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Functional and toxicological characteristics of isolated renal mitochondria: Impact of compensatory renal growth

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Abstract

Mitochondria were isolated from renal cortical homogenates from control rats and rats that had undergone uninephrectomy and compensatory renal growth (NPX rats). Activities of selected mitochondrial processes, including key enzymes of intermediary metabolism, glutathione-dependent enzymes, and glutathione transport, were measured, and the effects of three mitochondrial toxicants were assessed to test the hypothesis that compensatory renal growth is accompanied by increases in mitochondrial metabolism and that this is associated with increased susceptibility to injury from oxidants or other mitochondrial toxicants. Activities of malic and succinic dehydrogenases were significantly higher in mitochondria from NPX rats than in mitochondria from control rats. Although the rates of state 3 respiration were significantly higher in mitochondria from NPX rats, the rates of state 4 respiration and respiratory control ratios were not different between mitochondria from control and NPX rats. Activities of glutathione redox cycle enzymes did not differ significantly between mitochondria from control and NPX rats. However, the rates of uptake of glutathione into mitochondria were approximately 2.5-fold higher in tissue from NPX rats than in tissue from control rats. Incubation of mitochondria from NPX rats with three mitochondrial toxicants [*tert*-butyl hydroperoxide, methyl vinyl ketone, and *S*-(1,2-dichlorovinyl)-L-cysteine] caused greater inhibition of state 3 respiration and larger increases in malondialdehyde formation than similar incubations of mitochondria from control rats. These results indicate that mitochondria from hypertrophied renal cells are more sensitive to oxidants or mitochondrial toxicants. Baseline levels of malondialdehyde were also significantly higher in mitochondria from NPX rats, suggesting that a basal oxidant stress exists in mitochondria from hypertrophied cells.

Keywords: Kidney; Glutathione transport; Oxidative stress; Compensatory renal cellular hypertrophy; Mitochondria

1. Introduction

In virtually all mammalian species, when renal mass is reduced significantly, a series of compensatory physiological, morphological, and biochemical changes occur in the remaining functional renal mass [1,2]. The compensatory response is characterized predominantly by cellular hypertrophy, which occurs primarily in the proximal tubules, and its acute phase in the remaining functional renal tissue of rodents is complete within 7–10 days. This hypertrophic response includes increases in cellular protein content, brush-border and basolateral membrane surface area, overall cell size, cellular energy metabolism, and activities of various enzymes. Although many processes increase in proportion to the increase in cellular protein, other processes increase disproportionately to protein. This suggests that these processes may have particular significance with regard to cellular function in the hypertrophied state.

At the levels of the intact proximal tubular epithelial cell and intact kidney, the hypertrophic response leads to changes having profound toxicological significance. Some of these changes include: increased production of GSH and increased activities of several GSH-dependent enzymes, increased transcription of metallothionein genes, and increased expression and/or activity of several ion and metabolite transport carriers, including ($Na^+ + K^+$)-stimulated ATPase [3–9]. It has been hypothesized that these changes and the increased workload imposed lead to increased en-

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Abbreviations: tBH, *tert*-butyl hydroperoxide; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; MDA, malondialdehyde; MVK, methyl vinyl ketone; NPX, uninephrectomized; RCR, respiratory control ratio; and TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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ergy demands on proximal tubular cells [10,11]. To accommodate these energy demands, there are increased rates of mitochondrial electron transport, which, in turn, produce a hypermetabolic state. The primary toxicological implication of these changes is that the hypertrophied renal proximal tubular cells are more susceptible to the toxic effects of several diverse chemicals, including heavy metals such as inorganic mercury [12–17].

The goals of the present study were to characterize selected alterations in mitochondrial function that occur as a result of compensatory renal growth and to assess the impact of these changes on susceptibility to mitochondrial toxicants. Suspensions of isolated mitochondria from renal cortical homogenates derived from control and NPX rats were used as the model system. The activities of selected mitochondrial enzymes and of substrate-stimulated oxygen consumption were measured as indicators of mitochondrial function. Toxicity was assessed by measurements of the rates of state 3 and state 4 oxygen consumption (respiration) and formation of MDA, an indicator of lipid peroxidation. Mitochondria were incubated with one of three chemicals, tBH [18,19], MVK [20,21], or DCVC [20], to probe susceptibility to intoxication. The three agents were chosen because they are well-established toxicants in renal mitochondria and because they each act by different mechanisms. tBH is an oxidant that causes toxicity by depletion of GSH through the action of GSH peroxidase. MVK is a direct-acting (i.e. there is no need for metabolism) alkylating agent that causes oxidant stress by alkylating soft nucleophiles, such as GSH. DCVC, in contrast, is metabolized to reactive electrophiles that produce an oxidant stress by both alkylation of soft nucleophiles and by oxidation of thiols, including GSH, perhaps in part by inhibition of GSSG reductase.

In addition to assessing mitochondrial function and toxicity, transport of GSH into isolated renal cortical mitochondria (from control and NPX rats) was also characterized. Transport of GSH from cytoplasm into mitochondrial matrix is the major, if not sole, determinant of mitochondrial GSH status [22,23]. In a series of studies [19,24,25], we have demonstrated that two of the anion carriers responsible for the transport of citric acid cycle dicarboxylate intermediates across the mitochondrial inner membrane also transport GSH. Furthermore, activity of these two carriers (the dicarboxylate and 2-oxoglutarate carriers) can account for nearly all of the transport of cytoplasmic GSH into the mitochondria [25]. The previously described observation that activity of the mitochondrial respiratory chain is elevated in renal proximal tubules, after compensatory renal growth, suggests that activities of carriers that deliver carbohydrate substrates to the mitochondrial matrix will also have to be elevated. Consequently, we expect that transport of GSH would be increased. This would have important implications for defense against oxidant stress and maintenance of intramitochondrial redox status.

Results from the present study show that mitochondrial metabolism and respiration are indeed increased after uninephrectomy and compensatory renal growth and demonstrate that mitochondria isolated from NPX rats are more susceptible to injury from oxidants and alkylating agents than are isolated mitochondria from control rats. The marked alterations in mitochondrial function may help provide a mechanism for the increased susceptibility of NPX rats to several nephrotoxicants. Additionally, targeting of protective agents to preserve mitochondrial function and, in particular, redox status may help minimize chemically induced renal injury in patients with reduced renal mass.

2. Materials and methods

2.1. Chemicals

Rotenone, 1,1,3,3-tetraethoxypropane, ferricytochrome *c*, tBH, and MVK were purchased from the Sigma Chemical Co. DCVC was synthesized from trichloroethylene and L-cysteine as described previously [26]. Purity (>95%) was determined by HPLC analysis, and identity was confirmed by proton NMR spectroscopy. All other chemicals were of the highest purity available and were obtained from commercial sources.

2.2. Animals and surgical procedures

Male Sprague–Dawley rats (175–200 g at the time of surgery; Harlan Sprague–Dawley) were used in the present study. Animals were housed in the Wayne State University vivarium, were allowed access to laboratory chow and water *ad lib.*, and were kept in a room on a 12-hr light–dark cycle. The rats were divided into two surgical groups, one that served as a non-surgical control and the other that consisted of animals that underwent uninephrectomy. Animals were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg body weight; 0.1 mL of a 50 mg/mL solution per 100 g body weight) before surgery. Each uninephrectomy involved removal of the right kidney as described previously [7]. Previous studies have shown that there are no differences in responsiveness between cells or tissue isolated from non-surgical control and sham-operated rats; hence, non-surgically treated rats were used to obtain control mitochondria.

Mitochondria were isolated from renal cortical homogenates by differential centrifugation [27]. The buffer used for isolating mitochondria contained 20 mM triethanolamine/HCl (pH 7.4), containing 225 mM sucrose, 10 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 20 mM KCl, and 0.1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. EGTA (2 mM) was included in all preparatory stages, except the final resuspension, to remove calcium ions. This final buffer (i.e. no EGTA) is subsequently referred to as "mitochondrial buffer."

As reported previously [5,7,14], the remnant kidney in

NPX rats increased markedly in size and weight relative to those of the corresponding kidney in control rats. The weight of the remnant, left kidney from NPX rats used in the present study was 1.55 ± 0.06 g (mean ± SEM, N = 12), whereas the weight of the left kidney from control rats was 1.16 ± 0.06 (mean ± SEM, N = 12), which represents a statistically significant ($P < 0.05$) difference in mass of 33.6%. The yield of mitochondrial protein from the remnant, left kidney of NPX rats was 101 ± 5 mg protein (mean ± SEM, N = 6), whereas that from the left kidney of control rats was 76.4 ± 12.4 (mean ± SEM, N = 7). Although the difference in the yield of mitochondrial protein per kidney between control and NPX rats was > 30%, it was not statistically significant ($P = 0.0603$).

2.3. Enzyme assays and protein determination

GSH peroxidase (EC 1.11.1.9) activity was measured with 0.25 mM H_2O_2 as substrate and was equated to NADPH oxidation as detected by the decrease in absorbance at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) [28]. GSSG reductase (EC 1.6.4.2) activity was measured as NADPH oxidation by the decrease in absorbance at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) [29]. Glutamate dehydrogenase (EC 1.4.1.2) activity was measured spectrophotometrically by coupling 2-oxoglutarate reduction to NADH oxidation, and activity was quantitated by determining the consequent decrease in absorbance at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) [30]. Malic dehydrogenase (EC 1.1.1.37) activity was measured by coupling malate reduction to NADH oxidation, and activity was quantitated by determining the consequent decrease in absorbance at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) [31]. Succinate: cytochrome *c* oxidoreductase (EC 1.3.99.1) activity was measured by coupling succinate oxidation to ferricytochrome *c* reduction, and activity was quantitated by determining the increase in absorbance at 550.5 nm ($\epsilon = 18{,}500\ M^{-1}\ cm^{-1}$) [32]. The protein content in suspensions of isolated mitochondria was determined by a Coomassie Blue G dye-binding assay [33], using bovine serum albumin (0.1 to 0.6 mg/mL) as a standard.

2.4. Mitochondrial oxygen consumption

Oxygen consumption in suspensions of isolated mitochondria was measured with a Gilson 5/6H oxygraph (Gilson) in a thermostated, air-tight, 1.6-mL chamber at 25°. State 3 rates of oxygen consumption were measured by the addition of 0.3 mM ADP and respiratory substrate (4 mM glutamate + 2 mM malate for coupling site I; 3.3 mM succinate in the presence of 5 μM rotenone [in ethanol; final concentration = 0.3%, v/v] for coupling site II; 1 mM ascorbate + 0.2 mM TMPD for coupling site III) to the chamber containing 0.5 mL of mitochondrial sample (1.5 to 3.0 mg protein/mL) and 1 mL of mitochondria buffer. State 4 rates of oxygen consumption were measured after depleting ADP. Respiratory control ratios (RCR = state 3 rate/state 4 rate) > 3.0 were used as the criterion for functional integrity.

2.5. Lipid peroxidation

MDA content was measured as an index of lipid peroxidation, and was quantitated as thiobarbituric acid-reactive material, according to Ref. 34. Aliquots of mitochondrial suspensions (0.5 mL) were mixed with 0.5 mL of 10% (w/v) trichloroacetic acid and 1.0 mL of 0.76% (w/v) 2-thiobarbituric acid, and the mixtures were heated in a boiling water bath for 15 min. After cooling to room temperature, insoluble material was removed by centrifugation, and absorbance of supernatants was measured at 532 nm. Acid hydrolysates of 1,1,3,3-tetraethoxypropane were used as MDA standards. Standard curves were generated and were linear (regression line: $y = 0.00280 + 0.05624x$; $r^2 = 1.000$) between 0.2 and 10 nmol MDA/mL.

2.6. GSH uptake

Uptake of GSH into isolated mitochondria was measured by the "centrifugation-resuspension method" described previously [24]. Suspensions of isolated mitochondria (2 mg protein/mL) were incubated in mitochondrial buffer containing 0, 1, 2.5, 5, 7.5, or 10 mM GSH for up to 20 min. At indicated times, 0.5-mL aliquots of the mixtures were placed in microcentrifuge tubes and centrifuged at 13,000 *g* for 30 sec at 4°, the supernatants were decanted, and pellets were resuspended in 0.5 mL of ice-cold mitochondrial buffer and centrifuged again. Pellets were then resuspended in 0.5 mL of 10% (v/v) perchloric acid, and acid extracts were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene. *S*-Carboxymethyl-*N*-dinitrophenyl derivatives of GSH were analyzed by HPLC [18,35].

2.7. Data analysis

All values are means ± SEM of measurements made on the indicated number of separate mitochondrial preparations. Significant differences among means for data were first assessed by a one-way analysis of variance. When significant "F values" were obtained, the Fisher's protected least significance *t*-test was performed to determine which means were significantly different from one another, with two-tail probabilities < 0.05 considered significant. To assess statistical differences in kinetic parameters for GSH transport between NPX and control mitochondria, analysis of covariance was applied to the Eadie–Hofstee plot data.

3. Results

3.1. Mitochondrial enzyme and respiratory activities

Activities of three enzymes involved in mitochondrial carbohydrate metabolism and two enzymes involved in the

Table 1
Effect of compensatory renal cellular hypertrophy on mitochondrial enzyme activities

	Control (mU/mg protein)	NPX
Glutamate dehydrogenase	195 ± 16 (6)	183 ± 10 (9)
Malic dehydrogenase	55.5 ± 3.1 (9)	210 ± 16* (9)
Succinate:cytochrome *c* oxidoreductase	252 ± 14 (9)	347 ± 18* (9)
GSH peroxidase	75.4 ± 5.5 (9)	62.0 ± 7.1 (9)
GSSG reductase	46.3 ± 1.5 (9)	50.0 ± 1.5 (9)

Enzyme activities were measured by spectrophotometric assays, as described in "Materials and methods." Results are means ± SEM of measurements from the indicated number of separate mitochondrial preparations shown in parentheses. NPX = mitochondria from rats that have undergone uninephrectomy and compensatory renal cellular hypertrophy.

* Significantly different ($P < 0.05$) from value in mitochondria from control rats.

GSH redox cycle were measured in renal cortical mitochondria from control and NPX rats (Table 1). Because total content of cellular protein [1,5,7,8] and mitochondrial content of protein [10] increase as a consequence of uninephrectomy and compensatory renal growth, values for mitochondrial enzyme activities that are unchanged when normalized to protein content are, in fact, increased when normalized to cell number. In other words, the amount of certain enzymes per cell may be increased, but only in proportion to any increases in overall cellular protein content. No significant differences in glutamate dehydrogenase activity were detected between renal mitochondria from control and NPX rats. In contrast, both malic dehydrogenase and succinate:cytochrome *c* oxidoreductase activities were significantly increased (by 378 and 38%, respectively) in mitochondria from NPX rats relative to mitochondria from control rats, even when normalized to protein content. There was no significant difference in activity of GSH peroxidase or GSSG reductase between renal mitochondria from control rats and renal mitochondria from NPX rats.

Results of analysis of respiratory function with substrates that donate electrons at the three different coupling sites in renal mitochondria from control and NPX rats are presented in Fig. 1. No significant difference in state 3 respiration, with either glutamate + malate (site I) or ascorbate + TMPD (site III) as respiratory substrates, was detected between renal mitochondria from NPX rats and renal mitochondria from control rats. However, succinate-stimulated state 3 respiration (coupling site II) was 68% higher in renal mitochondria from NPX rats than in those from control rats. Similarly, the RCR value (which provides an indication of how well oxygen consumption is coupled to ADP phosphorylation) was elevated significantly with succinate, but not with glutamate + malate, as respiratory substrate in renal mitochondria from NPX rats relative to that in renal mitochondria from control rats. Additionally, the RCR value with ascorbate + TMPD as respiratory substrates was significantly greater in renal mitochondria from NPX rats than in those from control rats.

3.2. Chemically induced toxic effects in isolated mitochondria

To assess the susceptibility of isolated mitochondria to chemically induced injury, mitochondria were incubated with one of three known mitochondrial toxicants, and the effects on respiration and lipid peroxidation were determined. The three chemicals, tBH, MVK, and DCVC, produce mitochondrial toxicity by GSH oxidation and formation of reactive oxygen species, alkylation of thiol groups, and a combination of thiol oxidation and alkylation, respectively [18–21].

Incubation of renal mitochondria from control rats for 15 min with up to 2 mM tBH had no effect on state 4 respiration, whereas the highest concentration of tBH tested (2 mM) produced a statistically significant increase in state 4 respiration in isolated renal mitochondria from NPX rats (Fig. 2A). In contrast, tBH produced concentration-dependent inhibition of state 3 respiration in renal mitochondria from both control and NPX rats. As shown in Fig. 1, the rate of state 3 respiration in untreated mitochondria (i.e. 0 mM tBH) from NPX rats was 2.1-fold higher than that in untreated mitochondria from control rats. Maximal inhibition of state 3 respiration was 38% in mitochondria from control rats and 59% in mitochondria from NPX rats. The greater amount of inhibition of state 3 respiration and the modest stimulation of state 4 respiration induced by tBH in renal mitochondria from NPX rats (relative to those from control rats) resulted in only modest differences in RCR values (Fig. 2B). The maximal decrease in RCR value in mitochondria from control rats was 26% with 1 mM tBH and that in mitochondria from NPX rats was 44% with 0.5 mM tBH.

State 4 respiration was unaffected in renal mitochondria from either control or NPX rats incubated for 15 min with up to 2 mM MVK (Fig. 3A). In contrast, MVK markedly inhibited state 3 respiration in renal mitochondria from both control and NPX rats. The rate of state 3 respiration in untreated mitochondria (i.e. 0 mM MVK) from NPX rats was 51% higher than that in untreated mitochondria from control rats. As with tBH, the maximal amount of inhibition of state 3 respiration caused by MVK was greater in mitochondria from NPX rats (69%) than in mitochondria from control rats (42%). Unlike the results with tBH, MVK caused a marked decrease in RCR values in mitochondria from NPX rats (60% decrease at 2 mM MVK) and no significant change in the mitochondria from control rats (Fig. 3B).

Effects of DCVC and MVK on state 3 and state 4 respiration were similar in incubations of renal mitochondria from control and NPX rats: DCVC exhibited no effect on state 4 respiration in mitochondria from either control or NPX rats. However, 2 mM DCVC caused significantly more inhibition of state 3 respiration in mitochondria from NPX rats (82%) than in mitochondria from control rats (64%) (Fig. 4A). Similar to the pattern of effects of DCVC

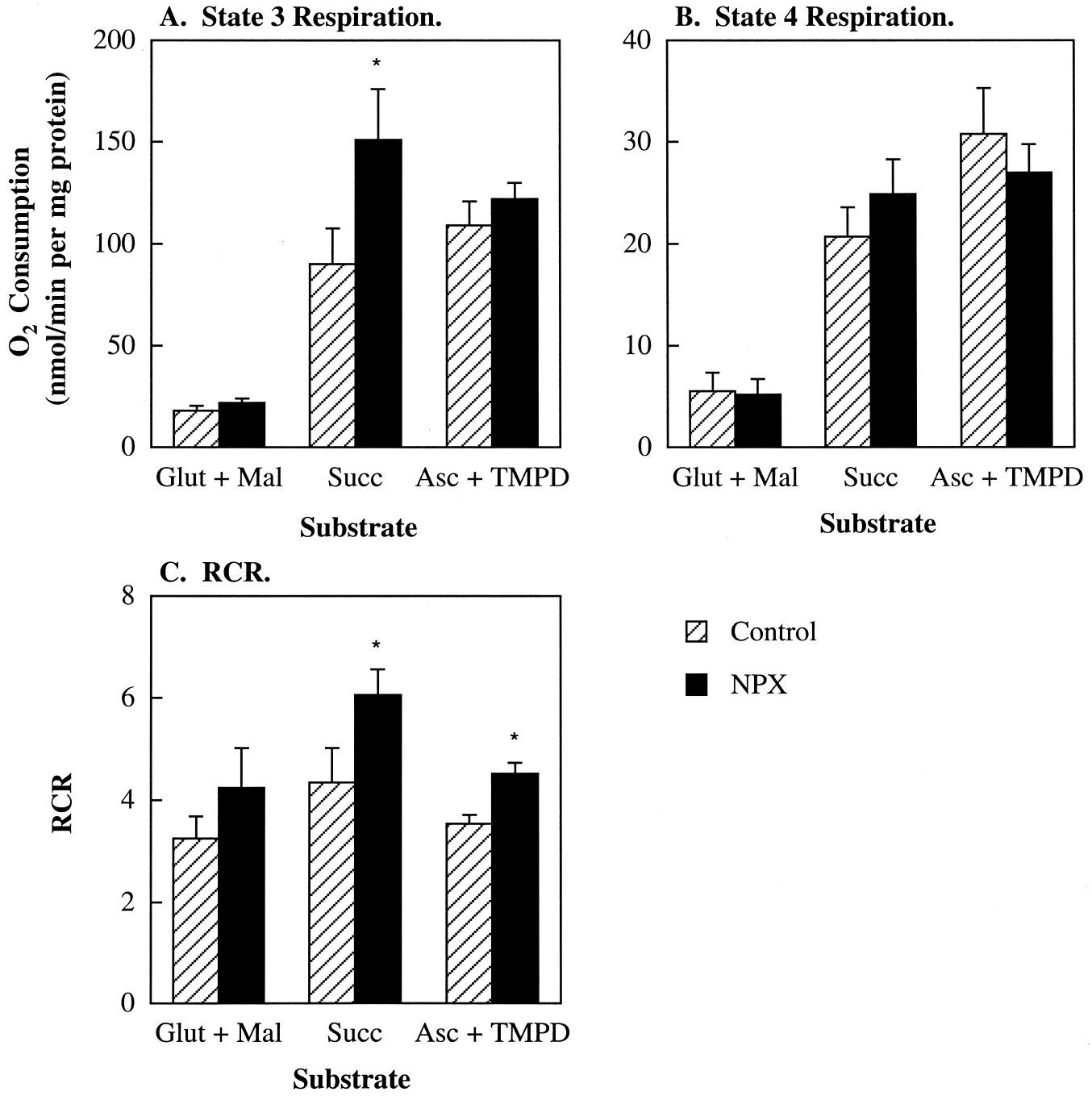

Fig. 1. Effect of compensatory renal cellular hypertrophy on mitochondrial respiratory function. Mitochondrial oxygen consumption was measured in isolated renal cortical mitochondria from control rats and rats that had undergone uninephrectomy and compensatory renal hypertrophy (NPX rats). State 3 respiration (A) was measured in an oxygraph after addition of mitochondrial suspension, buffer, respiratory substrate [1 mM glutamate + 1 mM malate; 3.3 mM succinate (in the presence of 5 μM rotenone); 1 mM ascorbate + 0.2 mM TMPD], and 0.3 mM ADP. State 4 respiration (B) was measured after consumption of ADP. RCR values (C) were calculated as the ratio of state 3/state 4 rates of respiration. Results are the means ± SEM of measurements from 8 separate mitochondrial preparations. Key: (*) significantly different ($P < 0.05$) from the corresponding control. Abbreviations: Glut, glutamate; Mal, malate; Succ, succinate; Asc, ascorbate.

on state 3 respiration, RCR values were decreased to a significantly greater extent in mitochondria from NPX rats (77% decrease at 2 mM DCVC) than in mitochondria from control rats (54% decrease at 2 mM DCVC) (Fig. 4B).

Isolated mitochondria from both control and NPX rats incubated with tBH exhibited concentration-dependent increases in MDA formation (Fig. 5), indicating lipid peroxidation and oxidative stress. Basal levels of MDA in untreated mitochondria from NPX rats were 2.1-fold higher than those in untreated mitochondria from control rats. Over

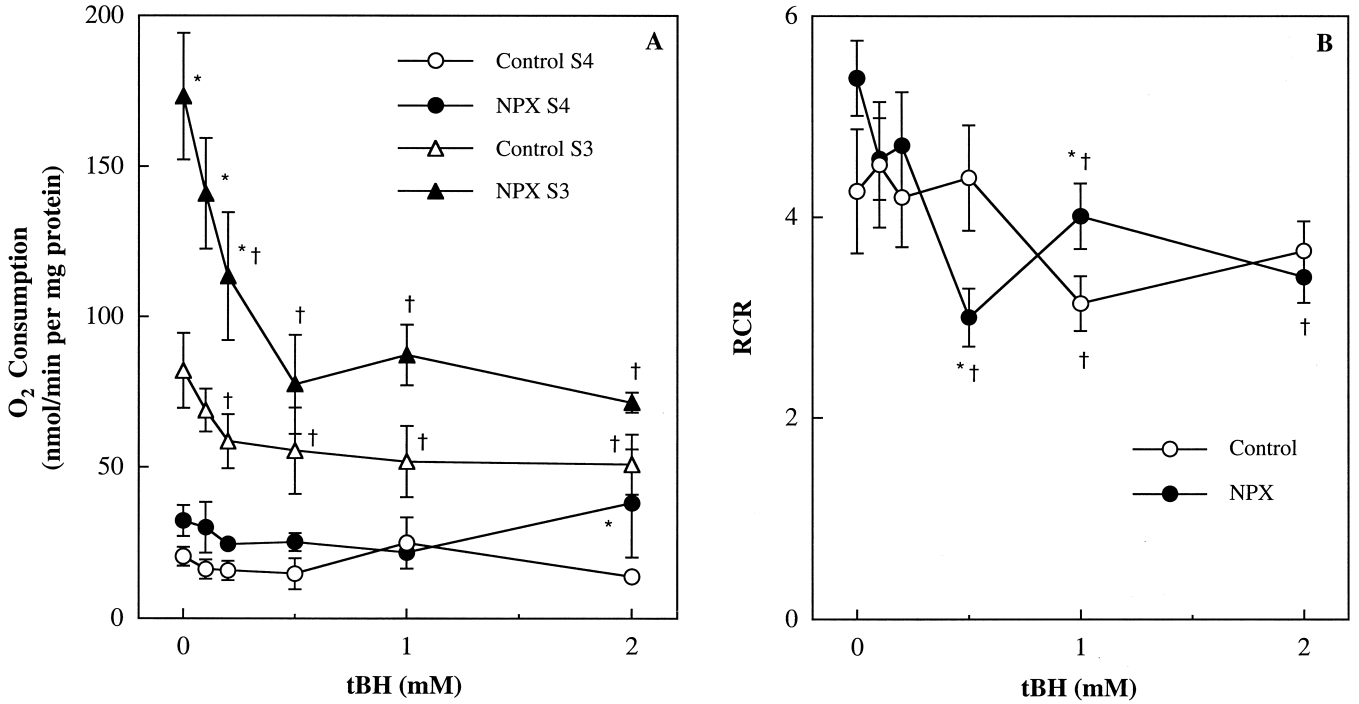

Fig. 2. Effect of tBH on respiratory function in isolated mitochondria from control and NPX rats. Renal cortical mitochondria from control or NPX rats were incubated with the indicated concentration of tBH for 15 min. Oxygen consumption was measured in the presence of 3.3 mM succinate + 5 μM rotenone and 0.3 mM ADP. State 3 and state 4 rates of respiration (A) and RCR values (B) were determined as described in "Materials and methods." Results are means ± SEM of measurements from 8 separate mitochondrial preparations. Key: (*) significantly different ($P < 0.05$) from control at the same concentration of tBH, and (†) significantly different ($P < 0.05$) from 0 mM tBH within the same surgical group.

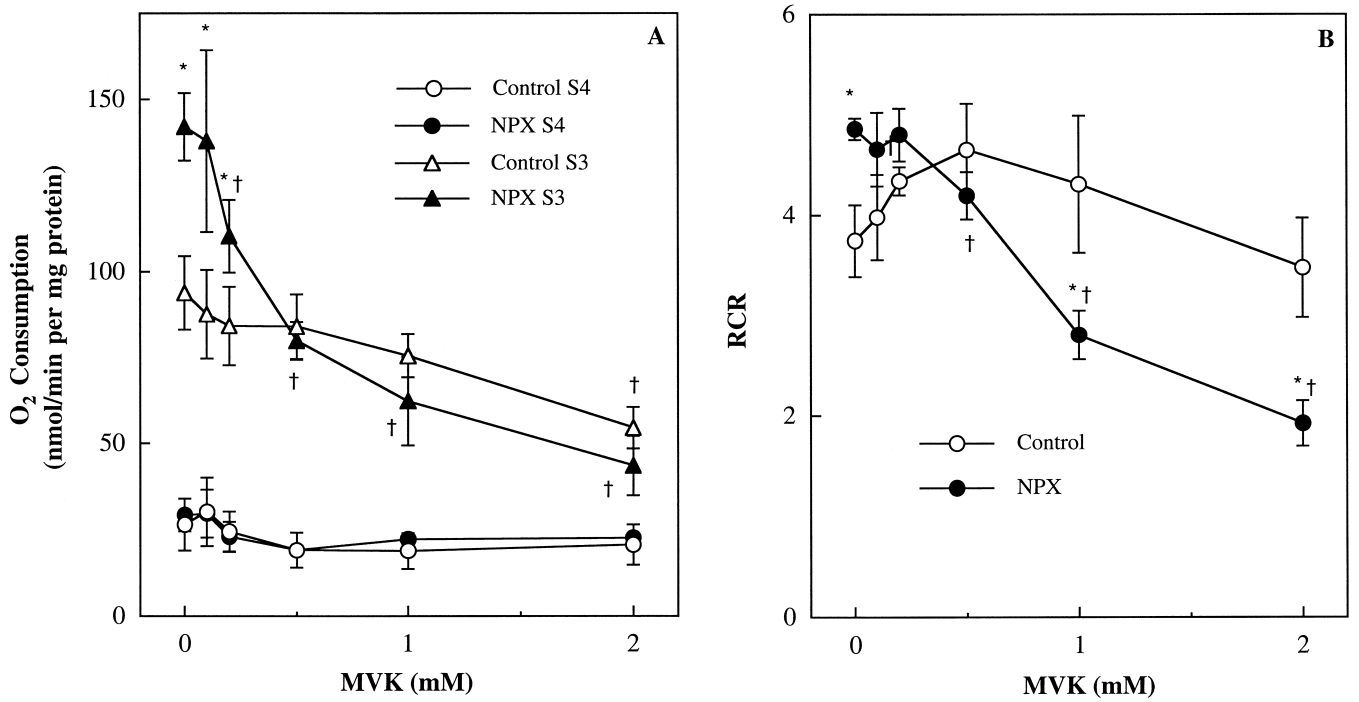

Fig. 3. Effect of MVK on respiratory function in isolated mitochondria from control and NPX rats. Renal cortical mitochondria from control or NPX rats were incubated with the indicated concentration of MVK for 15 min. Oxygen consumption was measured in the presence of 3.3 mM succinate + 5 μM rotenone and 0.3 mM ADP. State 3 and state 4 rates of respiration (A) and RCR values (B) were determined as described in "Materials and methods." Results are means ± SEM of measurements from 8 separate mitochondrial preparations. Key: (*) significantly different ($P < 0.05$) from control at the same concentration of MVK, and (†) significantly different ($P < 0.05$) from 0 mM MVK within the same surgical group.

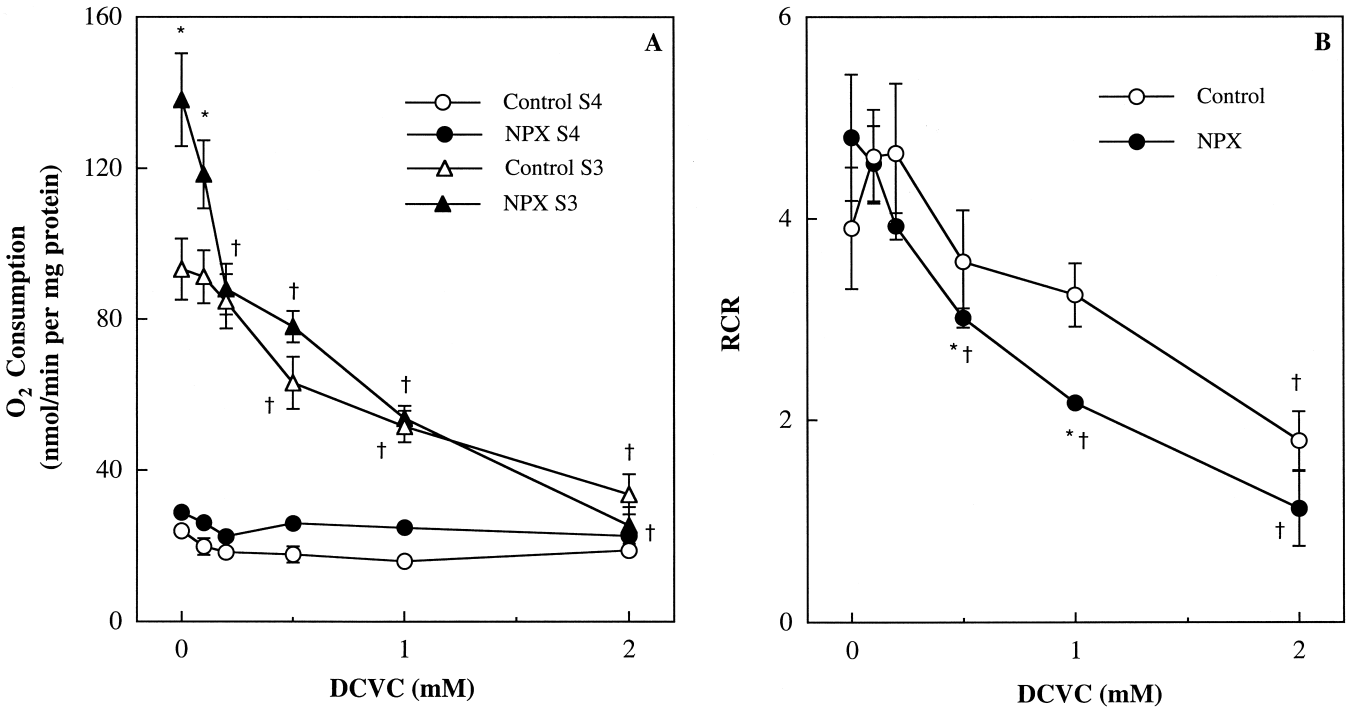

Fig. 4. Effect of DCVC on respiratory function in isolated mitochondria from control and NPX rats. Renal cortical mitochondria from control or NPX rats were incubated with the indicated concentration of DCVC for 15 min. Oxygen consumption was measured in the presence of 3.3 mM succinate + 5 μM rotenone and 0.3 mM ADP. State 3 and state 4 rates of respiration (A) and RCR values (B) were determined as described in "Materials and methods." Results are means ± SEM of measurements from 8 separate mitochondrial preparations. Key: (*) significantly different ($P < 0.05$) from control at the same concentration of DCVC, and (†) significantly different ($P < 0.05$) from 0 mM DCVC within the same surgical group.

the range of tBH concentrations tested, contents of MDA increased 3.4-fold in mitochondria from control rats and 2.4-fold in mitochondria from NPX rats.

Results of MDA formation in incubations of mitochondria from control or NPX rats containing buffer, 2 mM DCVC, or 2 mM MVK were qualitatively similar with those obtained with tBH. Both DCVC and MVK increased MDA levels by a similar amount (1.7- to 2.1-fold) in mitochondria from both control and NPX rats (Fig. 6).

3.3. GSH transport in isolated mitochondria

Maintenance of redox status is critical to mitochondrial function, and GSH status is an important component in that process. Time- and concentration-dependence of GSH uptake in isolated mitochondria from control and NPX rats were measured to determine whether the capacity of mitochondria to accumulate GSH is altered as a result of compensatory renal growth. GSH was transported rapidly into isolated renal mitochondria from both control and NPX rats, reaching equilibration at 5–10 min at all GSH concentrations tested (Fig. 7). The levels of GSH reached a plateau that was markedly higher in mitochondria from NPX rats than in mitochondria from control rats (25.2 vs 9.22 nmol GSH/min per mg protein; 2.7-fold higher).

Initial rates of GSH uptake (at each concentration tested) were plotted against substrate concentration to generate a Michaelis–Menten plot and were used to construct an Eadie–Hofstee plot to derive kinetic parameters (Fig. 8). As with the time–course data, both plots show that compensatory renal growth significantly affected mitochondrial transport of GSH: mitochondria from NPX rats exhibited a 2.3-fold lower K_m and a 1.9-fold higher V_{max} than mitochondria from control rats. Analysis of covariance showed that kinetic parameters for GSH transport were significantly different between mitochondria from control and NPX rats. More specifically, the K_m for GSH was 8.62 and 3.76 mM in mitochondria from control and NPX rats, respectively, and the V_{max} was 10.6 and 20.6 nmol GSH/min per mg protein in mitochondria from control and NPX rats, respectively. Calculation of the ratio V_{max}/K_m shows that mitochondria from NPX rats catalyzed GSH uptake with a 4.5-fold higher efficiency than mitochondria from control rats ($V_{max}/K_m = 1.23$ and 5.48 in mitochondria from control and NPX rats, respectively).

4. Discussion

The present study was designed to test the hypothesis that renal mitochondria from NPX rats exhibit increased rates of intermediary metabolism and respiration and that this change is associated with increased basal and stimulated oxidative stress. We then tested the hypothesis that

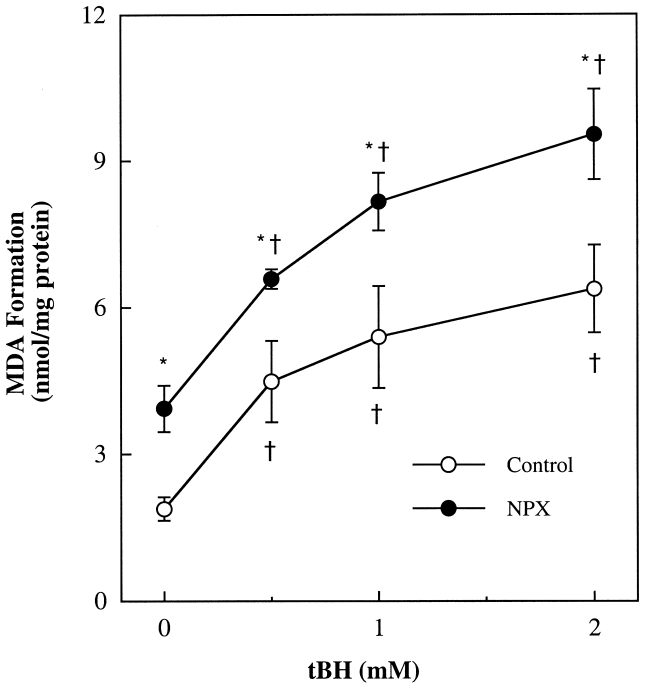

Fig. 5. Concentration dependence of tBH-induced MDA formation in isolated mitochondria from control and NPX rats. Suspensions of isolated mitochondria from control and NPX rats were incubated with 0, 0.5, 1, or 2 mM tBH for 15 min at 25°. Lipid peroxidation was assessed by measurement of MDA formation. Results are the means ± SEM of measurements from 3–4 separate mitochondrial preparations. Key: (*) significantly different ($P < 0.05$) from the control at the same concentration of tBH, and (†) significantly different ($P < 0.05$) from 0 mM tBH within the same surgical group.

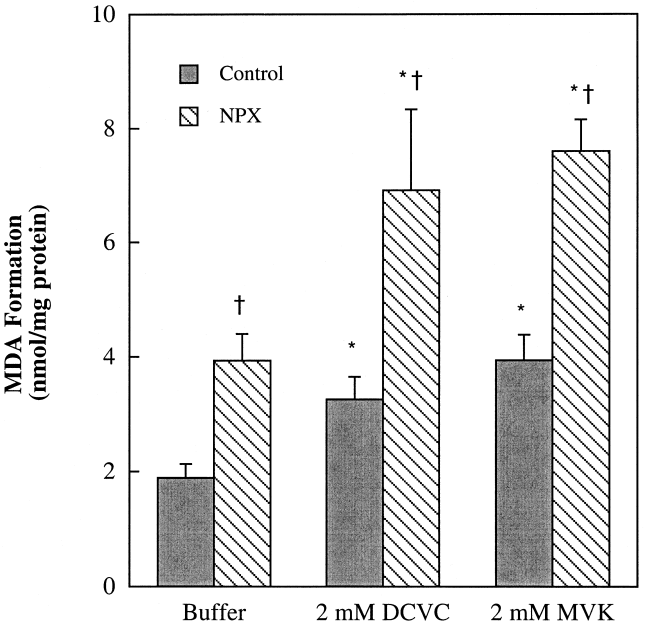

Fig. 6. DCVC- and MVK-induced lipid peroxidation in isolated mitochondria from control and NPX rats. Suspensions of isolated mitochondria from control and NPX rats were incubated with either buffer, 2 mM DCVC, or 2 mM MVK for 15 min at 25°. Lipid peroxidation was assessed by measurement of MDA formation. Results are the means ± SEM of measurements from 3–4 separate mitochondrial preparations. Key: (*) significantly different ($P < 0.05$) from buffer-treated sample in the same surgical group, and (†) significantly different ($P < 0.05$) from the correspondingly treated sample from control rats.

mitochondria from hypertrophied kidneys are more susceptible to chemically induced oxidant injury. Our results show for the first time that mitochondria from hypertrophied kidneys exhibit increased rates of respiration, particularly when a site-II respiratory substrate (i.e. succinate) is used as the electron donor. It is important to note that although there were several processes or reactions that did not increase as a result of compensatory renal growth when they were normalized to protein, the fact that they did not decrease relative to protein is significant, inasmuch as cellular and mitochondrial protein contents are known to increase by as much as 30–75% under these conditions [1,5,7,8,10]. In the current study, overall mitochondrial content of protein increased by approximately 33%.

Two interesting questions arise from the data on enzyme activities. First, some dehydrogenases increase relative to changes in total mitochondrial protein as a consequence of compensatory renal growth (e.g. malic and succinate dehydrogenases), whereas other dehydrogenases (e.g. glutamate dehydrogenase) do not change relative to total mitochondrial protein. The mechanism for this selectivity is unknown. However, rates of state 3 oxygen consumption with a site II substrate were increased selectively, which is consistent with the large increase, relative to protein, of succinate dehydrogenase. Additionally, the lack of an increase in glutamate dehydrogenase relative to protein is also consistent with the absence of an increase in state 3 oxygen consumption with a site I substrate. The second question pertains to whether the increase in total mitochondrial protein is due to specific and selective induction of protein synthesis in mitochondria or the production of more mitochondria. Although there are no direct data addressing this point, the selective increase in activity of some mitochondrial dehydrogenases and the lack of a large increase in the yield of mitochondrial protein relative to total left kidney protein suggest that specific induction of certain mitochondrial proteins may play a role in the increase in mitochondrial protein. An overall increase in the number of mitochondria within the hypertrophied proximal tubular cell also may occur.

Neither mitochondrial GSH peroxidase nor mitochondrial GSSG reductase activities differed between mitochondria from control and NPX rats when the activities were normalized to protein. However, these two enzymes did increase in mitochondria from NPX rats, although in proportion to mitochondrial protein. It has been suggested that the higher rates of mitochondrial respiration result in a hypermetabolic state induced by the increased workload on the remaining functional nephrons [10]. Such a state would be expected to be accompanied by increased rates of generation of reactive oxygen species resulting from electron flow through the respiratory chain. Evidence showing that

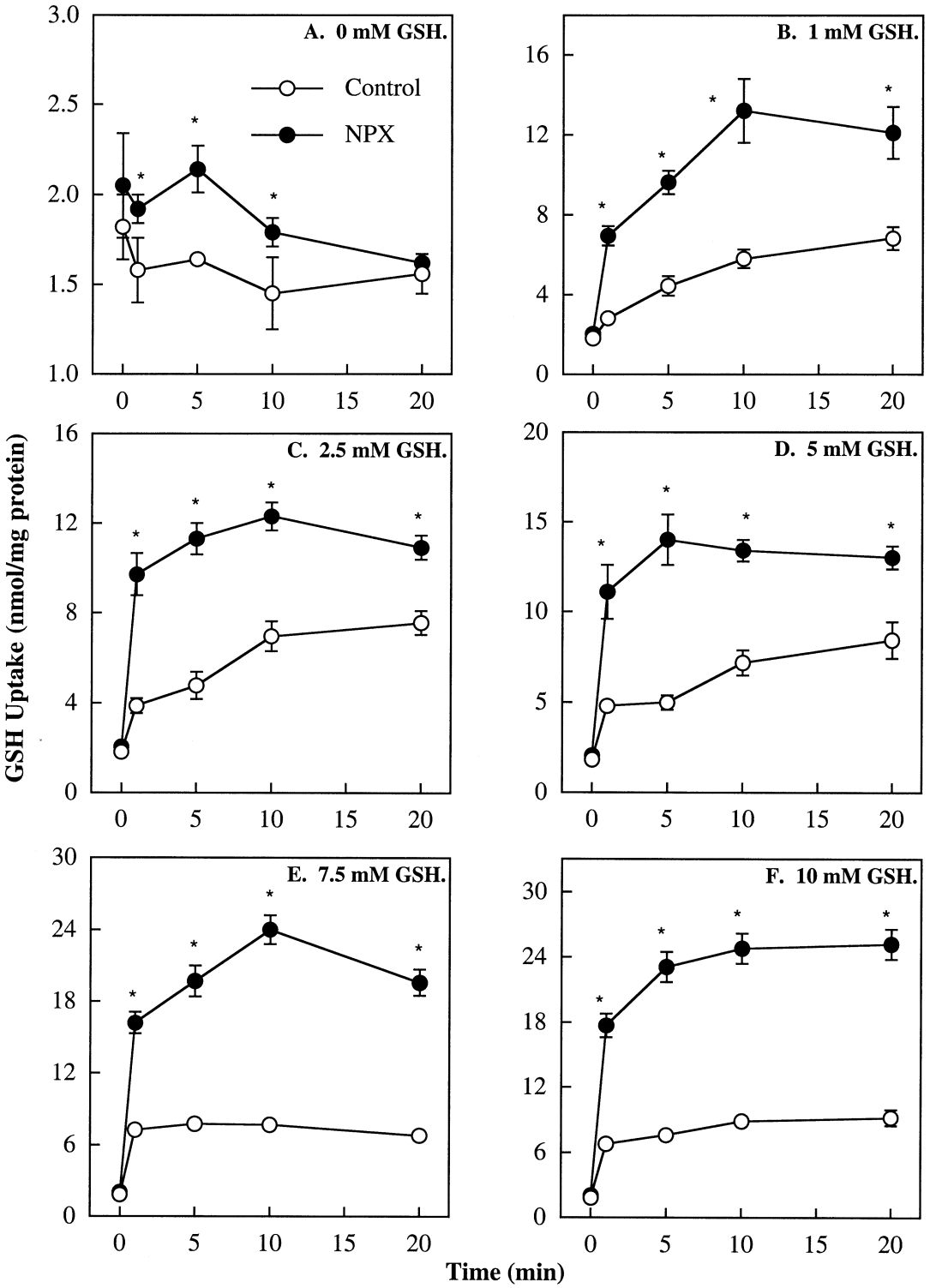

Fig. 7. Time- and concentration-dependence of GSH uptake in isolated mitochondria from control and NPX rats. Time courses of uptake of GSH [0, 1, 2.5, 5, 7.5, and 10 mM] were measured in suspensions of isolated mitochondria from control or NPX rats. At the indicated times, aliquots of mitochondrial suspensions were centrifuged, resuspended in ice-cold buffer, and recentrifuged; pellets were resuspended in perchloric acid. Acid extracts were derivatized with iodoacetate and 1-fluoro-2,4-dinitrobenzene and were analyzed for GSH content by HPLC. Results are the means ± SEM of measurements from 6 and 3 separate mitochondrial preparations for control (=0 mM) and each concentration of GSH, respectively. Where error bars are not shown, SEM values were within the size of the data points. Key: (*) significantly different ($P < 0.05$) from values in the corresponding control samples.

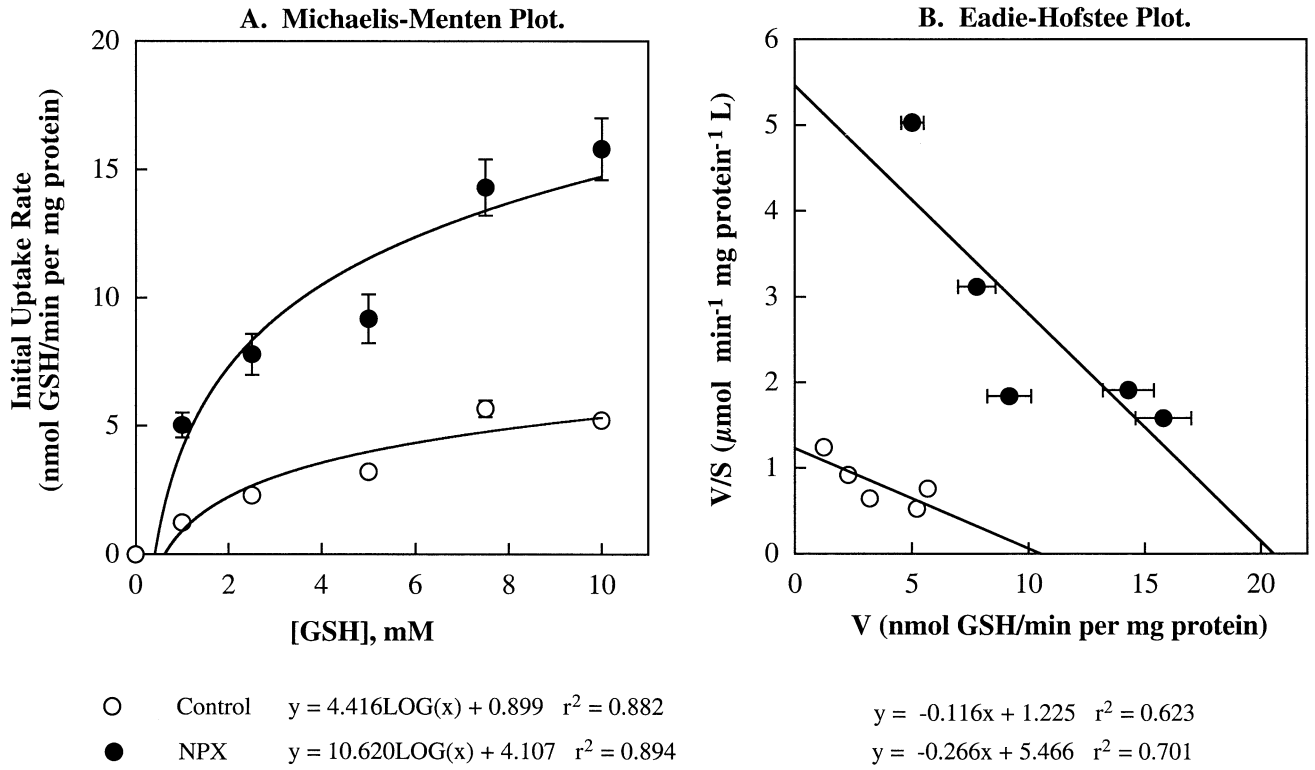

Fig. 8. Kinetics of GSH uptake in renal mitochondria from control and NPX rats. Initial rates of uptake for GSH were obtained from the data in Fig. 7, and these were plotted versus GSH concentration: Michaelis–Menten plot (A) or Eadie–Hofstee plot (B). Curves in panel A were obtained by logarithmic regression, whereas those in panel B were obtained by linear regression. Regression equations and correlation coefficients are shown below each plot.

this is indeed the case in renal mitochondria from NPX rats comes from data showing that basal levels of MDA were approximately 2-fold higher in mitochondria from NPX rats than in mitochondria from control rats (cf. Figs. 5 and 6). Although measurement of MDA formation is only an indirect indicator of oxidative stress, it is used routinely by many investigators who study oxidative stress. In the present study, MDA formation was measured in paired incubations with either buffer or oxidant. Hence, any change in measured levels of MDA in samples incubated with oxidant (i.e. tBH, MVK, or DCVC) is considered relative to levels of MDA determined in samples incubated with buffer (i.e. control samples), and is thus a valid indicator of relative oxidative stress.

One would also expect that rates of metabolite transport into mitochondria would be elevated as a consequence of compensatory renal growth. Although transport of citric acid cycle intermediates was not measured directly, transport of GSH, which occurs by way of the dicarboxylate and 2-oxoglutarate carriers [19,24,25], was measured, and was found to be markedly and significantly increased in mitochondria from NPX rats relative to mitochondria from control rats. Overall transport of GSH into mitochondria from NPX rats exhibited a greater than 2-fold lower K_m and a nearly 2-fold higher $V_{max.}$ These data indicate that GSH transport in mitochondria from NPX rats is approximately 4-fold more efficient than that in mitochondria from control rats. The dicarboxylate and 2-oxoglutarate carriers are involved in the transport of key metabolic intermediates, such as succinate, malate, and inorganic phosphate, across the mitochondrial inner membrane [36]. Increases in the activities of these carriers would be necessary to deliver substrate to the complexes of the respiratory chain. Direct measurement of rates of dicarboxylate transport would, of course, support this hypothesis, but this is beyond the scope of the present study. Our purpose in measuring GSH transport was not to provide an indication of dicarboxylate transport, but to provide an indication of the ability of renal mitochondria to regulate redox status by increasing delivery of GSH to the mitochondrial matrix.

To test the second hypothesis concerning the toxicological consequences of the increased rates of metabolism and respiration associated with compensatory renal growth, three chemicals that are well-characterized mitochondrial toxicants were studied. In each case, greater inhibition of state 3 respiration and greater increases in MDA formation were observed in mitochondria from NPX rats than in mitochondria from control rats. Some minor differences were noted in the patterns of the effects produced by the three agents, and these seem consistent with the differences in their mechanisms of action. For example, tBH was the most effective agent in increasing MDA formation, and this makes sense as tBH produces reactive oxygen species more

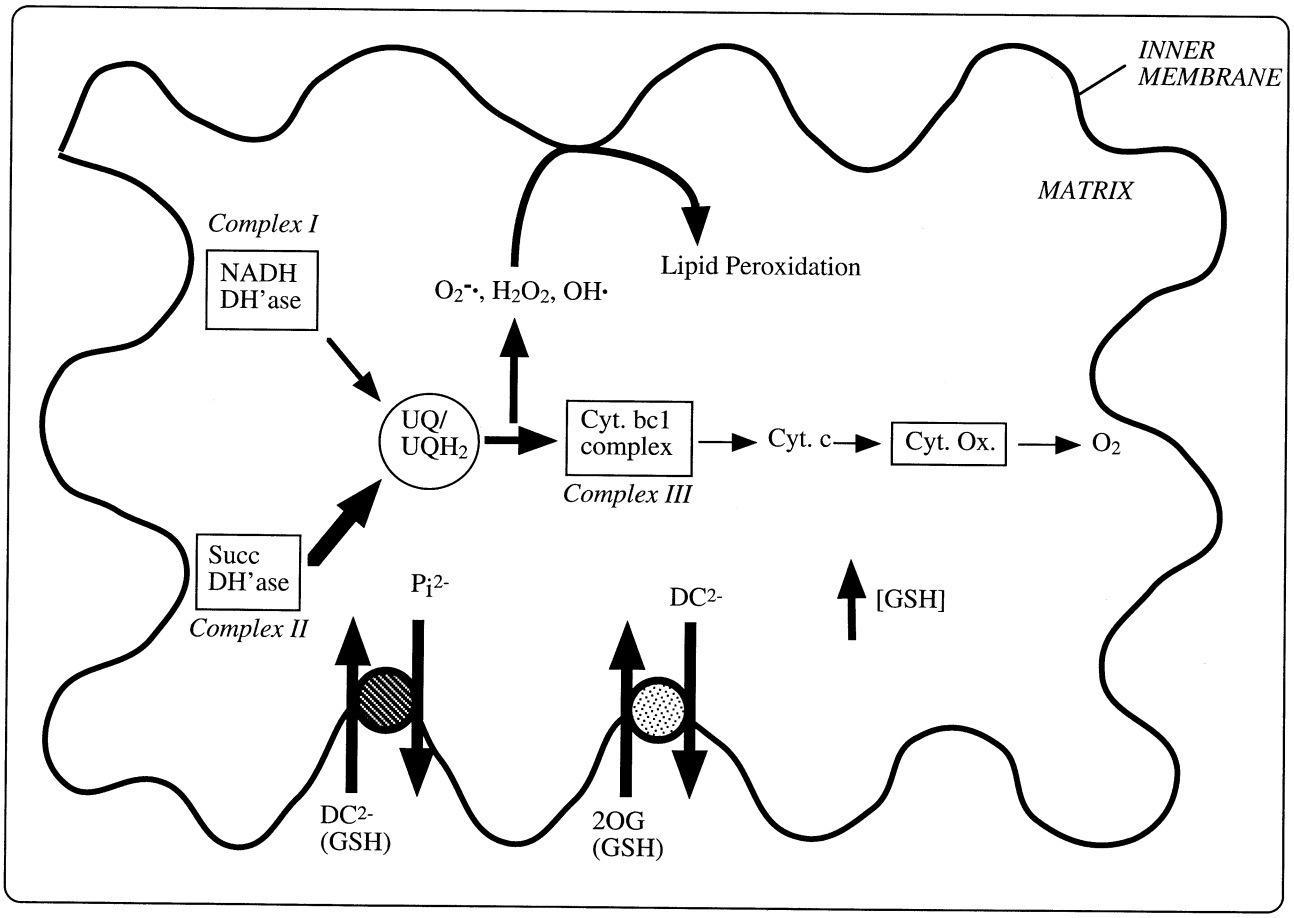

Fig. 9. Summary scheme of processes in isolated renal mitochondria that are altered in NPX rats. The scheme summarizes the processes of metabolite and electron transport in renal mitochondria. The thickness of arrows is used to indicate relative flux of each step, with the thicker arrows indicating higher flux. The data from the present study show that rates of metabolite and GSH transport into renal mitochondria and rates of electron flow, particularly through complexes I–III, were increased significantly after uninephrectomy and compensatory renal growth. The increased electron flow resulted in increased release of reactive oxygen species, presumably at the ubiquinone-to-cytochrome bc_1 step, resulting in increased rates of lipid peroxidation. Abbreviations: DH'ase, dehydrogenase; Succ, succinate; UQ and UQH_2, oxidized and reduced ubiquinone; Cyt., cytochrome; Ox., oxidase; DC^{2-}, dicarboxylate; 2OG, 2-oxoglutarate; and P_i^{2-}, inorganic phosphate.

directly than either DCVC or MVK, both of which act primarily by thiol alkylation.

We have demonstrated previously in isolated rat renal proximal tubular cells [14,15] and in whole animal studies [7] that the inherent susceptibility of proximal tubular cells to the toxic effects of inorganic mercury is increased significantly after uninephrectomy and compensatory renal growth. Similarly, we find that renal mitochondria from NPX rats are far more sensitive to the toxic effects of three well-characterized mitochondrial toxicants than renal mitochondria from normal rats. The finding is surprising in one sense, in that some of the detoxification reactions (i.e. GSH peroxidase and GSSG reductase) maintained their activity levels relative to protein. However, the increase in respiratory activity, particularly with coupling site II substrates such as succinate, was increased markedly relative to protein content, suggesting that the generation of reactive oxygen species may increase substantially to levels that are beyond the capacity for detoxification.

The toxicological significance of these results in terms of understanding the mechanism of the altered susceptibility to chemically induced renal injury in rats that have undergone uninephrectomy and compensatory renal growth is illustrated by the diverse array of nephrotoxic chemicals whose effects are altered significantly as a result of uninephrectomy and compensatory renal growth. In addition to the severity of the nephropathy induced by inorganic mercury, which is increased in NPX rats relative to control rats [7,12,16,17], the nephropathy induced by analgesics [37,38] and cadmium-metallothionein [39] has also been shown to be enhanced following uninephrectomy and compensatory renal growth. Enhanced mitochondrial metabolism, and the accompanying hypermetabolic state of the hypertrophied renal proximal tubular cell, may thus play a key role in their altered susceptibility to numerous drugs and other chemicals.

One adaptation that has occurred as a consequence of compensatory renal growth, and that can possibly be used

therapeutically to protect mitochondria from oxidant injury, is the increase in transport of GSH from the cytoplasm into the mitochondria. We did not specifically quantify the normal, endogenous concentrations of GSH within the mitochondria because the *in vitro* measurements would not likely reflect the actual concentrations within the intact cell. Upon isolation by differential centrifugation, renal mitochondria lose some of their endogenous metabolites, including GSH [19,24]. The incubations without added GSH (cf. Fig. 7) showed similar values for initial contents of GSH in the mitochondria from both NPX and control rats. A similar type of phenomenon was observed in isolated renal proximal tubular cells from control and NPX rats, namely that the isolated cells from control and NPX rats show no differences in GSH content [14]. However, it is well-documented by us and others that levels of GSH in renal proximal tubular cells increase significantly following uninephrectomy and compensatory renal growth [7,12,13]. It is likely, therefore, that proximal tubular mitochondria in the intact, remnant kidney of NPX rats also have higher concentrations of GSH than corresponding mitochondria from the kidneys of control rats. We know that the increases in GSH content that occur in proximal tubular cells of NPX rats are due to increased expression and activity of γ-glutamylcysteine synthetase (EC 6.3.2.2) [5,7], which is the rate-limiting enzyme for the synthesis of GSH. The source of mitochondrial GSH, however, is not *de novo* synthesis from precursor amino acids, but is the cytoplasm; mitochondria appear to lack the enzymes for synthesis of GSH and, therefore, must obtain it by transport of cytoplasmic GSH across the mitochondrial inner membrane [19,22,40]. Hence, the mechanism for increased intramitochondrial content of GSH is the increased catalytic efficiency (decreased K_m and increased V_{max}) in transporting GSH from the cytoplasm into the mitochondrial matrix.

In conclusion, we demonstrated that renal mitochondria isolated from NPX rats exhibit significantly higher rates of substrate metabolism and respiration than those from control rats. These changes were associated with or accompanied by an increased basal level of oxidants, as seen in the MDA measurements. A summary scheme, illustrating the processes in the renal mitochondrion that are altered as a result of uninephrectomy and compensatory renal growth, is shown in Fig. 9. The scheme highlights the suggested, enhanced rates of delivery of substrates to the respiratory chain, increased rates of transport of GSH by the dicarboxylate and 2-oxoglutarate carriers, and the enhanced rates of electron flow along selected portions of the respiratory chain. The data from this study suggest that these increased rates of electron flow then lead to increased rates of release of reactive oxygen species, which may cause increased lipid peroxidation within the mitochondrion. Additionally, exposure to oxidants or thiol-alkylating agents produced greater inhibition of mitochondrial state 3 respiration and greater increases in MDA formation in isolated mitochondria from NPX rats as compared with those from control rats, suggesting that reduced renal mass is associated with greater risk to renal injury induced by oxidants and other potential mitochondrial toxicants. This increased risk may be modulated by more rapid and efficient transport of GSH into the mitochondria, which may directly protect against oxidative stress.

Acknowledgments

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