Elevated Formation of Nitrate and N-Nitrosodimethylamine in Woodchucks (Marmota monax) Associated with Chronic Woodchuck Hepatitis Virus Infection

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ABSTRACT

Nitrate balance and N-nitrosodimethylamine (NDMA) excretion were studied in woodchucks chronically infected with woodchuck hepatitis virus (WHV). Twenty-four-h urinary recovery of a bolus dose of [15N]nitrate was 54 ± 12% in woodchucks. WHV-infected animals formed 3-fold more nitrate endogenously than did control animals (P < 0.01). Treatment of WHV-infected animals with Escherichia coli lipopolysaccharide increased nitrate excretion 15-fold, while uninfected animals increased nitrate excretion 4-fold. The endogenous formation of NDMA was higher in WHV-infected woodchucks than in uninfected controls. After administration of L-[^15N]arginine, [15N]nitrate, and [15N]NDMA were detected in urine indicating that arginine is a precursor of biosynthesized nitrate and the hepatocarcinogen NDMA. NDMA probably were formed from the nitrosating agents during the oxidation of arginine to oxides of nitrogen and citrulline. Woodchucks chronically infected with WHV develop hepatocellular carcinomas with high frequency. Our observations suggest an additional mechanism that may be involved in the pathogenesis of hepatocellular carcinoma associated with chronic WHV infection.

INTRODUCTION

Nitrate exposure may be a risk factor in cancer due to the reduction of nitrate to nitrite with subsequent formation of carcinogenic N-nitroso compounds within the body (1, 2). Humans are exposed to nitrate from exogenous sources and from endogenous formation. Tannenbaum et al. (3) showed that nitrate excretion exceeded intake when humans were placed on a low-nitrate diet for several weeks. Germfree rats endogenously formed nitrate indicating that excess urinary nitrate is not produced by gut microflora (4). Nonspecific diarrheal disease in humans significantly increased urinary nitrate excretion (5). Experiments in animals treated with LPS from Escherichia coli suggested that endogenous synthesis of nitrate was related to immunostimulation (6). LPS-stimulated macrophages produced large amounts of nitrate and nitrite in culture (7). Macrophages convert the guanido nitrogen of L-arginine into nitric oxide (NO), with further oxidation to nitrite (NO2-), and nitrate (NO3-) in vitro (8, 9). Leaf et al. (10, 11) confirmed that L-arginine is the source of the nitrogen for nitrate formation in vivo in humans, rats, and ferrets. Immunostimulated macrophages also nitrosate amines such as morpholine, thioproline, and proline in culture (12). LPS caused an increase in urinary N-nitrosodiethylnitrosamine excretion in ascorbic acid-deficient rats (13). These findings suggest that immunostimulation resulted in increased nitrate and perhaps N-nitroso compound formation in vivo and, thus, may represent an increased cancer risk (14).

Woodchucks chronically infected with WHV, which is closely related to human hepatitis B virus, develop hepatocellular carcinoma with high frequency (15, 16). WHV-infected woodchucks also develop hepatocellular carcinoma when exposed to NDEA (17).

Our objective was to determine if endogenous nitrate formation was elevated as a result of the chronic hepatitis associated with WHV infection. We wanted to determine if carcinogenic nitroso compounds, such as the hepatocarcinogen NDMA, were formed endogenously because of chronic WHV infection. Previous studies monitored excretion of noncarcinogenic nitrosamines. We also wanted to know if l-arginine could serve as a precursor for the N-nitroso group in endogenously synthesized nitroso compounds.

MATERIALS AND METHODS

Reagents. NMOR, morpholine, and 4-MP were purchased from Aldrich Chemical Co. (Milwaukee, WI). LPS (E. coli, serotype 0.127:B8, phenol extract), NDMA, and NDPA were purchased from Sigma Chemical Co. (St. Louis, MO). Isotopically labeled Na[^15NO][99% atom purity] and [[^15N]guanido-L-arginine [99% purity] were purchased from Cambridge Isotope Laboratories, Woburn, MA. Furosemide (5%, Lasix) was purchased from Hoechst-Roussel Agri-Vet Company, Somerville, NJ; Ketamine (Ketaset) was from Aveco, Ft. Dodge, IA; and xyazine (Gelamine) was from Butler Co., Columbus, OH. All water was double distilled in glass. NDMA and nitrate were not detected in the distilled water, glucose, or reagent blanks.

Nitrate Balance and NDMA Excretion. Eight normal (3.3 ± 0.9 kg) and eight chronically WHV-infected 1-yr-old male captivity-born woodchucks (3.4 ± 0.9 kg) were housed individually in stainless steel metabolic isolation chambers. WHV infection was achieved by i.v. injection at 3 days of age with 100 μl of diluted woodchuck serum containing approximately 5 x 10⁶ woodchuck infection dose50 of WHV. WHV carrier became woodchuck hepatitis surface antigen positive at 2 to 3 mo of age, and antigenemia persisted thereafter. The animals consumed a low-nitrate diet (3.2 mg/kg) prior to and during 8-day nitrate balance experiments and were given access to double distilled water (<0.05 mg of nitrate/liter) ad libitum. Food and water intakes were measured. Urine was collected every 8 h in bottles containing 25 mg of phenylmercuric acetate and 40 mg of sodium azide as bacteria and nitrosation inhibitors. Nitrate balance was measured during Days 1 to 5. On the sixth day the animals were given 32.6 μmol of [15N]nitrate in 0.5 ml of water by stomach tube followed by 5 ml of water. Animals were anesthetized during the tube feeding by i.m. injection of 45 mg/kg of ketamine and 45 mg/kg of xylazine. The total amount of nitrate formed endogenously was calculated by correcting urinary nitrate excretion, less the dietary nitrate, for catabolic loss of [15N]nitrate (18). On the seventh and eighth days the animals were given 35 mg/kg of body weight 4-MP in 1 ml of 0.9% sterile saline solution by i.m. injection twice daily, and urine was collected for NDMA analysis.

Incorporation of L-[^15N]Arginine into Nitrate and NDMA. Two WHV-infected male woodchucks (4.2 ± 0.1 kg) and one uninfected male woodchuck (3.9 kg) were housed as described above, and urine...
was collected. The animals were given only 10% glucose in water ad libitum for 7 days, and nitrate excretion was monitored. Animals were dosed as follows: Day 3, 333 mg of L-[15N]arginine and 35 mg/kg of body weight 4-MP in 1 ml of 0.9% sterile saline, both by i.m. injection; day 5, LPS (1 mg/kg of body weight) in 0.5 ml of 0.9% sterile saline solution by i.m. injection, followed 6 h later by L-[15N]arginine and 4-MP as described above; Days 3 and 5, 8 h after L-[15N]arginine and 4-MP administration, furosemide (5 mg/kg of body weight) was given i.m.; and Day 6, 4-MP only was given as described above. Furosemide, a diuretic, was given to increase the amount of NDMA excreted by increasing urine volume. This was necessary so that sufficient NDMA could be collected for analysis. 4-MP was given to inhibit NDMA metabolism for the same reason.

Nitrate and [15N]Nitrate, and NDMA Analyses. Nitrate was quantified as described previously (19). [15N]Nitrate was measured as [15N]nitrobenzene by GC-MS (20). A 25-ml aliquot of urine was analyzed for NDMA using a GC-TEA (Hewlett-Packard Model 5890 gas chromatograph; Model 543 TEA) (21). Recovery of NDMA (10 ng) and NDPA (internal standard, 44 ng) from spiked urine averaged 88.4% and 99%, respectively. The limit of detection for NDMA was 80 ng/liter. Additional work (23) showed levels ranging from 2 to 5 ng/liter in healthy animals (23). Recovery of NDMA (10 ng/ml) to several samples did not result in the formation of NMOR during storage or analysis.

GC-MS Analysis for [15N]NDMA. Urine samples were steam distilled as described previously (21), and the distillate was washed 3 times with 30 ml of pentane. Pooled pentane was back-washed twice with 15 ml of water. The pooled aqueous portions were saturated with sodium chloride and extracted with 3 x 40 ml of DCM. The DCM was dried over anhydrous sodium sulfate, concentrated to 4 ml in a Kuderna-Danish concentrator, and slowly concentrated to 2 ml with N2. The concentration was confirmed by GC-MS (Fig. 1).

Incorporation of L-[15N]Arginine into NO3, L-[15N]Arginine, when given to the two groups along with the diuretic furosemide and 4-MP (Day 3), resulted in a 5- to 6-fold increase in nitrate excretion over Day 2 with no treatment (Table 2). LPS in conjunction with L-[15N]arginine, furosemide, and 4-MP (Day 5) increased 24-h nitrate excretion 4- and 15-fold over baseline levels (Day 2) in normal and WHV-infected animals, respectively (Table 2).

Following combined L-[15N]arginine, furosemide, and 4-MP treatment (Day 3; Table 3), controls excreted 379 nmol/24 h of urinary [15N]nitrate, which represented 6.8% of total urinary nitrate and 0.045% of the [15N]nitrogen available from the L-[15N]arginine (Table 3), assuming that only one labeled guanido nitrogen per L-arginine molecule could be incorporated (23). [15N]Nitrogen incorporation into urinary nitrate increased to 8.3% when LPS was added to the regimen (Day 5; Table 3) in controls. WHV-infected woodchucks excreted 500 nmol/24 h of urinary [15N]nitrate during the same period (Day 3; Table 3), for infected and uninfected animals was 2.25 ± 1.0 (SD) and 2.73 ± 0.44 µmol/day, respectively, and was not significantly different (P > 0.05) between WHV-infected and control groups. Infected woodchucks excreted 5.37 ± 2.02 µmol/day, while uninfected animals excreted 2.87 ± 0.77 µmol/day (P < 0.05). The mean 24-h urinary recovery of the 32.6-µmol bolus dose of [15N]nitrate was 48.7 ± 16.4%, and 57.5 ± 8.22% (SD, n = 6) for control and WHV-infected animals, respectively (combined mean = 54.0 ± 12.5%, n = 12). The percentage of recovery of [15N]nitrate was not different between WHV-infected and control groups (P > 0.05). When corrected for [15N]nitrate loss due to catabolism (46%) and for dietary nitrate intake (18), infected animals endogenously formed nearly 3-fold more nitrate than did control animals (P < 0.01), which resulted in a 1.9-fold greater nitrate excretion in the WHV-infected group compared with the control group (P < 0.05; Table 1).

NDMA Excretion. Both normal and WHV-infected woodchucks excreted only trace (undetectable to 0.05 nmol/day) amounts of NDMA. After 4-MP treatment, WHV-infected animals excreted an average of 0.28 nmol/day, while controls excreted only trace amounts. The structure of excreted NDMA was confirmed by GC-MS (Fig. 1).

Incorporation of L-[15N]arginine into NO3- was demonstrated by GC-MS Analysis for [15N]NDMA (Fig. 1) and NDMA isolated from woodchuck urine (2).

### RESULTS

Nitrate Balance. All animals excreted more nitrate than they took in (Table 1). The average nitrate ingestion (diet + water) was 2.25 ± 1.0 (SD) and 2.73 ± 0.44 µmol/day, respectively, and was not significantly different (P > 0.05) between WHV-infected and control groups. Infected woodchucks excreted 5.37 ± 2.02 µmol/day, while uninfected animals excreted 2.87 ± 0.77 µmol/day (P < 0.05). The mean 24-h urinary recovery of the 32.6-µmol bolus dose of [15N]nitrate was 48.7 ± 16.4%, and 57.5 ± 8.22% (SD, n = 6) for control and WHV-infected animals, respectively (combined mean = 54.0 ± 12.5%, n = 12). The percentage of recovery of [15N]nitrate was not different between WHV-infected and control groups (P > 0.05). When corrected for [15N]nitrate loss due to catabolism (46%) and for dietary nitrate intake (18), infected animals endogenously formed nearly 3-fold more nitrate than did control animals (P < 0.01), which resulted in a 1.9-fold greater nitrate excretion in the WHV-infected group compared with the control group (P < 0.05; Table 1).

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HEPATITIS AND IN VIVO NITRATE AND NDMA FORMATION

Table 2 Nitrate excretion for woodchucks given L-[15N]arginine, 4-MP, and furosemide with or without LPS

<table>
<thead>
<tr>
<th>Day</th>
<th>Animal</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (control)</td>
<td>10.3</td>
<td>1.07</td>
<td>5.56</td>
<td>0.18</td>
<td>4.21</td>
<td>11.42</td>
<td>14.93</td>
</tr>
<tr>
<td></td>
<td>B (infected)</td>
<td>1.24</td>
<td>1.18</td>
<td>8.91</td>
<td>1.53</td>
<td>25.56</td>
<td>20.73</td>
<td>4.45</td>
</tr>
<tr>
<td></td>
<td>C (infected)</td>
<td>0.71</td>
<td>1.97</td>
<td>8.95</td>
<td>1.2</td>
<td>20.87</td>
<td>4.42</td>
<td>10.95</td>
</tr>
<tr>
<td>Mean</td>
<td>(B and C)</td>
<td>0.98</td>
<td>1.58</td>
<td>8.93</td>
<td>1.37</td>
<td>23.2</td>
<td>12.58</td>
<td>7.7</td>
</tr>
<tr>
<td>Treatment</td>
<td>None</td>
<td>None</td>
<td>L-[15N]arginine, 4-MP, furosemide</td>
<td>None</td>
<td>LPS, L-[15N]arginine, 4-MP, furosemide</td>
<td>4-MP</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 [15N]/Nitrogen in urinary nitrate

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Control</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO3 excreted (nmol)a</td>
<td>^15N (%)</td>
<td>^15NO3 (nmol)</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>1.07</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>L-[15N]arginine, 4-MP, furosemide</td>
<td>5.56</td>
<td>6.8</td>
</tr>
<tr>
<td>5</td>
<td>LPS, L-[15N]arginine, 4-MP, furosemide</td>
<td>4.21</td>
<td>8.3</td>
</tr>
<tr>
<td>6</td>
<td>4-MP</td>
<td>11.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

 annotations: a Uncorrected for catabolic loss.
 f Assuming only one labeled guanido nitrogen per arginine molecule could be incorporated.
 f Corrected for catabolic loss.

Table 4 NMDA excretion for woodchucks given L-[15N]arginine with or without LPS (nmol/day)

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Control, A</th>
<th>B</th>
<th>C</th>
<th>Mean (B and C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-[15N]arginine, 4-MP, furosemide</td>
<td>0.13</td>
<td>0.21</td>
<td>0.73</td>
<td>0.47</td>
</tr>
<tr>
<td>5</td>
<td>LPS, L-[15N]arginine, 4-MP, furosemide</td>
<td>0.20</td>
<td>0.67</td>
<td>0.41</td>
<td>0.54</td>
</tr>
<tr>
<td>6</td>
<td>4-MP</td>
<td>0.23</td>
<td>0.31</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>7</td>
<td>No treatment</td>
<td>0.22</td>
<td>0.18</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>Mean</td>
<td>Total NMDA excretion</td>
<td>0.2</td>
<td>0.34</td>
<td>0.39</td>
<td>0.37</td>
</tr>
</tbody>
</table>

which represented 5.6% of the total urinary nitrate and 0.056% of the [15N]nitrogen from the L-[15N]arginine dose. Addition of LPS to the L-[15N]arginine, furosemide, and 4-MP treatments (Day 5; Table 3) increased urinary [15N]nitrate excretion to 1580 nmol/24 h, which was 6.8% of total urinary nitrate and 0.19% of the available [15N]nitrogen from the L-[15N]arginine dose (Table 3). The incorporation of [15N]nitrogen from L-[15N]arginine is 3.5-fold higher than the value without LPS treatment.

Incorporation of L-[15N]arginine into NDMA. NDMA was detected in the 24-h urine samples in both groups after the coadministration of 4-MP, diuretic, and L-[15N]arginine, but WHV-infected woodchucks excreted 4-fold more NDMA (Day 3; Table 4). Treatment with LPS (Day 5; Table 4) resulted in increased NDMA excretion in both groups, but infected woodchucks excreted 2.7-fold more NDMA than did untreated control animals.

Increased nitrate formation in WHV-infected animals has been related to chronic hepatitis, although portal and parenchymal hepatitis would be expected to be mild in 1-yr-old WHV carriers (16, 27). The liver has a large population of tissue macrophages or Kupffer cells, which have been shown to proliferate in vivo in inflammatory states (28). There is also marked infiltration by other inflammatory cells into the liver in woodchucks chronically infected with WHV (16, 29). Kupffer cells and hepatocytes as well as other macrophages have been shown in culture to metabolize L-arginine to citrulline, nitric oxide, nitrite, and N-nitrosoamino acids than did controls. However, they suggested that bacterial infection by nitrate-reducing bacteria and not immunostimulation were responsible for the higher urinary levels. Our data indicate that WHV infection, which in our animals produced no overt clinical symptoms, resulted in increased nitrate formation.

DISCUSSION

The 24-h urinary recovery of a bolus dose of [15N]nitrate was 54 ± 12% in woodchucks, which is similar to the 53% recovery of p.o. [15N]nitrate measured in humans (24). This suggests that the catabolism of nitrate is quantitatively similar in humans and woodchucks.

WHV-infected woodchucks endogenously formed 3-fold more nitrate than did control animals (P < 0.01) without an added stimulus such as LPS. Previous studies have shown that endogenous nitrate synthesis is elevated during immunostimulation (5). Tricker et al. (25, 26) reported that bilharzia patients with parasitic Schistosoma haematobium infection and patients with urinary diversions excreted more nitrite, nitrate, and N-nitrosoamino acids than did controls. However, they suggested that bacterial infection by nitrate-reducing bacteria and not immunostimulation were responsible for the higher urinary levels. Our data indicate that WHV infection, which in our animals produced no overt clinical symptoms, resulted in increased nitrate formation.

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The combination of L-[15N]arginine, furosemide, and 4-MP increased nitrate excretion in WHV-infected woodchucks 6-fold, suggesting that L-arginine may have been a limiting factor in nitrate synthesis when animals were given only glucose water. Treatment of WHV-infected animals with LPS increased nitrate excretion 15-fold over untreated WHV-infected animals. Leaf et al. (11) did not see an increase in nitrate excretion with L-arginine alone in rats fed laboratory chow but did find a 10-fold increase with a combination of LPS and L-arginine. Our animals may have responded to L-arginine alone because they did not have any dietary source of L-arginine, while those of Leaf et al. (11) consumed low-nitrate laboratory chow. The diuretic may have also been responsible for a portion of the increase in nitrate excretion due to the higher amount of water lost.

The finding that guanidino nitrogen from L-arginine is incorporated into urinary nitrate in woodchucks is similar to the results of Leaf et al. (11) in humans, rats, and ferrets. LPS induced an increase in incorporation of 15N from L-[15N2]arginine into nitrate in rats, but not in ferrets. Infected woodchucks had increased incorporation of 15N from L-[15N2]arginine into nitrate compared with uninfected controls, especially after LPS treatment. Animals received 10% glucose only in order to decrease the dilution of L-[15N2]arginine by dietary L-arginine. The body pool of L-arginine is, however, large and likely responsible for the relatively low rate (approximately 8%) of [15N]nitrogen incorporation into nitrate and NDMA.

Two problems limit the detection of NDMA in urine. (a) NDMA is rapidly metabolized and would not be expected to appear in the urine (33, 34). (b) NDMA equilibrates quickly with the body water because of its miscibility and may not concentrate appreciably in urine because of its ability to cross lipid membranes (35). NDMA excretion would, therefore, not be expected to be much greater than the proportion of body water excreted. In order to overcome these limitations and to provide sufficient NDMA for GC-MS analysis, we dosed animals with 4-MP, which is a short-term inhibitor of NDMA metabolism (21). We also dosed animals with the diuretic furosemide, which increased the urine volume approximately 4-fold. The result of this was an increase in NDMA excretion. This NDMA had to come from endogenous nitrosation, because animals were consuming only glucose in water which had tested negative for NDMA.

For the reasons given above, data on the endogenous formation of carcinogenic N-nitroso compounds are limited. The present data are the first to suggest that a chronic infection can increase the endogenous formation of a carcinogenic N-nitroso compound in vivo. The 3-fold increase in the M1+ ion (and m/z 30, 31) in the mass spectrum of NDMA from infected animals compared with unlabeled NDMA extracted from control woodchuck urine indicates that a portion of the N-nitroso group in the NDMA contained [15N]nitrogen from L-[15N2]arginine. The incorporation of [15N]nitrogen from L-[15N2]arginine indicates that L-arginine is a precursor of the nitroso group in NDMA. Miwa et al. (36) have shown that immunostimulated macrophages nitrosate morpholine in vivo. Nitrosation does not, in this case, appear to be acid catalyzed via nitrite (9, 36) but is mediated via nitric oxide, which is an intermediate in the L-arginine to nitrate/nitrite pathway (32). Nitric oxide reacts rapidly with dissolved O2 to yield NO2, which exits in equilibrium with the effective nitrosating agents N2O3 and N2O4, both of which are capable of nitrosating dialkylamines in neutral aqueous solution to form nitrosamines or reacting with water to yield nitrate and nitrite (23). Assuming that NDMA is equally distributed in the body water, which is approximately 55% of body weight (37), and that NDMA is not metabolized after administration of 4-MP, infected woodchucks endogenously formed 5.4 nmol of NDMA/day/per animal while control animals formed 1.8 nmol/day/animal.

The development of primary hepatic neoplasms occurs with high frequency in woodchucks chronically infected with WHV (15, 16, 27). Tumors in woodchucks develop during persistent antigenemia, active viral replication, and in the presence of active hepatic inflammation (27). A characteristic influx of inflammatory cells appears in and around the tumor (necroinflammation) at the time of hepatocellular carcinoma development, and a tumor-promoting role for these inflammatory cells has been postulated (16). Long-term, severe, hepatic inflammation may stimulate macrophages (Kupffer cells) in the liver to produce nitric oxide, nitrate, nitrite, NDMA, and possibly other carcinogenic N-nitroso compounds. Baldwin et al. (17) demonstrated that NDEA causes hepatocellular carcinomas that are similar to those observed in WHV-infected woodchucks. NDMA is also a well-known liver carcinogen in a variety of animals (33).

There is irrefutable evidence that nitrate is endogenously synthesized in mammals (3–5, 24, 38). Studies have shown that macrophages stimulated with LPS can nitrosate secondary amines in vitro (36). Leaf et al. (39) recently reported the endogenous nitrosation of morpholine in rats treated with LPS. Our data indicate that woodchucks chronically infected with hepatitis virus endogenously synthesize more nitrate than do noninfected animals and confirm that L-arginine is a precursor of nitrate. Our data also show that the endogenous formation of NDMA is higher in infected animals and that at least a portion of the NDMA results from the process of forming nitrogen oxides from the oxidation of L-arginine in vivo. This means that the process of forming nitric oxide in response to immunostimulation also results in the formation of a potent hepatocarcinogen. This provides an additional mechanism for chronic hepatitis to influence the risk of liver carcinoma but does not prove that endogenous nitrosation is a causative factor in liver cancer.

REFERENCES

Hepatitis and in vivo nitrate and NDMA formation


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