Perinatal Development of Heart, Kidney, and Liver Mitochondrial Antioxidant Defense

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ABSTRACT. Development of the mitochondrial antioxidant defense system was studied to assess its potential role in the newborn mammal's tolerance to oxidative challenge and to gain insight into the fetal adaptation to a relatively hyperoxic adult environment. Isolated heart, kidney, and liver mitochondria from fetal, newborn, and adult guinea pigs were used. *In situ* function of the antioxidant enzymes was estimated in mitochondrial suspensions after the addition to selenite or tert-butyl hydroperoxide by determining NAD(P)H oxidation rates spectrophotometrically at 340–375 nm. Kidney and liver mitochondria from newborn animals were less susceptible to selenite and tert-butyl hydroperoxide-induced NAD(P)H oxidation. The pattern of change, however, varied widely with tissue type. Kidney mitochondria displayed the largest change with a 3- to 4-fold increase in rate from the fetal to adult period. NAD(P)H oxidation rates in intact mitochondria did not correlate consistently with glutathione reductase and peroxidase activities in sonicated mitochondria suggesting *in situ* regulation by other endogenous factors. Immediately after birth, mitochondrial glutathione reductase and peroxidase activities dropped 38–50% and 50–70%, respectively, in all tissues studied. Total glutathione content of heart and liver mitochondria did not change with age. Adult kidney mitochondrial glutathione, however, declined to 24% of fetal values. Mitochondrial superoxide dismutase activity increased 150–300% from the fetal to the adult period in all tissues studied. Perinatal changes in the mitochondrial antioxidant system and their relationship to mitochondrial calcium metabolism are discussed in terms of the newborn's resistance to oxidative stress. (*Pediatr Res* 26:220–226, 1989)

Abbreviations

GSH, reduced glutathione
GSSG, oxidized glutathione
SOD, manganese superoxide dismutase
tBOOH, tert-butyl hydroperoxide

Mammalian birth introduces a dramatic environmental change on the emerging organism. Altered circulation and oxygen availability necessitate adaptive changes in metabolism. Before birth, fetal oxygen tensions (PaO₂) are low (25 mm Hg). After birth, average PaO₂ rises (to 80–90 mm Hg) initiating the switch to a more aerobic metabolism (1–4). In the newborn

period, rising tissue Po₂ accompanies increased formation of reactive oxygen species which stimulate lipid peroxidation, as measured by ethane and pentane expiration (5, 6), as well as serum malondialdehyde (7) and lipoperoxide levels (8). As antioxidant defense systems develop, lipid peroxidation decreases to adult levels between 10–20 d of age (7, 8).

Despite increased lipid peroxidation at birth, newborn mammals are more resistant to a variety of oxidative challenges when compared to adult animals of the same species (9, 10). Although the biochemical mechanism of this tolerance remains unknown, the mitochondrial antioxidant system is likely to play a key role. When mitochondria metabolize oxidants, large quantities of NAD(P)H are consumed through glutathione dependent redox cycles (11, 12). An increase in the matrix NAD(P)/NAD(P)H ratio will induce Ca²⁺ efflux (12–14). Interestingly, isolated mitochondria from newborn animals are less susceptible to oxidant-induced NAD(P)H oxidation and Ca²⁺ release (15). Therefore, tolerance to oxidative stress in newborn animals may stem from differences in the mitochondrial antioxidant defense system.

Mitochondria generate reactive oxygen species at rates dependent on the surrounding Po₂ (16, 17). Under normal physiologic conditions, the respiratory chain produces superoxide radical and hydrogen peroxide (H₂O₂), accounting for 1–4% of the total O₂ consumed (18). Production rate increases linearly with Po₂ (16, 17). Therefore, a rise in average tissue Po₂ at birth may enhance *in vivo* superoxide and H₂O₂ production by the mitochondrial respiratory chain.

Superoxide radicals and H₂O₂ liberated by the mitochondrial respiratory chain are reduced to H₂O by antioxidant enzyme systems within the matrix. Superoxide radical can dismutate to H₂O₂ spontaneously or enzymatically (19). Glutathione peroxidase reduces H₂O₂ to H₂O forming GSSG from GSH. Glutathione reductase then regenerates GSH from GSSG consuming NAD(P)H (20). Failure to remove H₂O₂ and superoxide increases the likelihood of forming hydroxyl radicals, highly reactive intermediates that can initiate the lipid peroxidative chain reaction (19). Conversely, effective removal of H₂O₂ will oxidize NAD(P)H and induce Ca²⁺ efflux (11–14). This type of efflux can uncouple oxidative phosphorylation via Ca²⁺ cycling involving the Ca²⁺/H⁺ antiport and Ca²⁺-uniport systems (21, 22). In addition, oxidation of mitochondrial GSH and NAD(P)H may modulate substrate oxidations in mitochondria (23).

The development of antioxidant defense systems have been studied extensively in newborn animals, using tissue homogenates (9, 10, 24–26). Little is known, however, about the development of the mitochondrial antioxidant defense system (25). This system is of paramount importance because: 1) mitochondria are a known source of reactive O₂ species under normal physiologic conditions (16–18), 2) the rate of mitochondrial superoxide radical and H₂O₂ generation increases linearly with Po₂ (16, 17), 3) mitochondrial metabolism of peroxides can increase free intracellular Ca²⁺ (11, 21) and deplete cellular ATP by inducing Ca²⁺ efflux and futile Ca²⁺ cycling, respectively, and 4) it may play a role in modulating substrate entry into oxidative pathways (23).

Received January 27, 1989; accepted May 8, 1989.

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Supported in part by a grant from the National Institutes of Health Grant GM-33267 and the Medical Research Foundation of Oregon.

The purpose of this study was to characterize the mitochondrial antioxidant defense system during the perinatal period and to assess its potential role in the newborn mammal's tolerance to oxidative stress. To do this, mitochondria from fetal, newborn and adult guinea pigs were studied using two approaches. First, the ability of intact, isolated mitochondria to reduce the oxidants selenite and tBOOH were determined. Second, NAD(P)H oxidation rates were compared to the mitochondrial glutathione reductase and peroxidase activities, as well as the endogenous substrate levels. Mitochondrial manganese-containing SOD was assayed as an indirect indicator of *in vivo* superoxide production.

MATERIALS AND METHODS

Animals. Topeka guinea pigs, bred and housed at this institution, were used as experimental animals. Term fetuses were obtained by cesarean section 63 d (full gestation) after a 1 wk breeding period. Newborn animals in which the time of birth could not be accurately recorded were not used. Adult animals were maintained on *ad libitum* guinea pig food and water. Newborns were allowed to nurse until the time of sacrifice.

Mitochondrial preparations. Mitochondria were isolated as described by Mela *et al.* (4). Mitochondrial suspensions were analyzed for protein (27) and used to make the various determinations described below. Heart and kidney mitochondrial respiratory control ratios were consistently more than 10; those of the liver were more than 6.

Oxidation of mitochondrial NAD(P)H. NAD(P)H oxidation was monitored kinetically at 340–375 nm in intact mitochondria using a Hitachi 557 dual wavelength spectrophotometer (13). Isolated mitochondria were suspended in 65 mM KCl, 125 mM sucrose, 20 mM Tris-Cl, 0.2 mM Tris-PO₄, 10 μ M rotenone, and 2 mM succinate at pH 7.4. NAD(P)H oxidation rates were calculated after addition of 500 μ M sodium selenite or 500 μ M tBOOH. These concentrations elicit maximal oxidation rates (12, 15).

Nucleotide assay. Nucleotides were assayed according to Stocchi *et al.* (28). Mitochondria were suspended (at 25°C) in 200 μ L of 120 mM KCl, 10 mM Tris-Cl, 10 mM Tris-PO₄, 5 mM succinate, 10 μ M rotenone at pH 7.4. The suspension was alkalinized with 400 μ L of 0.5 M KOH, vortexed for 15 s and cooled for 1 min on ice. The pH was adjusted to 6.5–7.0 with 180 μ L 1 M KH₂PO₄ and the resulting solution was filtered (at 4°C) using a Rainin 0.45- μ m filter. Nucleotides in the filtrate were separated on a RESOLVE C₁₈₋₅ column (3.9 mm $\times$ 15 cm) using the following solvent gradient: 0–9 min, flow 0.8 mL/min with 100% 0.1 M KH₂PO₄ (pH 6.0); 9–15 min, flow increased linearly to 1.3 ml/min and the mobile phase changed linearly to 2% methanol and 98% 0.1 M KH₂PO₄; 15–20 min, mobile phase changed linearly to 10% methanol and 90% 0.1 M KH₂PO₄. Nucleotides were detected at 254 and 340 nm. Standard curves were constructed using external standards containing known quantities of nucleotides. Quantitation was based on peak area.

Glutathione reductase and peroxidase assays. Mitochondria were sonicated under buffered hypotonic (60 mM KCl, 10 mM Tris-Cl, and 10 mM Tris-PO₄ at pH 7.4) conditions in a saline ice bath and assayed immediately. For the reductase assay (29), 50–200 μ L aliquots of the sonicate were mixed with 800–950 μ L of 120 mM KCl, 10 mM Tris-Cl, 10 mM Tris-PO₄, and 5 mM GSSG at pH 7.4. The reaction was started by adding 0.3 mM NADPH and its disappearance was monitored kinetically at 340–375 nm. Glutathione peroxidase was assayed by the method of Tappel (30). Aliquots (100–250 μ L) of sonicate were mixed with 750–900 μ L of 120 mM KCl, 50 mM Tris-Cl, 0.1 mM EDTA, 0.25 mM GSH, 0.3 mM NADPH, and glutathione reductase (2 U/mL) at pH 7.4. The disappearance of NADPH was monitored kinetically after the addition of 1.2 mM tBOOH. All measurements were done at 25°C in a Hitachi 557 dual wavelength spectrophotometer.

Glutathione assay. Total glutathione concentration was determined enzymatically using glutathione reductase and 5-5'-di-

thiobis-2-nitrobenzoate (31). Mitochondria were sonicated as described above and frozen at –70°C until assayed. Aliquots (100–200 μ L) of the thawed sonicate were mixed with 800–900 μ L of 120 mM KCl, 30 mM Tris-Cl, 6.3 mM EDTA, 1 mM NADPH, and 0.6 mM 5-5'-dithiobis-2-nitrobenzoate at pH 7.4. The reaction was initiated by addition of glutathione reductase (4 U/mL). Change in optical density at 412–540 nm was directly proportional to GSH concentration. Sample concentrations were calculated from a standard curve constructed with known concentrations of GSH.

SOD assay. SOD in the sonicate was assayed by its ability to inhibit the reaction of nitroblue tetrazolium with superoxide anion radical (32, 33).

Ubiquinone assay. Heart, kidney, and liver mitochondrial suspensions were analyzed for ubiquinone by high pressure liquid chromatography using a RESOLVE C₁₈₋₅ column as previously described (34).

Statistical analysis. Animals were grouped according to age and the results of the fetal and adult groups were compared individually with all other groups by ANOVA. Significant differences were confirmed using Dunnett's test. Additional information is provided in "Results" and the figure legends.

RESULTS

NAD(P)H oxidation. Heart, kidney, and liver mitochondria from fetal and newborn animals were assayed for their ability to reduce selenite and tBOOH. Figure 1 illustrates selenite and tBOOH induced oxidation of endogenous NAD(P)H in isolated mitochondria. Selenite catalyzes the nonenzymatic oxidation of GSH-forming GSSG (35). The metabolism of tBOOH involves glutathione peroxidase which uses GSH to reduce tBOOH to an alcohol, also forming GSSG. Once formed, GSSG is reduced by glutathione reductase consuming mitochondrial NADPH. NADH is also oxidized via the transhydrogenase reaction (20).

Age-related differences in mitochondrial NAD(P)H oxidation rates with selenite and tBOOH are shown in Figure 2. Fetal kidney mitochondria (day 0) exhibited low rates of selenite and tBOOH metabolism. Rates declined further during the first day and then rose steadily with age resulting in adult values that were 3- to 4-fold higher than fetal values. Heart mitochondria displayed low NAD(P)H oxidation rates with both selenite and tBOOH at all ages. Liver mitochondria displayed a much differ-

Fig. 1. Oxidation of endogenous NAD(P)H by selenite and tBOOH in isolated liver mitochondria from a 7.2-d-old animal. Mitochondria were suspended in buffered medium at 1.55 mg protein/mL and endogenous NAD(P)H was monitored kinetically at 340–375 nm as described in "Materials and Methods." Two tracings are shown superimposed in which either selenite (500 μ M) or tBOOH (500 μ M) were added to initiate NAD(P)H oxidation. Rates were calculated from the slope of a line drawn through each tracing. A simplified schematic of the metabolic pathways leading to NAD(P)H oxidation are shown for each oxidant.

Fig. 2. Rate of *A* selenite and *B* tBOOH-induced NAD(P)H oxidation by isolated heart, kidney, and liver mitochondria from fetal (day 0), newborn, and adult animals. Rates were calculated as described in the legend to Figure 1. Each symbol depicts the mean NAD(P)H oxidation rate and age of each group. The bracket above or below each symbol includes one standard deviation from the mean. The *n* values averaged five with a range of three to eight animals in each group. * and † denotes a significant difference of at least $\alpha < 0.05$ from the fetal and adult group, respectively.

ent pattern. Fetal NAD(P)H oxidation rates with selenite and tBOOH were high and declined rapidly during the first day. Rates with tBOOH remained low up to 8 d with adult rates persisting approximately 32% below fetal values. Rates with selenite were lowest at d 2 then increased slightly up to d 8–9;

adult rates were comparable to those of the fetal group. Note that selenite-induced NAD(P)H oxidation rates were consistently higher than those obtained with tBOOH (Figs. 1 and 2), suggesting that glutathione peroxidase, and not GSSG reduction, was rate-limiting in tBOOH metabolism. To summarize, isolated mitochondria exhibited large age and tissue dependent differences in selenite and tBOOH-induced NAD(P)H oxidation rates with similar patterns of change for both oxidants.

Glutathione reductase and peroxidase activities. To better understand the age-dependent differences in NAD(P)H oxidation, mitochondrial glutathione reductase and peroxidase activities were determined. Several important differences require emphasis. First, fetal mitochondria possessed the highest glutathione reductase activities in all tissues tested (Fig. 3*A*). Activity drops in all tissues during the first day with adult values remaining 15–33% below fetal values. Kidney mitochondria exhibited higher glutathione reductase activities than liver or heart at all ages studied. Glutathione peroxidase activity of all mitochondria followed a similar age-dependent pattern, although significant tissue specific differences were evident (Fig. 3*B*). Most notably, mito-

Fig. 3. Glutathione reductase *A* and peroxidase *B* activity in sonicated heart, kidney, and liver mitochondria from fetal (day 0), newborn, and adult animals. Each symbol depicts the mean enzyme activity and age for each group. The brackets above or below each symbol includes 1 SD from the mean. The *n* values averaged six with a range of three to nine animals in each group. * and † denotes a significant difference of at least $\alpha < 0.05$ from the fetal and adult group, respectively.

chondrial glutathione peroxidase activities in fetal and newborn liver were higher than those of kidney or heart. Adult values in liver and kidney were similar, whereas the activities in heart mitochondria remained much lower.

An interesting paradox emerges when the mitochondrial glutathione reductase activities are compared with the selenite-induced NAD(P)H oxidation rates in intact mitochondria. Inasmuch as selenite rapidly oxidizes GSH-forming GSSG, the rate of endogenous NAD(P)H oxidation with selenite should reflect mitochondrial glutathione reductase activity (Fig. 1). As expected, mitochondrial glutathione reductase activity (Fig. 3A) showed some correlation with the selenite-induced NAD(P)H oxidation rate (Fig. 2A) in liver ($r = 0.73$) and heart ($r = 0.81$). In sharp contrast, glutathione reductase activities in kidney mitochondria do not correlate ($r = 0.21$) with the selenite-induced NAD(P)H oxidation rates. In fetal kidney mitochondria (Fig. 2A), when NAD(P)H oxidation rates were low, glutathione reductase activities were highest (Fig. 3A). Conversely, in adult kidney mitochondria, glutathione reductase activities were 80% of fetal values and selenite-induced NAD(P)H oxidation rates were paradoxically 300% higher. Changes in glutathione reductase activities may explain age-related differences in selenite-induced NAD(P)H oxidation rates in heart and liver but other factors are clearly involved in kidney mitochondria.

Another paradox exists between tBOOH-induced NAD(P)H oxidation rates in intact mitochondria and mitochondrial glutathione peroxidase activities. NAD(P)H oxidation rates with tBOOH should reflect endogenous glutathione peroxidase activity. The peroxidase uses GSH to reduce tBOOH to an alcohol-forming GSSG. GSSG is then reduced (via glutathione reductase) consuming NAD(P)H (Fig. 1). For heart mitochondria, this assumption appears to hold true; tBOOH-induced NAD(P)H oxidation rates (Fig. 2B) parallel endogenous glutathione peroxidase activity (Fig. 3B). Additionally, mitochondrial glutathione peroxidase activities in fetal kidney were 41% of values in fetal liver and, as expected, tBOOH-induced NAD(P)H oxidation rates in fetal kidney mitochondria were equally low (42%) when compared to fetal liver mitochondria. This assumption fails when liver mitochondria from older animals are compared to those of kidney. Kidneys from animals more than 2 d of age have mitochondrial glutathione peroxidase activities consistently lower than those of liver (Fig. 3B), yet kidney mitochondria displayed paradoxically high tBOOH-induced NAD(P)H oxidation rates; up to 280% of values in liver mitochondria. The rates of tBOOH metabolism in intact mitochondria reflect glutathione peroxidase activities for heart, as well as, fetal kidney and liver mitochondria. Other factors appear to regulate tBOOH metabolism in older (>2 d) kidney and liver mitochondria.

Mitochondrial glutathione concentrations. Inasmuch as the selenite and tBOOH-induced NAD(P)H oxidation rates could be limited by substrate availability, the endogenous glutathione concentration of the isolated mitochondria were determined (Table 1). Heart and liver mitochondria show no consistent age-related change in total glutathione content. In contrast, kidney mitochondria exhibited a steady decline in total glutathione as the age of the animal increases. Adult values for kidney mitochondria are 24% of fetal values, significantly lower than those of adult heart (39%) or liver (13%) mitochondria ($\alpha < 0.025$). Therefore, glutathione concentrations did not limit tBOOH or selenite-induced NAD(P)H oxidation rates because adult kidney mitochondria had the lowest glutathione concentrations and yet exhibited the highest NAD(P)H oxidation rates.

Adenine and pyridine nucleotides. Several nucleotides are known inhibitors of glutathione peroxidase (36–38). NADPH and NADP⁺ inhibit activity 30 to 55% at concentrations of 0.26 and 0.7 mM, respectively. Figure 4 displays the total NADP(H) concentrations in isolated heart, kidney, and liver mitochondria as a function of age. Note that liver mitochondria contained 2.5- to 5.6-fold higher NADP(H) levels than heart or kidney. ATP is also known to affect glutathione peroxidase activity, causing 30%

Table 1. Total glutathione content of isolated kidney, heart, and liver mitochondria from animals of various ages*

Age (d)	Kidney	Heart	Liver
Fetus (term)	5.1 ± 0.3 (3)	3.6 ± 0.3 (3)	9.8 ± 3.4 (4)
0.5 ± 0.4	4.0 ± 1.6 (7)	4.2 ± 0.9 (6)	10.6 ± 2.4 (7)
1.1 ± 0.1	4.0 ± 1.9 (4)	3.7 ± 2.0 (4)	7.5 ± 2.3 (3)
3.0 ± 0.5	3.6 ± 0.5 (3)†	4.5 ± 1.2 (4)	6.9 ± 2.4 (5)
5.2 ± 0.3	3.2 ± 1.7 (7)	4.5 ± 1.4 (6)	8.9 ± 2.9 (7)
8.1 ± 1.8	1.7 ± 0.8 (5)‡	2.4 ± 1.0 (5)	8.2 ± 0.4 (5)
Adult	1.2 ± 0.8 (7)‡	3.2 ± 1.6 (6)	9.7 ± 1.6 (6)

* Values are expressed as means ± 1 SD. Numbers in parentheses are the numbers of animals in each group.

† and ‡ denote a significant difference of $\alpha < 0.01$ and $\alpha < 0.001$ from the fetal group, respectively.

Fig. 4. NADP(H) concentration in isolated heart, kidney, and liver mitochondria from fetal (day 0), newborn, and adult animals. Each symbol depicts the mean NADP(H) concentration and age for each group. The bracket above or below each symbol includes 1 SD from the mean ($n =$ four or five animals in each group). * and † denotes a significant difference of at least $\alpha < 0.05$ from the fetal and adult group, respectively.

inhibition at 0.7 mM concentrations (36). Table 2 shows adenine nucleotide concentrations in isolated heart, kidney, and liver mitochondria as a function of age. Heart and liver showed significant increases in mitochondrial adenine nucleotides with age, however, no changes in kidney mitochondria were detected.

SOD activity. Heart, kidney, and liver SOD activities are significantly ($\alpha < 0.01$) higher in adults than in fetal animals (Fig. 5). Higher SOD activities suggest augmented superoxide radical formation by the mitochondrial respiratory chain. Inasmuch as the rate of mitochondrial superoxide generation increases linearly with ubiquinone concentration (17), the ubiquinone content of isolated heart, kidney and liver mitochondria from animals of various ages were determined. Animals were placed into groups according to age. Mitochondrial ubiquinone increased 1.6-, 2.0-, and 2.9-fold from the fetal to adult period in heart, kidney, and liver, respectively (data not shown). Figure

Table 2. Adenine ribonucleotide content (ATP + ADP + AMP) of isolated kidney, heart, and liver mitochondria from animals of various ages (values expressed as nmol/mg protein)*

Age (d)	Kidney	Heart	Liver
Fetus (term)	10.2 ± 1.7 (4)	14.6 ± 1.7 (4)	13.9 ± 0.6 (4)
0.5 ± 0.3	10.8 ± 1.4 (4)	14.6 ± 1.6 (4)	17.8 ± 0.7 (4)†
3.2 ± 0.3	11.8 ± 3.0 (4)	14.5 ± 1.9 (4)	14.8 ± 1.8 (4)
9.0 ± 1.0	10.3 ± 0.8 (5)	14.1 ± 1.4 (5)	15.5 ± 1.3 (5)
Adult	12.1 ± 1.1 (4)	22.1 ± 1.3 (4)‡	15.3 ± 0.5 (4)

* Values are expressed as means ± 1 SD. Numbers in parentheses are the numbers of animals in each group.

† and ‡ denote a significant difference of $\alpha < 0.025$ and $\alpha < 0.001$ from the fetal group, respectively.

Fig. 5. Mitochondrial SOD activity in sonicated mitochondria from fetal (day 0), newborn, and adult animals. Each symbol depicts the mean SOD activity and age for each group. The bracket below each mean includes 1 SD. The n values averaged five and ranged from three to seven animals in each group. * and † denotes a significant difference of $\alpha < 0.01$ and < 0.05 respectively, from the fetal group.

6 shows the ubiquinone content *versus* SOD activity for each age group. The changes in ubiquinone correlate well with the changes in SOD activity in heart ($r = 0.97$) and kidney ($r = 0.94$) mitochondria. The correlation in liver mitochondria was insignificant ($r = 0.52$, $\alpha = 0.14$).

DISCUSSION

Isolated mitochondria showed large age and tissue specific differences in both selenite and tBOOH-induced NAD(P)H oxidation rates (Fig. 2). The similarity in the pattern of change for both oxidants is remarkable, however, the changes in NAD(P)H oxidation rate were not accompanied by corresponding alterations in antioxidant enzyme activities (Fig. 3). For example, oxidant-induced NAD(P)H oxidation rates in kidney mitochondria were 3- to 4-fold higher in the adult than in the fetus. This large difference in rates cannot be explained by elevated glutathione reductase or peroxidase activities. In fact, adult kidney mitochondrial glutathione reductase activities were 70% of fetal values, yet selenite-induced NAD(P)H oxidation rates were 300% higher. Similarly, kidney glutathione peroxidase activities were similar in the fetal and adult period despite 460% higher tBOOH-induced NAD(P)H oxidation rates in the adult. A similar discrepancy existed between tBOOH-induced NAD(P)H oxidation rates and glutathione peroxidase activity in liver and kidney. Newborn (>2 d) and adult liver mitochondria had elevated or similar glutathione peroxidase activities when compared to kidney mitochondria of the same age. Intact kidney mitochondria, however, consistently display higher NAD(P)H oxidation rates

Fig. 6. Mitochondrial ubiquinone content versus SOD activity in heart and kidney mitochondria from animals of various ages. Animals were placed into groups according to animal age. Each symbol depicts the mean value obtained for each age group. The brackets below and left of each symbol include 1 SD from the mean SOD activity and ubiquinone content respectively. The n values for each group averaged five and ranged from three to seven animals in each group. Both correlations are significant at $\alpha < 0.002$.

than age-matched liver mitochondria. Therefore, mitochondrial glutathione reductase and peroxidase activities in sonicated mitochondria were not necessarily indicative of the antioxidant function of the intact organelle. Estimation of antioxidant function merely on the basis of enzyme activities in disrupted mitochondria may be misleading because other factors appear to be regulating the activities of these antioxidant defense enzymes *in situ*.

Several factors present in the mitochondrial matrix are known regulators of glutathione peroxidase and reductase activity. NADPH and NADP⁺ inhibit glutathione peroxidase activity 30–50% at 0.26 and 0.7 mM concentrations, respectively (36, 37). Figure 4 reveals 2.5–5.6 fold higher NAD(P)H levels in liver mitochondria than those of kidney or heart. This corresponds to matrix NAD(P)H concentrations of 4.2 to 5.0 mM in liver mitochondria, assuming a matrix volume of 1 μ L/mg mitochondrial protein. Therefore, NAD(P)H inhibition of glutathione peroxidase *in situ* may be responsible for lower tBOOH-induced NAD(P)H oxidation rates despite higher glutathione peroxidase activity in older (>2 d) liver *versus* kidney mitochondria. Incubation of glutathione reductase with NADPH or NADH causes reversible inactivation of the enzyme. This type of inhibition may have an important regulatory role (20, 29). Adenine ribonucleotides are competitive inhibitors of glutathione peroxidase with increased hydroperoxide levels enhancing the inhibitory effect (36). No correlation, however, was found between the age-related changes in mitochondrial ATP content (Table 2) and tBOOH-induced NAD(P)H oxidation rates. Coenzyme A is also a potent inhibitor of glutathione peroxidase with a K_i of $< 5 \mu$ M. Binding the sulfhydryl group of coenzyme A reduces the inhibitory effectiveness 6-fold (37). All these potential regulators are present within the mitochondria at concentrations known to affect the enzymatic rate. Therefore, mitochondrial antioxidant function, estimated solely by enzyme activities in disrupted mitochondria, should be interpreted with caution.

SOD activities were higher in adult heart, kidney, and liver mitochondria when compared to the fetus (Fig. 5) suggesting heightened superoxide radical production in adult mitochondria. Similar increases in rat brain mitochondrial SOD after birth have been described (25). Two known factors that influence the rate of superoxide formation by mitochondria require emphasis.

First, the rate of superoxide formation increases linearly with the PO_2 at the mitochondrial inner membrane (16, 17). This depends, in part, on changes in organ blood flow and incoming PaO_2 , factors that vary with perinatal age and the tissue in question. Second, the concentration of mitochondrial ubiquinone, the predominate site of superoxide radical formation (17), increases in heart, kidney, and liver postbirth (see "Results"). Assuming the PO_2 at the inner membrane does not decrease, mitochondrial superoxide formation should increase with ubiquinone concentration. In fact, mitochondrial SOD activity correlates well with ubiquinone concentration in heart and kidney (Fig. 6). The rise of mitochondrial SOD from the fetal to adult period is a complex phenomenon that may suggest heightened mitochondrial superoxide radical generation. The etiology remains speculative, however, the rise in heart and kidney mitochondrial ubiquinone may lead to elevated SOD activity indirectly by increasing the rate of superoxide radical formation (17). In liver mitochondria, the lack of correlation between ubiquinone concentration and SOD activity suggests that other factors (e.g. PO_2 , other sources of superoxide, translobular heterogeneity) may be operative in liver.

Selenite or tBOOH-induced oxidation of endogenous NAD(P)H in intact mitochondria may be a more reliable indicator of mitochondrial antioxidant function than determination of enzyme activities in disrupted mitochondria. Nevertheless, other yet unstudied pathways may interfere with interpretation of data obtained by this method. In certain cell types, GSSG formed during oxidant metabolism can be actively extruded from the cell (40). Although preliminary results in our laboratory are negative, a similar GSSG transport system may exist in mitochondria which could compete with glutathione reductase for GSSG, thereby decreasing the NAD(P)H oxidation rate. In addition, succinate with rotenone, used as substrate in these experiments, may give falsely low NAD(P)H oxidation rates in the presence of NADP⁺-malate dehydrogenase. Heart mitochondria are known to contain substantial levels of this enzyme (41), possibly accounting for the low rates of oxidant-induced NAD(P)H oxidation (Fig. 2). NADP⁺-malic enzyme may also be an important source of reducing equivalents necessary for mitochondrial oxidant detoxification. The significance of mitochondrial GSSG transport and NADP⁺-malate dehydrogenase activity in the mitochondrial antioxidant defense system are currently under investigation.

Mitochondrial antioxidant function may provide resistance to oxidative stress in the newborn period by stabilizing the mitochondrial Ca^{2+} pool. Mitochondria continually cycle Ca^{2+} through separate uptake and efflux systems. Under normal conditions, a low steady-state extramitochondrial Ca^{2+} concentration is maintained against a continual slow efflux by an active Ca^{2+} uniporter (21). Oxidation of mitochondrial NAD(P)H, by a variety of substances, accelerates Ca^{2+} efflux, possibly through activation of a $\text{Ca}^{2+}/\text{H}^+$ antiporter (14, 22). Efflux initiates a futile Ca^{2+} cycle as the uniporter system actively takes up released Ca^{2+} (42). ATP levels drop because Ca^{2+} uptake continues at the expense of ATP production (43). If necessary, extramitochondrial ATP may also be hydrolyzed to maintain mitochondrial Ca^{2+} uptake (42). During the newborn period, the mitochondrial antioxidant system displayed significantly lower oxidant-induced NAD(P)H oxidation rates than those of adult animals (Fig. 2). Lower NAD(P)H oxidation should prevent mitochondrial Ca^{2+} efflux. To support this, liver mitochondria from newborn animals are indeed less susceptible to oxidant-induced Ca^{2+} efflux than those of adult animals (15). The ability of the mitochondrial antioxidant system to resist NAD(P)H oxidation in the newborn period may provide tolerance to oxidative challenge by stabilizing the mitochondrial Ca^{2+} pool.

This report characterizes, for the first time, the mitochondrial antioxidant defense system during perinatal development in three important tissues. The results demonstrate that: 1) intact mitochondria from newborn animals are less susceptible than

those of adults to oxidant-induced NAD(P)H oxidation. Inasmuch as mitochondrial Ca^{2+} efflux has been linked to oxidant-induced NAD(P)H oxidation, the newborn's resistance to oxidant-induced NAD(P)H oxidation, and thus Ca^{2+} efflux, may underlie their tolerance to hyperoxia (9, 10) and other exogenous oxidants (15). 2) Age- and tissue-specific differences in mitochondrial glutathione reductase do exist. In the term fetus, mitochondrial glutathione reductase is high. Activity drops during the first day in all tissues studied with adult values remaining below those of the fetus. Mitochondrial glutathione peroxidase shows a similar age-dependent pattern with a significant decline in activity occurring post-birth in all tissues. 3) Mitochondrial SOD activity is increased in adult mitochondria as compared to fetal or newborn animals. 4) NAD(P)H oxidation rates in intact mitochondria do not consistently correlate with glutathione reductase and peroxidase activities in disrupted mitochondria. These findings suggest involvement of additional endogenous regulatory factors in antioxidant-related responses *in situ*.

Acknowledgment. The authors thank Debbie Nelson for her help in the preparation of the manuscript.

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