Decreased NADH-Oxidoreductase Activities as an Early Response in Rat Liver to the Carcinogen 2-Acetylaminofluorene

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ABSTRACT

Reduced nicotinamide adenine dinucleotide (NADH):ferricyanide reductase and DT-diaphorase specific activity in total homogenates of rat liver are markedly decreased as a very early biochemical event of hepatocarcinogenesis induced by the carcinogen 2-acetylaminofluorene (AAF). A 50 to 75% decrease in NADH:ferricyanide reductase was observed after 1 day of AAF (0.025% in the diet) feeding and persisted throughout a 7-week continuum of AAF administration. Carcinogen added directly to cell extracts had no effect. Similar results were obtained with single injections of either AAF or diethylnitrosamine. Xanthine dehydrogenase was also reduced in liver following AAF administration to nearly the same extent as NADH:ferricyanide reductase and DT-diaphorase. Total NADH:cytochrome c reductase and mitochondrial activity as estimated from succinic dehydrogenase and DT-diaphorase. Total NADH:cytochrome c reductase activity showed maximum fluctuations of ±15% among groups. The synthesis of poly(ADP-ribose) is stimulated by molecular damage to DNA caused by MNNG (21, 32) and other oncological reagents. The reduced nicotinamide adenine dinucleotide phosphate:cytochrome c reductase that functions in drug detoxification was elevated. With livers of animals fed 4-acetamido-phenol, a hepatotoxin chemically related to AAF, small decreases were noted in NADH:ferricyanide reductase, but not in xanthine dehydrogenase nor in DT-diaphorase. Initial lowering of these activities in the livers of the carcinogen-treated animals is preceded by or concomitant with a reduction in the levels of extramitochondrial pyridine nucleotides known from other studies to result from DNA damage.

INTRODUCTION

Changes in oxidoreductase enzymes have been reported as a result of chemical transformation (1), or in Morris hepatomas compared to normal liver (28, 31). Additionally, the carcinogens N-methyl-N-nitrosourea (19), and MNNG* (21, 32), as well as the acetyloxime derivative of AAF (32) have been shown to cause a rapid reduction of cellular levels of NAD(H) in a variety of cell types. Studies of the mechanism of action of MNNG in 3T3 cells (21) suggest that lowering of NAD(H) is due to a greatly enhanced rate of utilization of NAD(H) for the synthesis of poly(ADP-ribose). The synthesis of poly(ADP-ribose) is stimulated by molecular damage to DNA caused by MNNG (21, 32) and other oncological agents, including radiation (18). The findings suggest a causal relationship between DNA damage and acute depression of cellular NAD(H) pools.

Herein we describe early consequences of administration of AAF and DEN to rats involving several extramitochondrial enzymes that utilize NAD(H). Specific activities of NADH:ferricyanide reductase, DT-diaphorase, and xanthine dehydrogenase in liver homogenates are reduced within the first day of carcinogen administration and are preceded or accompanied by a reduction in the total cellular pools of NAD(H).

MATERIALS AND METHODS

Animals and Diets. Male Fischer 344 rats weighing 100 to 125 g were supplied by either Charles River Breeding Laboratories (Wilmington, MA), or Harlan Laboratory Animal Supply (Indianapolis, IN). Weight-matched animals were divided into 3 groups and fed basal diet (15), basal diet plus carcinogen, or normal laboratory chow (Wayne Lab Blox; 24.52% protein, 4.15% fat, 3.2% fiber, 59.68% carbohydrate, and 8.45% ash), and were housed individually with a 12-hr light-dark cycle and given food and water ad libitum. The carcinogen, AAF, was blended into the basal carcinogenic diet at a concentration of 0.25 g/kg by the diet supplier (Teklad Mills, Madison, WI) (Charts 1 to 4), or was administered as a single dose of 5 mg/kg body weight (Chart 5). The DEN was administered i.p. as a single dose of 40 mg/kg body weight (Chart 5). AAF was supplied at 18.7 g/kg to achieve approximately the same level of hepatotoxicity (Chart 6). All animals were preconditioned for 2 weeks prior to the experiment by feeding basal or normal diet, as indicated. Those animals receiving normal rat chow for the initial 2 weeks received normal chow for the duration of the experiment. The groups receiving basal diet or basal diet plus AAF or AAP received normal laboratory chow for 1 week and basal diet for 1 week prior to addition of carcinogen or AAP. Animals receiving the basal diet plus AAF for greater than 14 days (Chart 1) were fed according to a modified Farber schedule (26, 27). This was essential to prevent animal mortality. The measured average carcinogen dose for each of the 3 cycles of carcinogen feeding was 10.2 mg AAF/day/kg body weight. AAF is used as a complete carcinogen to induce both nodules and hepatomas (12, 15, 16, 34). AAF was fed continuously.

Each table and chart represents one experiment, with individual experiments spaced several months apart. Average normal and basal enzyme activity showed maximum fluctuations of ±15% among groups of animals within a given experiment (e.g., Charts 1, 2, 5, and 6), but varied by more than a factor of 2 among different lots of animals. Those from Charles River (Chart 1; Table 2) exhibited consistently higher specific activities than did those from Harlan Laboratory Animal Supply (Table 1; Charts 2 and 5).

Chemicals. The carcinogen, AAF, and the AAF analogue, AAP, were provided by the Aldrich Chemical Co. (Milwaukee, WI). NADH, NADPH, cytochrome c, hypoxanthine, DCPIP, sodium succinate, and dehydroascorbate were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available.

Tissue Preparation. Animals were anesthetized with carbon dioxide and sacrificed by cervical dislocation. Three test animals, one on basal...
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diet and one on control diet, were sacrificed at each time point. Sacrifice was 1 to 2 hr after the initiation of the light cycle to minimize influences of circadian rhythms. The livers were perfused in situ with ice-cold 0.15 M KCl. Liver samples were taken for histological analysis, and the remaining liver was then excised and 30% total homogenates were prepared in a medium consisting of 50 mM Tris-HCl (pH 7.2), 50 mM KCl, 5 mM MgCl₂, and 250 mM sucrose containing 10 mM 2-mercaptoethanol, using a Polytron 20 ST (Kinematica, Lucerne, Switzerland) at 8000 rpm for 45 sec at 0-4°C.

NADH:Ferricyanide Reductase [EC 1.6.99.3, Reduced-NAD⁺ (Acceptor) Oxidoreductase]. Assays were modified from the basic procedure of Crane et al. (7), using 0.05 mM sodium phosphate (pH 7.0), and about 35 μg protein in a final volume of 2.8 ml. The concentration of NADH was 15 μM, and the ferricyanide concentration was 100 μM. Absorbance was monitored at 420 nm, with reference at 500 nm.

NADH:Cytochrome c Reductase (EC 1.8.99.3). The procedure described by Crane and Low (8) was used with cytochrome c as acceptor. Assays were in the presence of 0.05 mM sodium phosphate (pH 7.0), 0.5 mM KCN, and about 35 μg protein in a final volume of 2.8 ml. The cytochrome c concentration was 0.033%. Absorbance was measured at 550 nm, with reference at 541 nm.

Xanthine Dehydrogenase (EC 1.2.1.37). Measured by reduction of indophenol, xanthine dehydrogenase was assayed in 50 mM Tris-HCl (pH 7.8), 0.36 mM hypoxanthine, 1.3 μM DCPIP, and about 35 μg protein in a final volume of 2.8 ml, as modified from Morton (30). Absorbance was monitored at 500 nm, with reference at 500 nm.

DT-Diaphorase [EC 1.6.99.2, Reduced NAD(P)⁺ (Acceptor) Oxidoreductase]. DT-diaphorase activity was assayed in the presence of 20 mM sodium phosphate, pH 7.8, and about 35 μg protein at a final volume of 2.8 ml (13). The concentration of NADH was 15 μM and that of DCPIP was 1.3 μM. Absorbance was monitored at 600 nm, with reference at 500 nm.

NADH:Monodehydroascorbate Reductase (EC 1.6.5.4). Assays were in 50 mM Tris-HCl (pH 7.4), and a final volume of 2.8 ml (40). The NADH concentration was 0.17 mM. To this was added 3.3 mM of an equivalent mixture of ascorbate and dehydroascorbate, adjusted to pH 6.5 with 10 mM imidazole. Absorbance was read at 340 nm, with reference at 500 nm.

NADPH:Ferricytochrome c Oxidoreductase (EC 1.6.2.4). Assays were the same as for NADH-cytochrome c reduction, with the substitution of NADPH for the NADH in the reaction mixture.

Succinate Oxidase (EC 1.3.99.1). Succinate oxidase was assayed from the rate of oxygen consumption by the use of an oxygen electrode (39). The assay was in 1.5 ml of 50 mM phosphate buffer (pH 7.0), with 100 μM succinate and 0.2 mg cytochrome c from horse heart (type IV).

Succinate → Phenazine Methosulfate plus DCPIP (EC 1.3.99.1). The reduction of indophenol was measured spectrophotometrically at 620 nm in the presence of 30 to 50 μg protein, 50 mM sodium phosphate (pH 7.0), 10 mM KCN, 100 μM phenazinemethosulfate, and 500 μM DCPIP (23).

All enzyme reactions were at 37°C under conditions of linearity with respect to time, and protein concentration and zero order with respect to substrates. The following molar extinction coefficients (E(1%1 cm⁻¹)) were used in calculating specific activities: indophenol, 20; ferricyanide, 1; and cytochrome c, 19.5. Protein was estimated by the Lowry et al. (24) procedure, with bovine serum albumin as standard.

Extraction and Determination of NAD⁺ plus NADH. The NAD⁺ plus NADH were extracted by the addition of cold 0.5 M HClO₄ to homogenates, followed by neutralization with 1 M KOH plus 0.33 M KH₂PO₄-KH₂PO₄ (pH 7.5) and by the addition of 0.25 M NaOH followed by neutralization with 0.37 M H₃PO₄, respectively, as described by Jacobson and Jacobson (20). The extracted NAD⁺ plus NADH was then quantitated using a cycling assay involving alcohol dehydrogenase (25).

Statistical Analyses. All enzyme assays and nucleotide determinations were in duplicate or triplicate for each homogenate. Standard deviations were calculated for the average specific activities comparing 3 test animals or 3 control animals. Significance was determined using Student's t test.

RESULTS

Gross Morphology and Light Microscopy. The first histological change observed in livers of AAF-treated animals was an increase in cellularity adjacent to the bile ducts at about Day 9. Cellularity increased substantially by 14 days, with marked ductular proliferation and increased mitotic figures. Surface nodules appeared on the livers between Days 28 and 35, increased in size, and eventually replaced much of the normal liver parenchyma. In some livers, hepatomas were observed by the end of the longest experiments (77 days).

NADH:Ferricyanide Reductase. The NADH:ferricyanide reductase activities of livers of animals fed the AAF diet decreased markedly (66% reduction from the initial specific activities) within the first 3 weeks of carcinogen administration (Chart 1). Animals fed basal diet alone tended to show a slight but statistically insignificant decline (Chart 1; Table 1). The decrease was rapid, a nearly 50% reduction relative to control diet was observed within 1 day of carcinogen administration (Chart 2A). To show that the effect was sustained, 3 groups of animals were maintained on the Farber schedule for 11 weeks (Chart 1). NADH:ferricyanide reductase remained at a diminished level for up to 7 weeks, after which there was a partial recovery of activity to about 50% of control levels by the 11th week (Chart 1).

AAF (17 to 170 μg/ml, final concentration) had no effect on the enzymatic activities of the homogenates, and incubations of up to 3 hr at 85 μg/ml also were without effect compared to control preparations incubated in the absence of AAF.

DT-Diaphorase. DT-diaphorase activity also was decreased by about 50% as an early response to AAF (Table 1). When estimated as the fraction either inhibited by dicumarol or stimulated by addition of Tween (Chart 3), this activity was nearly eliminated from the livers of rats receiving AAF by Day 1.
Activities had returned to near control levels after 11 weeks (Table 2), although homogenates prepared from livers of animals receiving AAF still retained a somewhat elevated level of activity measured in the presence of dicumarol.

**Xanthine Dehydrogenase.** Xanthine dehydrogenase activities decreased to about 40% of control activities by Day 3 of carcinogen administration, with most of the inhibition coming within the first day (Chart 4). This effect of AAF persisted through the 11-week feeding regimen (Table 2). The xanthine dehydrogenase activity of homogenates was decreased substantially by the feeding of the basal diet lacking carcinogen in some trials (Table 1; Chart 4), but not in others (Chart 5B; Table 2).

**Other Enzymes.** Unaffected by carcinogen administration were NADH:cytochrome c reductase, NADH:monodehydroascorbate reductase, and succinate oxidase (Table 1). These activities were not significantly affected by any of the treatments, although values for animals fed either basal diet or basal diet plus carcinogen tended to be slightly lower than those for animals fed control diets, but within the normal range. Specific activities of NADPH:cytochrome c reductase increased rather than decreased (Chart 2B).

**Control Experiments Comparing Single Injections of AAF and DEN.** In order to eliminate altered feeding patterns as a source of the response and to provide information on a second hepatocarcinogen, animals were provided with a single i.p. dose of either AAF or DEN, and enzymatic activities were monitored for 16 days (Chart 5). Nearly 50% reductions in activities of NADH-ferricyanide reductase (Chart 5A), xanthine dehydrogenase (Chart 5B), and DT diaphorase (Chart 5C) were observed by Day 3, with a significant reduction already measured on Day 1. These same enzymatic activities for animals fed normal laboratory chow and basal diet were not significantly different. The activities remained low at Day 10, with some tendency to recover by Day 16.

**Control Experiments with AAP.** Rats fed the hepatotoxin, AAP, provide further evidence for specificity of the responses. Over a 2-week period of continuous feeding, a delayed reduction in NADH:ferricyanide reductase of about 20% was observed in homogenates from livers of treated animals by Day 14 (Chart 6A), compared to 50 to 60% for AAF (compare with Chart 1 and Chart 2A). Xanthine dehydrogenase was statistically unchanged from basal levels in livers of animals fed the AAP diet (Chart 6B), except at Days 10 and 14. With DT-diaphorase, activities were either unchanged or slightly elevated as a result of the AAP feeding (Chart 6C, in contrast to results with AAF.

**Subcellular Distribution.** Xanthine dehydrogenase and DT-diaphorase are cytosolic enzymes in liver. NADH:ferricyanide reductase activities are more widely distributed. While both particulate (mitochondria plus microsomes) and supernatant fractions prepared from homogenates of liver of animals fed AAF showed reductions in enzymatic activities, the greatest proportionate response was with the supernatant (soluble) fraction (Table 3).

**NAD(H) Levels.** NADH plus NADH levels were determined for one feeding trial after 1, 2, 3, 6, and 9 days of AAF feeding.

### Table 1

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>NADH: ferri-cyanide reductase (nmol ferricyanide reduced/min/mg protein)</th>
<th>NADH: cytochrome c reductase (nmol cytochrome c reduced/min/mg protein)</th>
<th>NADH: monodehydroascorbate reductase (nmol reduced/min/mg protein)</th>
<th>Succinate oxidase (nmoles O2 consumed/min/mg protein)</th>
<th>Succinate → Phe. methosulfate + DCPIP (μmol DCPIP reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1108 ± 76⁶</td>
<td>267 ± 35</td>
<td>415 ± 48</td>
<td>791 ± 15</td>
<td>420 ± 160</td>
</tr>
<tr>
<td>Basal control</td>
<td>898 ± 81</td>
<td>250 ± 38</td>
<td>275 ± 21⁶</td>
<td>676 ± 86</td>
<td>440 ± 200</td>
</tr>
<tr>
<td>AAF</td>
<td>580 ± 143⁵</td>
<td>231 ± 29</td>
<td>139 ± 33⁶</td>
<td>417 ± 107⁶</td>
<td>36 ± 8</td>
</tr>
</tbody>
</table>

*Average values ± S.D.

*Statistically different from normal controls (p < 0.01).

*Statistically different from normal (p < 0.01) and basal (p < 0.05) controls.

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Homogenates of livers from animals fed the control diet contained a constant amount of 9.6 ± 0.6 (S.D.) nmol/mg protein. For animals fed basal diet alone, the values were 9.7 ± 1.2 nmol/mg protein. With basal diet plus carcinogen, the values were 5.5 ± 0.2 after 1 day of carcinogen feeding, and then remained constant thereafter at 8.4 ± 0.1 nmol/mg protein.

**DISCUSSION**

The effects of AAF on the specific activities of NADH-ferricyanide reductase (28) and xanthine oxidase (42) during hepatocarcinogenesis agree well with levels of activities of this enzyme observed in transplantable Morris hepatomas. Prajda and Weber (31) report xanthine oxidase activities to be much reduced in the specific activities of the livers from animals fed AAP plus basal diets similar to those fed basal diet alone. For the DT-diaphorase, specific activities were unaffected or even increased slightly in livers of animals fed AAP plus basal diets similar to those fed basal diet alone. For the DT-diaphorase, specific activities were unaffected or even increased slightly in livers of animals fed AAP, whereas in livers of animals fed AAF this activity was virtually eliminated.

DT-diaphorase is a soluble flavoenzyme (not mitochondrial) of wide distribution that catalyzes the oxidation of NADH:NADPH by difference. AAF added directly to liver homogenates at concentrations of 17 to 170 μg/ml did not reduce enzyme activity, nor was there an inhibition in the presence of an activating fraction from rat liver. Åström and DePierre (1) also tested AAF directly on microsomal dehydrogenases and soluble DT-diaphorase and found no effect.

To evaluate the specificity of the response, hepatotoxicity equal to or exceeding that produced by AAF was induced by the hepatotoxin, AAP. The AAP-treated livers showed a reduced and somewhat delayed lowering of the specific activities of the NADH:ferricyanide reductase but, for xanthine dehydrogenase, the specific activities of the livers from animals fed AAP plus basal diets were similar to those fed basal diet alone. For the DT-diaphorase, specific activities were unaffected or even increased slightly in livers of animals fed AAP, whereas in livers of animals fed AAF this activity was virtually eliminated.

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Chart 4. Chronology of change of xanthine dehydrogenase activity during the first 6 days of continuous AAF feeding. O, normal laboratory chow (points, averages of 3 animals; bars, S.D.); A, basal diet; •, basal diet plus 0.025% AAF. Test points are significant (p < 0.05) from basal controls and (p < 0.01) from normal controls.

Chart 5. Chronology of change of NADH:ferricyanide reductase (A), xanthine dehydrogenase (B), and DT-diaphorase (C) during 16 days following a single i.p. injection of either AAF or DEN. O, normal laboratory chow (control; one animal per value); A, basal diet (points, means of 3 animals; bars, S.D.); •, basal diet plus 1.87% AAF (points, means of 3 animals; bars, S.D.). Significant differences were not obtained except for NADH:ferricyanide reductase at Days 3 and 14 (p < 0.05), and DT-diaphorase at Days 10 and 14 (p < 0.01), compared to livers of control animals fed basal diet.

Chart 6. Chronology of change of NADH:ferricyanide reductase (A), xanthine dehydrogenase (B), and DT-diaphorase (C), during 14 days of continuous feeding of the AAF analogue, AAP. O, normal laboratory chow (control; one animal per value); A, basal diet (points, means of 2 animals; bars, S.D.); •, basal diet plus 1.87% AAP (points, means of 3 animals; bars, S.D.). Significant differences were not obtained except for NADH:ferricyanide reductase at Days 3 and 14 (p < 0.05), and DT-diaphorase at Days 10 and 14 (p < 0.01), compared to livers of control animals fed basal diet.

Table 3

<table>
<thead>
<tr>
<th>Specific activity (µmol/min/10 g liver)</th>
<th>Total homogenate</th>
<th>Total particulate</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1836</td>
<td>1397</td>
<td>368</td>
</tr>
<tr>
<td>Basal control</td>
<td>1758</td>
<td>1279</td>
<td>369</td>
</tr>
<tr>
<td>AAF-treated</td>
<td>1078</td>
<td>825</td>
<td>148</td>
</tr>
<tr>
<td>(59)</td>
<td>(56)</td>
<td>(35)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of basal control.

and potent and specific inhibition by dicumarol (13), were utilized in the present study to verify that the activities inhibited as determined by the standard DT-diaphorase assay were, in fact, attributable to an activity with properties characteristic of DT-diaphorase. These criteria are well established and documented in the paper by Ernster (13). After 1 day of AAF administration, evidence of Tween stimulation and dicumarol inhibition was absent. However, the decrease in DT-diaphorase did not persist to the extent observed for NADH:ferricyanide reductase and xanthine dehydrogenase. Also, Åström and DePierre (1) report a stimulation of cytoplasmic DT-diaphorase after i.p. injection of 50 mg AAF/kg body weight once daily for 5 days. This may possibly be a response to increased hepatotoxicity, as we have observed with AAP.
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The results we report are not due to a general effect on inhibition of protein synthesis. Many other enzymes monitored during the course of these studies (γ-glutamyltranspeptidase, ornithine decarboxylase, nucleoside diphosphatase, casein kinase, CTP synthetase, UDP kinase, CMP-sialic acid synthetase, galactosyl transferase, CMP-sialic acid-lactosylceramide sialyltransferase, NADPH:cytochrome c reductase, succinate oxidase, and 5'-nucleotidase) were either unaffected or, at later times, were elevated in specific activity as a result of a AAF treatment (9–11, 37). With perfused livers from rats fed hepatocarcinogens, Burke and Miller (2) found increased rather than decreased incorporation of amino acids into plasma and liver proteins relative to normal livers (3). In tissue slice experiments, Zamecnik et al. (44) and Campbell (3) reported increased amino acid incorporation by liver tumors at stages where the NADH-ferricyanide- and xanthine dehydrogenase-specific activities remain depressed.

NADH or NADPH may increase (38, 41) or decrease (22) as a result of transformation. A consistent finding in chemical carcinogenesis, however, is that the levels of NADH are reduced to below levels found in the tissues or origin (21, 32). Regenerating and fetal liver also show lower than normal pools of pyridine nucleotides (17). The carcinogens MNNG (21), and N-methyl-N-nitroso urea (19) have been shown to rapidly lower total NAD(H) pools. Similarly, the acetoxy derivative of 2-AAF has been shown to reduce total pools of NAD(H) (32). Lowering of NADH pools may be a general consequence of DNA damage (18, 21, 32), and suggest a significant quantitative requirement for the vitamin-derived molecule for DNA repair and related processes (21). The lowering of NAD(H) following carcinogen administration is rapid. NAD(H) has a half-life of 40 to 80 min in mammalian cells (33), and the reduction in NAD(H) following carcinogen administration observed in the present investigation appears to precede the onset of marked changes in oxidation-reduction activities. Such alterations and attendant regulatory changes might influence, as well, the synthesis of soluble NAD(H)-requiring enzymes to account for the changes in enzymatic activities observed. NADH:cytochrome c reductase, a microsomal enzyme, and various mitochondrial activities, however, are unaffected. Even if the lowering of NAD(H) pools and the decreased levels of enzymatic activities are related sequentially in this system, an attempt to establish a causal relationship must await a better understanding of the molecular details of how the synthesis of the affected oxidoreductase enzymes is regulated. In this regard, it is of interest to note that cultured hepatocytes respond to 10 μM STZ by extensive DNA damage and reduced NAD(H) pools (4, 19, 35, 36), as well as by a lowering of NADH:ferricyanide reductase activity. In our studies, a reduction in NADH:ferricyanide reductase was observed in cultured hepatocytes within the first 2 hr after STZ addition (control cells, 243 ± 11 nmol/min/mg protein; STZ-treated cells, 190 ± 17 nmol/min/mg protein).

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