Nitrite and Nitrosamine Synthesis by Hepatocytes Isolated from Normal Woodchucks (Marmota monax) and Woodchucks Chronically Infected with Woodchuck Hepatitis Virus

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ABSTRACT

Hepatocytes isolated from woodchucks (Marmota monax) were shown to produce nitrite in vitro from L-arginine after stimulation with lipopolysaccharide (LPS). Hepatocytes isolated from woodchucks that were chronic carriers of woodchuck hepatitis virus formed twice as much nitrite as hepatocytes from noninfected animals. Nitrite synthesis by hepatocytes was directly related to L-arginine and LPS concentrations in the tissue culture medium and reached a plateau at 0.5 mM L-arginine and 1.0 μg/ml LPS. LPS-stimulated hepatocytes nitrosated morpholine to form N-nitrosomorpholine in the presence of L-arginine at a physiological pH of 7.4. There was a 10-fold increase in N-nitrosomorpholine production when hepatocytes were stimulated with LPS compared to unstimulated hepatocytes under similar conditions when both nitrite and morpholine were directly added to the medium. N'-monomethyl-L-arginine, a selective inhibitor of nitric oxide synthase, inhibited formation of both nitrite and N-nitrosomorpholine. These results demonstrate that nitrosating agents are formed in hepatocytes via the L-arginine-nitric oxide pathway. This suggests that endogenous formation of carcinogenic N-nitroso compounds could influence the process of hepatocarcinogenesis in woodchucks with chronic woodchuck hepatitis virus infection.

INTRODUCTION

Woodchucks chronically infected with WHV, which is closely related to human hepatitis B virus, develop hepatocellular carcinoma with high frequency (1-3). We recently demonstrated that [15N]nitrate and [15N]NDMA could be detected in woodchuck urine after administration of L-[15N2]-arginine and LPS (4). This indicated that L-arginine is a precursor of both nitrate and the hepatocarcinogenic NDMA, most probably as a result of the formation of nitrosating agents during the oxidation of L-arginine to NO and citrulline. Woodchucks chronically infected with WHV formed more nitrate and NDMA than control animals which suggested that endogenous production of N-nitroso compounds including NDMA may represent an etiological factor in woodchuck hepatocarcinogenesis (5).

Materials and Methods

Reagents. NMOR and morpholine were purchased from Aldrich Chemical Co. (Milwaukee, WI). LPS (E. coli, serotype 0127: B8, phenol extract), NDMA, N,N-di-(2-hydroxyethyl)enediamine dihydrochloride, sulfanilamide, and sodium azide were purchased from Sigma Chemical Co. (St Louis, MO). Minimum essential media (free of L-arginine, without phenol red), Hanks' balanced salt solution (Ca2+/Mg2+ free), Williams' Media E, fetal bovine serum, HEPES, and penicillin-streptomycin solution were purchased from Gibco Laboratories (Grand Island, NY). LPS (E. coli, serotype 0127:B8, phenol extract) and endotoxin fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). All water was double distilled in glass. NMOR and nitrite were not detected in the distilled water or reagent blanks.

Hepatocyte Isolation. Three normal male woodchucks weighing 4.5 ± 0.7 (SD) kg and four WHV chronic carrier male woodchucks (4.3 ± 0.5 kg) were utilized in these studies. Chronic WHV carrier woodchucks had been infected with the virus at birth as described previously (2, 4). Animals were tested for woodchuck hepatitis surface antigen as an indicator of infection status. Hepatocyte cultures derived from WHV chronic carrier woodchucks were also monitored for WHV surface antigen secretion by an enzyme-linked immunosorbent assay. The WHV-infected cultures remained surface antigen positive throughout the culture period, indicating continued viral expression. Hepatocyte isolation and culture were by modification of a previous described procedure (18). Animals were anesthetized with pentobarbital and the left lateral lobe of the liver was removed surgically. The liver lobe was perfused with Hanks' balanced salt solution [Ca2+/Mg2+ free, with 10 mM HEPES, 1.0 mM ethylenebis(oxyethylenenitriilo)tetraacetic acid, pH 7.4] at 37°C for 10 min, followed by perfusion with 0.06% collagenase D (Boehringer Mannheim Biochemical, Indianapolis, IN) at 37°C for 20 min. The liver capsule was removed and hepatocytes were dissociated by gentle agitation. The cell suspension was filtered through two layers of gauze pads into 5% fetal bovine serum/Williams' Media E (4°C), followed by centrifugation at 50 × g for 4 min at 4°C. The resulting hepatocytes were washed and pelleted for three additional cycles to achieve a hepatocyte purity of >96%. The cells were enumerated and viability was >85% as determined by trypan blue exclusion. Nonparenchymal cell (endothelial cells, Kupffer cells, and fat-storing cells) content was found to be <4% by Giemsa and Wright's stain on initial cell isolations and cell cultures 3 days postplating cytospin smear. Cell cultures were also Giemsa stained and counted before monolayer formation. All tests indicated a hepatocyte purity of >96% and it was assumed that less than 40% of the maximum 4%
contaminant were Kupffer cells. Hepatocytes were plated on collagen-coated flasks or wells at a density of 1 x 10^6 cells/cm² and incubated for adherence at 37°C and 5% CO₂. After 2.5 h incubation, nonadherent cells were removed by washing. Adherent cell monolayers were incubated with serum-free medium supplemented with growth factors and hormones as described by Lanford et al. (18). Seventy-two h after plating, the hepatocyte monolayers were washed with phosphate-buffered saline, followed by cultivation under the appropriate experimental conditions. In all experiments, the medium consisted of minimum essential media (l-arginine free, phenol red free) supplemented with 5% low-endotoxin fetal bovine serum (HyClone, Logan, UT), insulin 5 μg/ml, 10 mm HEPES, penicillin 50 units/ml, and streptomycin 50 μg/ml; 2 mm L-arginine, 1 mm L-NMMA, 10 μg/ml LPS, and 10 mm morpholine were added as indicated. The primary hepatocytes exhibited typical cuboidal cell geometry, containing a small nucleus, often binucleated, surrounded by a granular cytoplasm, characteristic of a parenchymal cell type.

The rat hepatocytes were isolated from two adult female Long-Evans rats (277 ± 12 g). This procedure was identical to that for hepatocyte isolation from woodchucks except that a 0.03% collagenase solution was utilized.

Nitrite Analysis. Nitrite was quantified in cell supernatants by colorimetric assay based on Griess reaction (19). Supernatant fluid (100 μl) was added to an equal volume of Griess reagent in microtiter plates. Absorbance at 550 nm was measured using a microplate reader (Bio-Tek EL312) and nitrite values were calculated from a standard curve after correcting for background.

NMOR Analysis. Sodium azide was added to samples used for NMOR analysis (final concentration, 0.01 M) as a nitrosation inhibitor. A 1-ml aliquot of supernatant was mixed with 1 g muffled Celite (1:1, w/v) and 35 ng NDPA and packed into a 10- x 100-mm glass column containing 5 g anhydrous sodium sulfate and glass wool at the top and bottom. Methylen chloride (8 ml) was passed through the column and 4 ml of eluate were collected and slowly concentrated to 0.5 ml under a stream of N₂. Samples were analyzed for NMOR by gas chromatography-Thermal Energy Analyzer (Hewlett-Packard Model 5890 GC; Model 543 TEA; 3-m x 2-mm inside diameter glass column containing 10% Carbowax 20 M on 80-100 Chromosorb WHP). Temperatures were: initial hold at 135°C for 2.2 min; 10°C/min to 180°C with an 8-min hold; injection port, 210°C; pyrolyzer, 550°C; interface, 200°C; and cold trap, 150°C. Peak heights were compared with external NMOR and NDPA standards. Recovery of NMOR (50 ng) and NDPA (internal standard, 35 ng) from spiked medium averaged 99.5 and 99%, respectively. Addition of dimethylmorpholine to several samples did not result in the detectable formation of N-nitrosodimethylmorpholine.

RESULTS

LPS-induced Nitrite Synthesis by Hepatocytes. Stimulation of woodchuck hepatocytes in culture with LPS resulted in the detection of significant amounts of nitrite when l-arginine was present in the medium (Fig. 1). Hepatocytes isolated from control animals in the presence of LPS and l-arginine formed 23.3 ± 13.37 μM nitrite/2.5 x 10⁶ cells/24 h, which was 12.5-fold increase over unstimulated cells (P < 0.01 (Fig. 1)). Hepatocytes isolated from WHV chronic carrier woodchucks, following LPS stimulation and l-arginine addition, formed 46.98 ± 25.8 μM nitrite/2.5 x 10⁶ cells/24 h, which was 25-fold more nitrite than unstimulated hepatocytes in the presence of l-arginine (P < 0.01). Hepatocytes isolated from WHV chronic carrier woodchucks produced significantly (Mann-Whitney test, P < 0.02) more nitrite than normal hepatocytes after stimulation by LPS in the presence of l-arginine. Hepatocytes from both control and WHV carrier woodchucks produced only small amounts of nitrite without LPS stimulation (Fig. 1). Without the addition of l-arginine, hepatocytes produced 6-7-fold more nitrite with LPS stimulation than without LPS in both normal (13.06 ± 8.14 μM nitrite/2.5 x 10⁶ cells/24 h) and WHV chronic carrier woodchucks (12.05 ± 13.37 μM nitrite/2.5 x 10⁶ cells/24 h) (Fig. 1). Correcting for nitrite production in the absence of l-arginine but with LPS stimulation, hepatocytes from WHV chronic carrier woodchucks produced 3.4-fold more nitrite than normal hepatocytes (34.93 ± 12.5 and 10.25 ± 9.7, P < 0.025, respectively).

L-NMMA, which inhibits the nitric oxide pathway (7), inhibited nitrite production by hepatocytes stimulated with LPS (Fig. 1). Indomethacin also inhibited nitrite production by hepatocytes (Fig. 2). Inhibition by indomethacin was observed at a threshold concentration of 0.2 μM. At higher concentrations, inhibition of nitrite synthesis increased with increasing indomethacin concentration and at an indomethacin concentration of 1.0 μM, there was 94% inhibition of nitrite synthesis. The amount of nitrite produced by woodchuck hepatocytes was directly related to the dose of LPS and l-arginine added to the medium (Figs. 3 and 4). Nitrite synthesis was stimulated by as little as 0.01 μg/ml LPS and began to plateau at a LPS concentration of ≥1.0 μg/ml (Fig. 3). Nitrite production by woodchuck hepatocyte-treated LPS increased with increasing l-arginine concentration and leveled off at an l-arginine concentration of 0.5 mm l-arginine (Fig. 4).

Hepatocytes isolated from Long-Evans rats formed 30.57 μM nitrite/2.5 x 10⁶ cells/24 h after stimulation by 10 μg/ml LPS and 2 mm l-arginine (Fig. 5). Without added l-arginine, hepatocytes produced 16.7 μM nitrite/2.5 x 10⁶ cells/24 h when cultured with LPS. L-NMMA also inhibited nitrite synthesis by rat hepatocytes.

Hepatocyte-mediated Nitrosation. LPS-stimulated hepatocytes nitrosated added morpholine to form NMOR in the presence of l-arginine under physiological conditions (pH 7.4, 37°C; Table 1). LPS-stimulated hepatocytes produced significantly more NMOR (336 nm) than unstimulated hepatocytes (55.5 nm) when the latter were incubated with 93.6 μM nitrite for

Fig. 1. Nitrite synthesis by hepatocytes isolated from WHV carriers and normal woodchucks. Hepatocytes (2.5 x 10⁶) were cultured for 24 h in 2 ml medium with (+) or without (−) 2 mm l-arginine, 10 μg/ml LPS, and 1 mm L-NMMA as indicated. Bars, SD; n = 3 animals for controls; n = 4 animals for WHV carriers; duplicate cultures were derived from each animal.)
HEPATOCYTES AND IN VITRO NITRITE AND N-NITROSAMINE FORMATION

DISCUSSION

Nitrite synthesis by rat hepatocytes and Kupffer cell cocultures, but not hepatocytes alone, has been reported. Kupffer cell-hepatocyte cocultures produced more nitrite than Kupffer cells alone in response to LPS. Hepatocytes exposed to medium transferred from LPS-stimulated Kupffer cells also produced nitrite and nitrate (15-17).

We found that neither woodchuck nor rat hepatocytes required the presence of Kupffer cells or medium transferred from LPS-stimulated Kupffer cells to produce nitrite. The reasons that our results differ from the previously reported requirement

72 h. This amount of nitrite (93.6 µmol) is greater than that found in the medium from stimulated hepatocytes. LPS treatment induced 10-fold greater nitrosation activity than untreated hepatocytes when corrected for background NMOR formation. The yield of NMOR from stimulated hepatocytes represented 0.6% of the total nitrite produced in culture and 0.003% of the morpholine added to the medium. L-NMMA not only inhibited formation of nitrite but also inhibited N-nitrosation of morpholine in culture, indicating that the nitrosating agents were derived from the L-arginine-nitric oxide pathway in hepatocytes.
for Kupffer cells or extracts are unknown. In our hands, hepatocytes isolated from Long-Evans rats produced 30.57 μM nitrite/2.5 × 10⁶ cells/24 h from L-arginine with stimulation by LPS (Fig. 5). In the absence of L-arginine, rat hepatocytes produced 16.9 μM nitrite/2.5 × 10⁶ cells/24 h when stimulated by LPS. These results in rats are similar to our results in woodchucks, thus excluding species difference.

The purity of our hepatocytes was >96% which is comparable to the >95% reported by Curran et al. (16). In addition, Billiar et al. (15) used a Kupffer cell:hepatocyte ratio of 5:1 in their cocultures. Therefore, the high concentration of nitrite produced in our >96% purity hepatocyte cultures could not be due to contamination with other cell types.

We also tested different matrices to which cells were attached. There was no difference between collagen-coated and gelatin-coated matrices (data not shown). Initially, our hepatocytes were incubated with serum-free medium. Later we tested the effect of culture medium and found that hepatocytes incubated with conventional medium produced more nitrite than the hepatocytes incubated with serum-free medium (data not show). We have recently developed an immortalized woodchuck hepatocyte cell line which produces nitrite from L-arginine upon stimulation by LPS. Data from these immortalized cells further support our observation that hepatocytes form NO and nitrite from L-arginine after stimulation by LPS without the requirement for Kupffer cells.

Hepatocyte cultures which did not contain added L-arginine produced 6–7-fold more nitrite after LPS stimulation than without LPS. Macrophages and endothelial cells have been reported not to form significant amounts of nitrite without L-arginine addition (6, 8, 12, 20). Although an L-arginine pool could make a partial contribution, formation of this small amount of nitrite without extracellular L-arginine may be due to synthesis of L-arginine from L-citrulline via the urea cycle (21). This L-arginine could then serve as a substrate for NO synthetase in hepatocytes.

Nitrite formation increased with the increasing amount of LPS in the medium (Fig. 3). The dose-response curve for the LPS-induced nitrite synthesis by hepatocytes was similar to that observed in macrophages (22). Nitrite synthesis by hepatocytes was initiated by as little as 0.01 μg/ml LPS and leveled off at a dose of ≥1.0 μg/ml (Fig. 3). γ-Interferon has been reported to be required or to be synergistic with LPS in triggering nitrite synthesis by macrophages (22). LPS alone, however, was sufficient to induce nitrite synthesis by hepatocytes under our experimental conditions.

Nitrite synthesis after LPS stimulation was dose related to the concentration of L-arginine (Fig. 4). Nitrite formation leveled off at a concentration of 0.5 mM L-arginine. Iyengar et al. (8) confirmed that L-arginine is the source of the nitrogen for nitrite using [15N]L-arginine. L-NMMA, which selectively inhibits the NO pathway, inhibited nitrite production by woodchuck hepatocytes (Fig. 1), indicating that hepatocytes produce nitrite from L-arginine through a similar pathway to macrophages. Preliminary experiments indicated that 4-dimethylaminopentynitrile inhibited nitrite production but exhibited significant cell toxicity. Indomethacin is used clinically as a nonsteroidal antiinflammatory and antipyretic agent. Indomethacin also inhibited, in a concentration-dependent manner, LPS-induced production of nitrite by hepatocytes with lower toxicity (Fig. 2). The inhibitory activity was observed at a threshold concentration of 0.2 μM, which is lower than found in plasma (2.8 μM) following therapeutic administration (23). These data suggest that antiinflammatory drugs may also inhibit NO synthetase.

Hepatocytes from WHV chronic carrier woodchucks produced twice the amount of nitrite from L-arginine as hepatocytes from normal animals after stimulation by LPS. These results are consistent with those obtained in vivo (4) in which unstimulated WHV-infected animals excreted 3-fold more nitrate than control animals. Treatment of WHV-infected animals with LPS increased nitrate excretion 15-fold, while uninfected animals increased nitrate excretion 4-fold.

The finding that LPS-stimulated hepatocytes form N-nitrosamines under physiological conditions (pH 7.4) when morpholine was added to the culture medium is similar to the results of Miwa et al. (9), utilizing immunostimulated macrophages. When nitrite was added to unstimulated hepatocytes at a concentration higher than that found in LPS-stimulated cultures after 72 h of incubation, only a small amount of NMOR (55.5 nmol) was observed. Significantly higher NMOR concentrations (336 nmol) were observed during stimulation with LPS. Iyengar et al. (8) also reported that when L-[15N₂]arginine was present in the medium, the enrichment of labeled nitrogen in NMOR was virtually identical to that in nitrate and nitrite. L-NMMA, which selectively inhibits the NO pathway (7), also inhibited NMOR formation in our experiments, which indicated that the nitrosating agents were from the L-arginine-nitric oxide pathway in hepatocytes. These in vitro data support our observation that the L-arginine-nitric oxide pathway results in the in vivo formation of NDMA (4).

These results indicate that formation of NMOR is not due to acid-catalyzed aqueous nitrosation via nitrite, but rather to a nonacidic, cell-mediated, neutral nitrosation via NO, which is an intermediate in the L-arginine to nitrite/nitrate pathway (8, 9, 24). LPS-stimulated hepatocytes convert the guanido nitrogen of L-arginine into NO. NO reacts with dissolved oxygen (O₂) to produce NO₂, which exists in equilibrium with the potent nitrosating agents N₂O₃ and N₂O₄. Both of these intermediates are capable of nitrosating dialkylamines in neutral aqueous solution to form nitrosamines or reacting with water to yield nitrite and nitrate (7, 8, 25).

Our data indicate that hepatocytes produce nitrite in vitro from L-arginine when stimulated by LPS and that WHV chronic carrier woodchucks form significantly more nitrite than normal hepatocytes. Hepatocytes also form NMOR under physiological conditions (pH 7.4). This indicates that NO formation from L-arginine in response to immunostimulation results in the endogenous formation of hepatocarcinogenic N-nitroso compounds and that such compounds could have an etiological role in hepatocarcinogenesis. This further supports our speculation that chronic hepatitis could increase the risk of liver carcinoma by stimulating the NO pathway (4). Nitric oxide has recently been shown to cause deamination of DNA and has also been shown to be mutagenic (26).

### Table 1 N-Nitrosation of morpholine by woodchuck hepatocytes

<table>
<thead>
<tr>
<th>Cell (10 μg/ml)</th>
<th>LPS (10 μg/ml)</th>
<th>MOR (10 nm)</th>
<th>NO₂⁻ (94 μM)</th>
<th>L-NMMA (1 mM)</th>
<th>Harvest</th>
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<tr>
<td>+</td>
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<td>–</td>
<td>336 ± 24*</td>
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<td>18.5 ± 3.8</td>
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<td>55.5 ± 8.75</td>
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<td>25.7 ± 3.35</td>
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* Mean ± SD.
REFERENCES


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