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RAPID COMMUNICATION

Time-Dependent Production of Nitric Oxide by Rat Hepatocyte Suspensions

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ABSTRACT. Isolated hepatocyte suspensions prepared by collagenase perfusion released high levels of nitrite into the extracellular medium during an 8-hr incubation. The release was time dependent, with increases first occurring by 4 hr and continuing throughout the remainder of the incubation period. Nitrite production was inhibited by the nitric oxide synthase (NOS) inhibitors aminoguanidine and N^G-nitro-L-arginine methyl ester (L-NAME), indicating that the nitrite is derived from nitric oxide (NO) production from NOS activity. Nitrite production was not related to bacterial or Kupffer cell contamination. The protein synthesis inhibitor cycloheximide and the transcription inhibitor actinomycin D also prevented nitrite production by parenchymal hepatocytes. Calcium-independent NOS enzyme activity increased with incubation times, and this increase coincided with the observed increases in nitrite production. Our results suggest that NOS is induced following the isolation of hepatocytes, and this induction results in the formation of high levels of NO. BIOCHEM PHARMACOL **57**;11:1223–1226, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. nitrite; nitric oxide; nitric oxide synthase; hepatocytes; iNOS; aminoguanidine

NO† is an important intercellular and intracellular messenger that serves numerous roles in controlling physiological functions. NO is synthesized by the enzyme NOS, which uses the amino acid L-arginine as an endogenous substrate. Hepatocytes have been shown to express the inducible form of NOS (iNOS) [1], and hepatocyte cultures can produce large amounts of NO in response to cytokines and/or endotoxin [1, 2]. Once synthesized, NO can exert a wide variety of biological effects on hepatocyte function. These effects include the inhibition of mitochondrial respiration, decreases in protein synthesis, decreases in cytochrome P450 activity, loss of glyceraldehyde-3-phosphate dehydrogenase activity, and activation of guanylate cyclase [3].

Although they are viable for only a limited time period, hepatocyte suspensions offer a convenient and simple method for assessing biochemical changes that occur in response to toxicants. Hepatocytes can be incubated in many different types of suspension media containing a variety of constituents and, as such, are used extensively in metabolic and toxicological studies [4]. In addition, recent studies have used the transplantation of isolated hepatocytes into animals as a strategy for maintaining liver function [5]. Several studies have examined NO production in cultured hepatocytes in response to cytokines. However, to our knowledge no studies have examined NO production in isolated hepatocyte suspensions. In the following study, we report that NO was produced by rat hepatocyte suspensions.

MATERIALS AND METHODS

Chemicals

2,3-Diaminonaphthalene was obtained from ICN Pharmaceuticals Inc. RPMI-1640, Krebs-Henseleit, and the components of modified Waymouth's medium were purchased from Sigma. Cycloheximide, actinomycin D, aminoguanidine, L-NAME, collagenase (type L), and all other chemicals also were obtained from Sigma.

Preparation of Isolated Hepatocyte Suspensions

Adult male Sprague–Dawley rats (180–220 g) were obtained from Simonsen Laboratories Inc., were housed in small groups, and were given food and water *ad lib.* in a 12-hr light/dark cycle for at least 1 week prior to use. Rat hepatocytes were prepared by collagenase (type L, Sigma) perfusion as previously described [6]. A yield of 4.5 to 5.5 $\times$ 10^8 was routinely obtained with over 93% viability as determined by trypan blue exclusion. In some experiments, parenchymal and nonparenchymal fractions were obtained by differential centrifugation through a Percoll gradient [7]. Hepatocyte suspensions (2 $\times$ 10^6 cells/mL, 12 mL total) were prepared in modified Waymouth's medium, as described in Fariss *et al.* [8]. After 15 min equilibration time, an aliquot of cells was taken as the 0 time point. Following

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† *Abbreviations*: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; and L-NAME, N^G-nitro-L-arginine methyl ester.

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collection at the 0 time point, chemical treatments (prepared in medium or as indicated) were immediately added. At hourly intervals thereafter, cells were sampled and pelleted, and supernatants were collected for nitrite and LDH leakage analysis. LDH determinations were performed as previously described [6].

Nitrite Determinations

Nitrite levels in medium were determined according to the procedures of Misko *et al.* [9] with slight modifications. Samples (100 μL) were mixed with 25 μL of 2,3-diaminonaphthalene reagent (0.5 mg/mL in 0.62 M HCl), and after a 10-min incubation 12.5 μL of 2.8 N NaOH was added. Nitrite standards were prepared fresh, and standard curves were constructed in the same medium as samples.

NOS Activity Assay

Hepatocyte calcium-independent NOS activity was determined by measuring the formation of nitrite. Hepatocytes were incubated in modified Waymouth's medium, and at 0, 2, 4, and 8 hr, aliquots were withdrawn and pelleted. Samples were resuspended in homogenization buffer [0.2 mM dithiothreitol in 50 mM Tris–HCl, pH 7.4, containing 1% (v/v) mammalian cell and tissue protease inhibitor mixture (Sigma)] and frozen rapidly in liquid nitrogen. After thawing, samples were sonicated briefly and centrifuged at 12,000 *g* for 20 min. Aliquots of the postmitochondrial supernatant were added to 50 mM Tris–HCl, pH 7.4, containing 1 μM FAD, 100 μM tetrahydrobiopterin, 1 mM NADPH, 2 mM arginine, 5 mM glutathione, 50 mM valine, and 0.1 mM EGTA and incubated at 37° for 4 hr for the determination of NOS activity. Reactions were stopped by boiling the samples for 5 min. After boiling, samples were centrifuged for 2 min at 12,000 *g*, and supernatants were collected for nitrite determinations. Nitrite levels were determined by adding 100 μL of samples to an equal volume of the Griess reagent [10] (0.5% sulfanilamide, 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid) and read at 550 nm after 10 min. Standards were prepared in the assay buffer using sodium nitrite. Proteins were determined by the BCA protein assay (Pierce Chemical Co.).

RESULTS AND DISCUSSION

Nitrite levels in culture medium are used frequently to assess NOS activity and the formation of NO in cell culture [9]. With isolated hepatocytes suspended at a concentration of 2×10^6 cells/mL in modified Waymouth's medium, increased nitrite levels were first detected after a 3-hr incubation and increased rapidly to 20–30 μM after 8 hr. The addition of the NOS inhibitors aminoguanidine or L-NAME to cell suspensions totally blocked nitrite formation (Fig. 1). These data indicate that the observed increases in extracellular nitrite were derived from NO production from NOS activity in hepatocyte suspensions. The capacity of hepatocytes to produce nitrite in other media was also examined (Fig. 1A). The highest nitrite levels (37 μM after 8 hr) were measured in hepatocytes

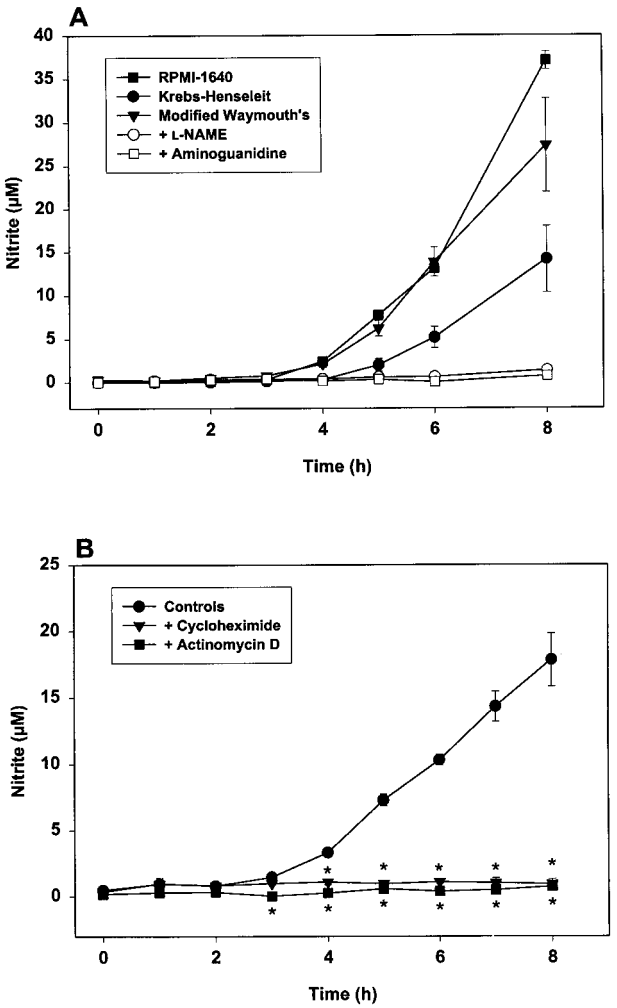

FIG. 1. Effects of media (panel A) and of cycloheximide and actinomycin D (panel B) on nitrite formation. (A) Following collagenase isolation using modified Waymouth's medium, rat hepatocytes were pelleted and resuspended at 2×10^6 cells/mL in RPMI-1640 (■), Krebs-Henseleit (●), modified Waymouth's medium (▼), modified Waymouth's medium containing 1 mM L-NAME (○), or modified Waymouth's medium containing 1 mM aminoguanidine (□). Hepatocytes were equilibrated for 15 min prior to the collection of the 0 time sample, and L-NAME and aminoguanidine were added at 0 time. (B) Inhibition of nitrite formation in isolated hepatocyte suspensions by cycloheximide or actinomycin D. Following a 15-min equilibration, 5 μM cycloheximide (▼) or 1 μM actinomycin D (■) was added to hepatocyte suspensions (2×10^6 cells/mL) at 0 time. The controls (●) received no drug treatment. Actinomycin D was dissolved in ethanol and added to the incubations. The final concentration of ethanol in the medium after the addition of actinomycin D was 0.1% (v/v). Samples were collected at the indicated times for 8 hr. Results are expressed as means ± SD for 3 separate incubations. Key: (*) statistically different from control at the indicated time point ($P < 0.01$) (analysis of variance with Dunnett's post hoc test).

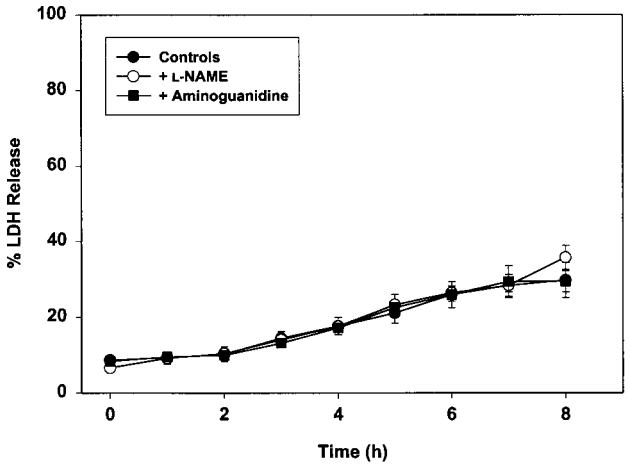

FIG. 2. Effects of NOS inhibitors on hepatocyte viability. Following collagenase isolation, rat hepatocytes were pelleted and resuspended at 2 × 10^6 cells/mL in modified Waymouth's medium containing no addition (●), 1 mM L-NAME (○), or 1 mM aminoguanidine (■). Hepatocytes were equilibrated for 15 min prior to the collection of the 0 time sample, and L-NAME and aminoguanidine were added at 0 time. Samples were collected at the indicated times for 8 hr. Results are expressed as means ± SD for 3 separate incubations.

incubated in RPMI-1640 medium. Hepatocyte suspensions in the amino acid free Krebs-Henseleit buffer exhibited a delayed onset of nitrite production and a decrease in the absolute amounts of nitrite formed after 8 hr. This attenuated production of nitrite in Krebs-Henseleit buffer may be due to the lack of the substrate arginine or other precursor amino acids for NOS activity. Hepatocytes suspended in Krebs-Henseleit buffer are known to have high rates of protein catabolism [11], and this may provide the substrate arginine for NOS activity at later time points. LDH analysis of these hepatocyte suspensions demonstrated that at least 65% of the hepatocytes were viable after an 8-hr incubation, and NOS inhibitors (L-NAME and aminoguanidine) did not affect cell viability significantly at the concentrations used in this study (Fig. 2). Experiments were conducted to examine whether parenchymal hepatocytes were responsible for nitrite production in the hepatocyte incubations. The addition of the antibiotics penicillin and streptomycin had no effect on nitrite production (data not shown), and thus bacterial contamination of media is not involved in nitrite formation. In addition to hepatocytes, Kupffer cells are another cell type found in the liver that expresses iNOS. To examine the potential involvement of Kupffer cells in nitrite production, parenchymal hepatocytes were centrifuged through a Percoll density gradient. This procedure has been shown to remove Kupffer cells and other nonparenchymal liver cells [7]. Enriched hepatocyte preparations generated nitrite at the same rate and extent as nonenriched hepatocyte preparations (data not shown). This suggests that parenchymal hepatocytes and not Kupffer cells are the major site of nitric oxide production in our hepatocyte suspensions.

If nitrite production is a result of the induction of iNOS in hepatocytes, then protein synthesis and transcription inhibitors would be expected to block nitrite formation. As shown in Fig. 1B, the addition of the protein synthesis inhibitor cycloheximide and the transcription inhibitor actinomycin D to hepatocyte incubations blocked the formation of nitrite over the entire 8-hr time period. As actinomycin D and cycloheximide treatments demonstrated no effect on cell viability, it is unlikely that cell toxicity could explain the inhibition of nitrite formation.

To investigate the induction of NOS activity, hepatocytes were incubated with and without cycloheximide for 8 hr. NOS activity measurements were performed on postmitochondrial supernatants in the absence of calcium and should therefore reflect primarily iNOS activity [3]. At 0 and 2 hr, NOS activity was less than 0.3 nmol nitrite formed/mg protein per hr. However, NOS activity levels were increased 14- and 33-fold over 0 time values at 4 and 8 hr, respectively, with NOS activity reaching 5.5 ± 0.2 nmol nitrite formed/mg protein per hr (N = 3) at 8 hr. The addition of cycloheximide to the incubation media inhibited NOS activity at both the 4 and 8 hr time points (less than 0.5 nmol nitrite formed/mg protein per hr). The increase in hepatocyte NOS activity closely paralleled the increase in nitrite levels observed in the extracellular medium. Collectively, this study suggests that iNOS is induced with time following hepatocyte isolations, and that this induction leads to the synthesis of large amounts of NO.

Recently, Wang *et al.* [12] demonstrated that iNOS mRNA levels are expressed in cultured murine hepatocytes. Increased iNOS mRNA levels were first observed 4 hr after isolation and peaked at 8 hr. However, the authors were unable to measure increased nitrite levels in the extracellular medium and speculated that the iNOS gene may be turned on in response to collagenase perfusions and culture conditions [12]. The rapid increase in nitrite formation in our hepatocyte suspensions suggests that collagenase perfusions may indeed activate iNOS expression. Collagenase preparations are, in general, poorly characterized and may contain endotoxin and other components that may trigger iNOS expression. In addition, the manipulations performed during collagenase perfusions (high flow rates, mechanical disruption, lack of calcium) may stress hepatocytes and alter gene expression. Our data agree with the hypothesis proposed by Wang *et al.* [12] that collagenase perfusions induce iNOS expression and extend these observations by demonstrating that culture conditions are not responsible for this event. We are currently investigating the mechanism of iNOS induction in hepatocyte suspensions following collagenase perfusions. Our report is the first evidence that isolated hepatocytes produce large amounts of NO in suspensions. This observation may have important implications for the isolated hepatocyte model system.

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