and LPS on HC total-protein synthesis. Increasing concentrations of rhIL-1 were added to HC cultures in combination with rmTNF, rrIFNg, and LPS for 24 hours and HC total-protein synthesis determined. Results are the mean ± SEM of three separate experiments each with quadruplicate cultures. Where error bars are not shown, they fall within the symbols. Control HC total-protein synthesis (—) and the protein synthesis following exposure to the combination of rrIFNg (100 U/mL) and the LPS-activated KC supernatant (---) are shown as a reference. When compared to medium controls, significant decreases ($p < 0.01$) in HC total-protein synthesis were observed at rhIL-1 concentrations ≥ 0.5 U/mL.

TABLE 3. *Effect of NMA on KC Supernatant and Recombinant Cytokine Induction of HC NO_2^-/NO_3^- Production and the Inhibition of HC Total Protein Synthesis*

HC Culture Conditions	NMA (1.0 mmol/L)	Protein Synthesis* (cpm ^{3}H-leu incorporation)	NO_2^-/NO_3^- (μmol/L)*
Medium	−	43796 ± 1847	3.1 ± 1.0
	+	43309 ± 3108	3.5 ± 1.9
KC supernatant	−	24564 ± 1612 (44)†‡	237.7 ± 20.4‡
	+	40606 ± 1241 (7)†	3.3 ± 0.4
rmTNF/rhIL-1/rrIFNg/LPS	−	27970 ± 2953 (36)†‡	248.8 ± 26.0‡
	+	39974 ± 2747 (9)†	3.8 ± 2.0

* Results are expressed as the mean ± SEM.
† The percent suppression compared to HC cultured in medium alone.
‡ $p \leq 0.05$ when compared to HC cultured in medium alone using the Student's t test.

induction of NO· synthase requires 4 to 18 hours of exposure to cytokines and/or bacterial endotoxin, following which larger quantities of NO· are produced. In this report we demonstrated that rTNF, rhIL-1, rrIFNg, and LPS act synergistically to induce maximal HC L-arginine metabolism to NO·. While these signals are similar to those required to induce maximal EMT-6 adenocarcinoma cell NO· production, they differ from that of macrophages. For example although rhIL-1 is a potent synergistic signal for HC, it has no effect on macrophage production of NO_2^-/NO_3^-.[35] Also dexamethasone, which inhibits the induction of macrophage NO· synthase,[36] has no effect on induction of HC NO_2^-/NO_3^- production (data not shown). Therefore, while these different cell types all produce NO· *via* a similar biochemical pathway, the specific stimuli for the induction of the NO· synthase enzyme within these cells appears to be relatively cell specific.

The physiologic significance of mammalian cell NO· biosynthesis is only beginning to be elucidated. *In vitro* endothelial cell-derived NO· has been shown to activate soluble guanylate cyclase in smooth muscle cells, leading to an increase in intracellular cGMP levels.[26] Through this mechanism, endothelial-derived NO· induces the relaxation of adjacent vascular smooth muscle, thereby decreasing vascular tone.[25-27] Consistent with these *in vitro* findings, Aisaka et al.[37] and Vallance et al.[38] showned that the *in vivo* administration of NMA, the competitive inhibitor of NO· synthase, results in arterial hypertension that is reversible with L-arginine. This suggests that ongoing endothelial NO· synthesis plays an important role in the regulation of vascular tone and blood pressure. Through the activation of soluble guanylate cyclase, NO· biosynthesis also appears to play a role in signal transduction within the central nervous system[31-33] and in the regulation of neutrophil chemotaxis.[11] *In vitro* NO· generated by macrophages has been shown to inactivate complex I and complex II of the electron transport chain[7,39] and the Krebs cycle enzyme aconitase.[21] This NO·-mediated inhibition of mitochondrial respiration is thought to be one mechanism by which activated macrophages inhibit the growth of some tumor cells[21,23,39] and microorganisms.[40,41] In this report we demonstrated that HC NO· synthesis is associated with a profound decrease in HC total-protein synthesis. We also showned that authentic NO· inhibits not only HC total protein synthesis but also the production of specific proteins (albumin and fibrinogen).[42] The exact mechanism by which NO· suppresses HC protein synthetic function is not known. Preliminary evidence indicates that the NO·-mediated suppression of HC protein synthesis is not dependent on the activation of soluble guanylate cyclase or the inhibition of mitochondrial respiration, but rather that it involves an undefined translational or post-translational regulatory mechanism.[42]

We originally established the KC:HC 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. Using this model, we demonstrated that multiple cytokines, produced by activated KCs, induce the conversion of L-arginine to NO· within HC, which, in turn, leads to the inhibition of HC total-protein synthesis. The biologic significance of this NO·-mediated inhibition of protein synthesis remains to be determined. *In vivo* experiments suggest that the increased NO· production seen in endotoxemia[22] may protect the liver from damage. In an *in vivo* model of endotoxin-induced hepatic necrosis, the administration of NMA with the endotoxin inhibited nitrogen oxide synthesis while it markedly increased the degree of hepatic damage.[43] The mechanism of the protective action of NO· in the liver and the *in vivo* relevance of the NO·-induced decrease in HC protein synthesis *in vitro* are under investigation.

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