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Influence of renal compensatory hypertrophy on mitochondrial energetics and redox status

Bavneet Benipal, Lawrence H. Lash *

Department of Pharmacology, Wayne State University School of Medicine, Detroit, MI 48201, United States

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ABSTRACT

A reduction in functional renal mass is common in numerous renal diseases and aging. The remaining functional renal tissue undergoes compensatory growth primarily due to hypertrophy. This is associated with a series of physiological, morphological and biochemical changes similar to those observed after uninephrectomy. Previous work showed that compensatory renal cellular hypertrophy resulted in an increase in susceptibility to several drugs and environmental chemicals and appeared to be associated with oxidative stress. Compensatory renal cellular hypertrophy was also associated with increases in mitochondrial metabolic activity, uptake of glutathione (GSH) across renal plasma and mitochondrial inner membranes, and intracellular GSH concentrations. Based on these observations, we hypothesize that the morphological, physiological and biochemical changes in the hypertrophied kidney are associated with marked alterations in renal cellular energetics, redox status and renal function *in vivo*. In this study, we used a uninephrectomized (NPX) rat model to induce compensatory renal growth. Our results show alterations in renal physiological parameters consistent with modest renal injury, altered renal cellular energetics, upregulation of certain renal plasma membrane transporters, including some that have been observed to transport GSH, and evidence of increased oxidative stress in mitochondria from the remnant kidney of NPX rats. These studies provide additional insight into the molecular changes that occur in compensatory renal hypertrophy and should help in the development of novel therapeutic approaches for patients with reduced renal mass.

1. Introduction

Significant reductions in functional renal mass involve pathophysiologic processes associated with alterations in electrolyte balance, proteinuria, abnormal carbohydrate metabolism and hypertension. Reductions in functional renal mass occur in humans during aging and severe kidney damage from diseases, injury or congenital conditions. Nephrectomy, or surgical removal of a kidney or a section of a kidney, is performed for treatment in these critical cases and for kidney transplantation. The incidence of reductions in renal mass is increasing in the U.S and represents a significant challenge to public healthcare. The number of patients treated with dialysis or kidney transplantation is projected to increase from 340 000 in 1999 to 651 000 in 2010 [1].

Reduction of renal mass due to nephrectomy results in increases in the size (hypertrophy) and function of the remnant kidney cells, most prominently in the proximal tubular (PT) region [2]. This compensatory hypertrophy is mediated by vasoactive molecules, cytokines, growth factors and increases in glomerular capillary pressure and flow [1–4]. The cellular characteristics of hypertrophy include increases in cellular volume, area and surface density of the basolateral (BLM) and brush-border plasma membranes (BBM), increased cellular synthesis and content of glutathione (GSH) [5], and mitochondrial proliferation within renal PT cells [6]. Physiological changes consist of increased renal blood flow, glomerular filtration rate, and water and electrolyte transport [4,7,8]. Biochemical changes include increases in cellular content of protein, rates of

Abbreviations: Acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; BLM, basolateral plasma membrane; BBM, brush-border plasma membrane; DIC, dicarboxylate carrier; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCS, L-γ-glutamyl-L-cysteine synthetase; GGT, γ-glutamyltransferase; GPX, glutathione peroxidase; GRD, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; MDH, malic dehydrogenase; Mrp, multidrug resistance associated protein; NaC3, sodium-dicarboxylate 3 carrier; NAG, *N*-acetyl-β-D-glucosaminidase; 3-NT, 3-nitrotyrosine; Oat, organic anion transporter; OGC, 2-oxoglutarate carrier; OPT, *o*-phthalaldehyde; PT, proximal tubular; SDH, succinate: cytochrome c oxidoreductase; Sod2, superoxide dismutase 2; tBH, tert-butyl hydroperoxide; Trx2, thioredoxin 2; NPX, uninephrectomized; VDAC, voltage-dependent anion channel (porin).

* Corresponding author at: Department of Pharmacology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201, United States. Tel.: +1 313 577 0476; fax: +1 313 577 6739.
E-mail addresses: bbenipal@med.wayne.edu (B. Benipal), l.h.lash@wayne.edu (L.H. Lash).

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cellular energy metabolism, Na^+/H^+ antiport activity in the proximal tubule [3], ($Na^+ + K^+$)-stimulated ATPase activity, and mitochondrial metabolism in proximal and distal tubules [9–11]. The toxicological implications of compensatory renal growth are an altered susceptibility to nephrotoxicants such as inorganic mercury, analgesics and cadmium metallothionein [12–20].

Our previous data demonstrated that cellular GSH content was significantly higher in renal homogenates prepared from whole kidney, cortex and outer stripe of the outer medulla from NPX rats as compared to those from control rats [5]. We also showed that renal PT cells from NPX rats are more susceptible than cells from control rats to many nephrotoxicants, including several oxidants [14–16], suggesting that the increased GSH content in NPX rats is not sufficient to counteract the renal oxidative stress, thereby leading to greater cell injury when a challenge exists.

Compensatory renal hypertrophy leads to an increase in energy demand, which is accompanied by an increase in the rate of mitochondrial electron transport [9–11]. Increased rates of electron transport, in turn, lead to what has been described as a hypermetabolic state, which likely includes higher levels of reactive oxygen species. These functional alterations may lead to mitochondrial damage, including oxidation of mitochondrial DNA, protein and lipids and opening of the mitochondrial permeability transition pore. Additionally, evidence suggests that altered mitochondrial GSH status contributes to mitochondrial dysfunction [21,22], suggesting that it may play a role in the altered energetic and biochemical phenotype of hypertrophied PT cells.

Based on all these previous observations, we hypothesize that reduction of renal mass due to uninephrectomy results in marked alterations in cellular energetics, redox status and renal function *in vivo*. To test this hypothesis, three sets of experiments were conducted. First, we assessed renal physiological parameters *in vivo* and measured mRNA and protein expression of renal plasma membrane organic anion transporters and mitochondrial GSH transporters. Second, we analyzed several parameters of renal mitochondrial redox status and cellular energetics. Third, we assessed and quantified both basal and toxicant-induced oxidative stress *ex vivo* in isolated mitochondria derived from renal cortex of control and NPX rats. Results from the present study show that compensatory renal hypertrophy causes alterations in renal physiological parameters and renal cellular energetics and upregulation of plasma membrane organic anion transporters. These changes are associated with modest renal injury and warrant further development of approaches to restore energetics and redox balance.

2. Materials and methods

2.1. Chemicals

Cis-parinaric acid was purchased from Molecular Probes (Eugene, OR, U.S.A.). Rotenone, tert-butyl hydroperoxide (tBH), hemin and 1-methyl-2-phenylindole were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available and were purchased from commercial sources.

2.2. Surgical procedure

Male Sprague–Dawley rats (150–175 g) were used in the present study. Animals that underwent surgical nephrectomy (removal of right kidney) were allowed a minimum 10-day recovery period prior to experiments [5]. Right-side nephrectomized rats were purchased from Harlan (Indianapolis, IN, U.S.A.). The surgical procedure was performed as described previously [5]. Control rats were surgically naïve, because previous studies showed that sham surgery has no observable effects on the biochemical and renal functional parameters being studied [5,15,18]. Control and NPX rats were age-matched for all studies.

2.3. In vivo assessment of renal physiological parameters

Rats were kept in metabolic cages and urine samples were collected every 24 h for 1 week. Basic parameters of renal function, including urine volume (U-Vol), urinary protein (U-Pr), urinary-GSH (U-GSH) and urinary glutathione disulfide (U-GSSG), urinary and serum creatinine (U-Cr, S-Cr), urinary albumin (U-Alb), urinary *N*-acetyl-β-D-glucosaminidase (U-NAG) and urinary γ-glutamyl-transferase (U-GGT) were measured. U-Pr (BCA protein assay kit from Pierce, Milwaukee, WI, U.S.A.), U-Cr and S-Cr (Quantichrom™ creatinine kit from BioAssay Systems, Hayward, CA, U.S.A.), U-Alb (Nephrat ii kit from Exocell, Philadelphia, PA, U.S.A.), U-GSH and U-GSSG (GSH-Glo® kit from Promega, Madison, WI, U.S.A.), and U-NAG (NAG assay kit from Diazyme, Poway, CA, U.S.A.) were measured using commercial kits. U-GGT was measured by a spectrophotomeric assay [23].

2.4. Determination of protein expression of renal plasma membrane organic anion transporters by Western blot analysis

Crude plasma membrane fractions were isolated according to the method of Scalera et al. [24] using a sucrose-triethanolamine (0.25 mM sucrose, 10 mM triethanolamine/HCl, pH 7.6, 0.1 mM phenylmethylsulfonyl fluoride) buffer. Protein expression of organic anion transporters was determined in isolated plasma membranes from rat kidney derived from control and NPX rats using commercially prepared antibodies. Polyclonal antibodies to organic anion transporter 1 and 3 (Oat1 and Oat3; *Slc22a6* and *Slc22a8*, respectively) were purchased from Alpha Diagnostic International (San Antonio, TX, U.S.A.). Monoclonal antibodies against multidrug resistance-associated protein 2 and 5 (Mrp2 and Mrp5; *Abcc2* and *Abcc5*, respectively) were purchased from Abcam (Cambridge, MA, U.S.A.). Monoclonal antibody against Na^+K^+-ATPase α1 subunit was purchased from Affinity Bioreagents, Inc. (Golden, CO, U.S.A.). Secondary antibodies (anti-rat, anti-rabbit, and anti-mouse) were purchased from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Polyclonal anti-actin antibody was purchased from Cell Signaling Technology (Danvers, MA, U.S.A.) for use as a control for loading of total cellular protein.

2.5. Determination of protein expression of renal mitochondrial GSH transporters and redox status proteins by Western blot analysis

Suspensions of isolated mitochondria were prepared from renal cortex from control and NPX rats by differential centrifugation as follows: rat kidneys were decapsulated and the medulla removed. Kidneys were then placed in 15 ml of ice-cold buffer (225 mM sucrose, 10 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 20 mM KCl, 20 mM triethanolamine, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM EGTA), homogenized, and centrifuged in 50-ml polycarbonate centrifuge tubes at $600 \times g$ (2250 rpm) for 10 min in a Sorvall SS34 rotor in a Sorvall RC2B centrifuge. Supernatant was decanted and saved. The pellets containing tissue fragments and mitochondria were washed with 30 ml of buffer and the resuspended material centrifuged at $600 \times g$ for 10 min. The supernatant fractions were combined and centrifuged at $15\,000 \times g$ for 5 min. The resultant pellet was resuspended in 2 ml of buffer without EGTA.

Protein expression of mitochondrial GSH transporters and redox enzymes were determined in isolated mitochondria with commercially prepared antibodies. Specific proteins were visualized by Enhanced Chemiluminescence (Pierce; Rockford, IL, U.S.A.).

Monoclonal antibodies against the 2-oxoglutarate carrier (OGC; *Slc25a11*) and prohibitin and polyclonal antibodies against the dicarboxylate carrier (DIC; *Slc25a10*), superoxide dismutase 2 (Sod2) and the voltage-dependent anion channel (VDAC or porin) were purchased from Abcam (Cambridge, MA, U.S.A.). Polyclonal antibody against thioredoxin 2 (Trx2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Expression of VDAC or prohibitin was used as mitochondrial loading controls.

2.6. Analysis of gene expression by real-time PCR

Total RNA from the renal cortex was isolated using Trizol® (Invitrogen; Carlsbad, CA, U.S.A.). First-strand cDNA was made from the isolated total RNA using a multiscribe reverse transcriptase with random hexamers from Applied Biosystems (Foster City, CA, U.S.A.). Taq Man Gene Assay kits containing primer/probe sets for Oat1 (GenBank accession no. NM_017224), Oat3 (GenBank accession no. NM_031332), DIC (GenBank accession no. NM_133418), OGC (GenBank accession no. NM_022398), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; loading control; GenBank accession no. NM_017008.3) were purchased from Applied Biosystems. All reactions were performed in triplicate. The relative amounts of mRNA were calculated by using the comparative C_T method.

2.7. Determination of mitochondrial GSH status

A fluorometric method [25] was used for determination of GSH and GSSG in mitochondrial suspensions. GSH content was measured by the fluorescent product formed on reaction with *o*-phthalaldehyde (OPT) with excitation at 350 nm and emission at 420 nm. GSSG content was measured after treatment with *N*-ethylmaleimide, followed by reduction with dithiothreitol, and measurement of the fluorescent product formed with OPT.

2.8. Enzyme assays and protein determination

Activities of several GSH- and energy metabolism-related enzymes were measured in cytoplasm and/or mitochondria from renal cortical homogenates from control and NPX rats, as reported previously [5,26]. GSSG reductase (GRD) activity was measured as NADPH oxidation by monitoring the decrease in A_{340} (ε = 6220 M^{-1} cm^{-1}). GSH peroxidase (GPX) activity was measured with 25 mM H_2O_2 as substrate and was equated to NADPH oxidation as detected by the decrease in A_{340} (ε = 6220 M^{-1} cm^{-1}). GSH *S*-transferase (GST) activity was measured with 1-chloro-2,4-dinitrobenzene as substrate and quantitation of *S*-2,4-dinitrophenyl-GSH formation by the increase in A_{340} (ε = 9600 M^{-1} cm^{-1}). Glutamate dehydrogenase (GDH) activity was measured spectrophotometrically by coupling 2-oxoglutarate reduction to NADH oxidation, and activity was quantified by determining the consequent decrease in A_{340} (ε = 6220 M^{-1} cm^{-1}). Malic dehydrogenase (MDH) activity was measured by coupling malate reduction to NADH oxidation, and activity was quantified by monitoring the decrease in A_{340} (ε = 6220 M^{-1} cm^{-1}). Succinate: cytochrome c oxidoreductase (SDH) activity was measured by coupling succinate oxidation to ferricytochrome c reduction, and activity was quantified by determining the increase in $A_{550.5}$ (ε = 18 500 M^{-1} cm^{-1}). Protein content of samples was determined by the bicinchoninic acid (BCA) protein assay method using bovine serum albumin as a standard [27].

2.9. Mitochondrial oxygen consumption

Oxygen consumption was measured in suspensions of isolated mitochondria with a Gilson 5/6H Oxygraph in a thermostated, air-tight, 1.6 ml chamber at 25 °C [22]. State 3 rates of oxygen consumption were measured by the addition of 0.3 mM ADP and respiratory substrate (3.3 mM succinate in the presence 5 μM rotenone) for coupling site II to the chamber containing 0.5 ml of mitochondrial sample and 1 ml mitochondrial buffer. State 4 rates of oxygen consumption were measured after depletion of ADP. Respiratory control ratio (RCR = State 3/State 4) was then calculated.

2.10. Assessment of basal and toxicant-induced mitochondrial oxidative stress

2.10.1. Lipid peroxidation by malondialdehyde assay

One method for assessment of lipid peroxidation was measurement of malondialdehyde (MDA) formation using 1-methyl-2-phenylindole [28]. Mitochondrial suspensions (200 μl) were treated with tBH (500 μM) or buffer for 1 h at 37 °C and then hydrolyzed using 0.1 M HCl and incubated for 60 min at 60 °C. An aliquot (200 μl) of the resulting supernatant was then incubated with 650 μl 1-methyl-2-phenylindole and 150 μl 37% HCl. After incubating the mixture for 30 min at 45 °C, A_{586} was determined.

2.10.2. Lipid peroxidation by cis-parinaric acid fluorescence assay

Cis-parinaric acid is considered a very sensitive marker for the initial stages of lipid peroxidation [29]. Isolated mitochondria (1 mg protein/ml) were incubated on ice with 6.4 μM cis-parinaric acid for 15 min. After pelleting mitochondria (11 200 g × 5 min) and discarding the supernatant, mitochondria were resuspended (0.5 mg protein/ml) in mitochondrial buffer. For anaerobic conditions, mitochondria were resuspended in mitochondrial buffer (prepared with nitrogen sparging) and treated with 5 μM hemin (Fe^{2+}). Samples were then added to a 24-well plate and incubated at room temperature for 10 min before treatment with tBH or buffer. Lipid peroxidation was measured as the loss of cis-parinaric acid fluorescence (excitation 324 nm, emission 413 nm) over time using a fluorescence plate reader [29,30].

2.10.3. Mitochondrial aconitase activity

Aconitase activity was assayed by following formation of NADPH at A_{340} at 25 °C, as described by Han et al. [31]. The reaction mixture contained 30 mM Tris–HCl, 30 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$, 1 U/ml isocitrate dehydrogenase and 0.1 mg of mitochondrial protein.

2.10.4. Analysis of oxidative stress markers by Western blot analysis

Proteins modified with 3-nitrotyrosine (3-NT) or 4-hydroxy-2-nonenal (HNE) were also assessed as markers of oxidative alterations in renal mitochondria. Monoclonal antibody to 3-NT was purchased from Abcam (Cambridge, MA, U.S.A.). Polyclonal antibody to HNE was purchased from Calbiochem (La Jolla, CA, U.S.A.).

2.11. Data analysis

Data for enzyme assays were normalized to the content of cellular protein. All measurements were performed at least 3–5 times. Results are expressed as means ± SEM unless specified. Densitometry of bands on Western blots were performed using GelEval 1.22 software for Mac OS X. When two or more parameters were varied and compared (e.g., tBH treatment vs. buffer in control and NPX rats), 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

Table 1
Physiological parameters of renal function *in vivo*. Parameters were measured in uninephrectomized (NPX) rats at 10 days post-surgery and in age-matched control rats. Results are means ± SEM of measurements from the indicated number of rats from each group.

Parameter (units; *n*)	Control	NPX
Kidney weight (g; 10)	0.95 ± 0.04	1.51 ± 0.06*
U-Vol (ml; 6)	3.08 ± 0.47	8.43 ± 1.63*
U-Pr (mg/24 h; 6)	11.1 ± 1.5	34.9 ± 3.2*
SCr (mg/dl; 3)	0.09 ± 0.04	0.24 ± 0.06*
U-Cr (mg/dl; 3)	168 ± 7	93.6 ± 11.2*
U-Cr (mg/24 h; 3)	7.63 ± 0.48	5.43 ± 0.30*
CCr (ml/min; 3)	6.67 ± 0.80	2.00 ± 0.54*
U-Alb (μg/24 h; 3)	150 ± 10	860 ± 110*
U-GSSG (nmol/24 h; 6)	1.53 ± 0.73	9.51 ± 0.32*
U-NAG (U/24 h; 9)	0.07 ± 0.01	0.14 ± 0.03*
U-GGT (mU/24 h; 6)	197 ± 9	327 ± 30*

Abbreviations: U-Vol, urinary volume; U-Pr, urinary protein; SCr, serum creatinine; U-Cr, urinary creatinine; CCr, creatinine clearance; U-Alb, urinary albumin; U-GSSG, urinary glutathione disulfide; U-NAG, urinary *N*-acetyl-β-D-glucosaminidase; U-GGT, urinary γ-glutamyltransferase.
* Significantly different ($P < 0.05$) from the corresponding value in control rats.

Table 2
Real-time quantitative PCR analysis of Oat1, Oat3, DIC, and OGC mRNA expression in renal cortex of control and NPX rats. Primers were designed with the aid of Oligo 6.76 and the cDNA sequences published in GenBank™. Primer and labeled probe sets were from Applied Biosystems. Results are based on triplicate measurements from three each of total RNA from kidney cortex of control and NPX rats and are means ± SEM. All C_T values are corrected to GAPDH C_T values. An optimum cDNA concentration of 30–300 ng DNA/well was determined for each gene.

Gene	Control	NPX
Plasma membrane Oats		
Oat1 (*Slc22a6*)	31.4 ± 0.9	32.8 ± 1.2
Oat3 (*Slc22a8*)	32.5 ± 0.9	32.8 ± 1.3
Mitochondrial GSH transporters		
DIC (*Slc25a10*)	32.9 ± 0.8	33.4 ± 1.1
OGC (*Slc25a11*)	33.6 ± 0.9	33.9 ± 1.2

Note: No values from NPX rats were significantly different from the corresponding value from control rats. *Significantly different ($P < 0.05$) from the corresponding sample from control rats.

considered significant. Otherwise, Student's *t*-test was performed to determine which means were significantly different from one another, using a two-tail probability of $P < 0.05$ as the criteria for significance.

3. Results

3.1. Renal physiological parameters in vivo

Examination of basic parameters of renal function indicates that there are some discernable changes in rats post-nephrectomy compared to age-matched control rats (Table 1). After uninephrectomy, the remaining kidney weighs significantly more than a single kidney from control rats, consistent with compensatory renal growth. NPX animals had markedly higher urine volume, indicating an increase in renal function to compensate for the loss of a kidney. However, we also observed increases in total U-Pr, U-Alb, SCr, U-NAG, U-GGT and U-GSSG, but lower U-Cr over a 24-h period and lower CCr, indicating significant impairment of renal function.

3.2. Protein and gene expression analysis of renal plasma membrane organic anion transporters

Based on previous observations of increases in intracellular GSH concentrations and GSH transport activity after compensatory renal hypertrophy [5,32], we hypothesized that these increases were due, at least in part, to increases in gene and/or protein expression of carriers involved in GSH transport across renal plasma membranes. To test this, we determined mRNA and protein expression of four renal plasma membrane transporters that are known or thought to be involved in GSH transport.

Real-time PCR analysis of Oat1 and Oat3 mRNA expression (Table 2) revealed no differences between control and NPX rats. In contrast, Western blot analyses of Oat1 and Oat3 protein levels showed nearly 2-fold increases in protein expression for both carriers in kidneys of NPX rats as compared to those of control rats (Fig. 1). Additionally, expression of two Mrp carriers that can transport GSH were examined. Whereas protein expression of Mrp2, which is localized to the BBM, was increased by approximately 30% in NPX rat kidneys compared to control rat kidneys, that of Mrp5, which is localized to the BLM, was unchanged. Inasmuch as both Oat1 and Oat3 are believed to mediate uptake of GSH into the renal PT cell whereas both Mrp2 and Mrp5 mediate efflux from the cell, the larger increase in Oat1/3 compared to that for Mrp2 are consistent with higher intracellular accumulation of GSH. We also examined protein expression of the $(Na^+ + K^+)$-ATPase, which provides the major

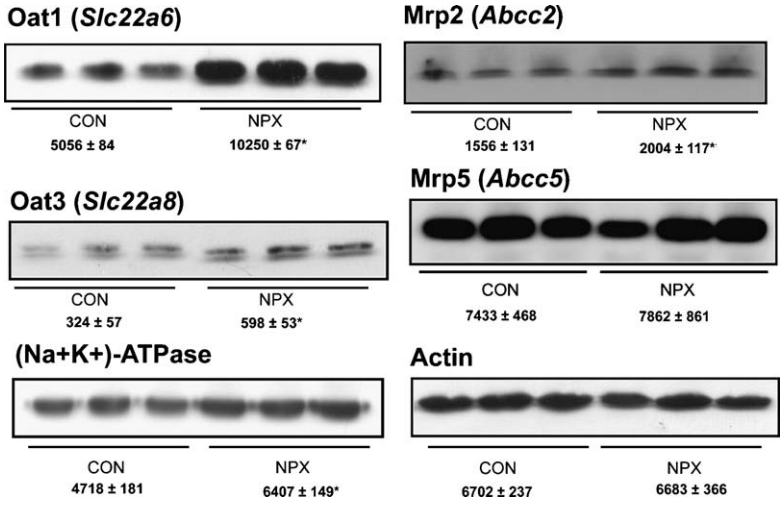

Fig. 1. Effect of compensatory renal growth on protein expression of renal plasma membrane organic anion transporters and the $(Na^+ + K^+)$-stimulated ATPase. Protein expression of Oat1, Oat3, Mrp2, Mrp5 and the $(Na^+ + K^+)$-stimulated ATPase were determined by Western blot analysis as described in Section 2. β-Actin protein was used as a loading control. Blots were scanned and quantified by densitometry using GelEval 1.22 software. Data represent means ± SEM of measurements from renal homogenates from three control and three NPX rats. *Significantly different ($P < 0.05$) from corresponding control sample.

Table 3
GSH and GSSG concentrations in renal mitochondria from control and NPX rats. Mitochondrial GSH and GSSG levels were measured in suspensions of isolated mitochondria from rat renal cortex from control and NPX rats using a fluorometric method. Results are nmol/mg protein and are means ± SEM of measurements from three rats from each group.

Parameter	Control	NPX
GSH	1.67 ± 0.08	1.97 ± 0.05*
GSSG	0.90 ± 0.04	1.05 ± 0.02*

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

driving force for many secondary active transport carriers, and found it to be significantly higher (1.4-fold) in kidneys of NPX rats as well.

3.3. Mitochondrial redox status

To determine whether or not compensatory renal hypertrophy causes alterations in renal mitochondrial redox status, we first measured mitochondrial GSH and GSSG levels in renal mitochondria from both control and NPX rats. Modest, but significant increases were observed in concentrations of both GSH and GSSG in renal mitochondria from NPX rats as compared to those from control rats (Table 3).

Inasmuch as GSH status in renal mitochondria is determined primarily, if not entirely, by the function of two inner membrane carrier proteins, the DIC and OGC [33,34], we next determined gene and protein expression of these carriers. Because previous work [22] showed markedly higher rates of GSH uptake by renal mitochondria from NPX rats as compared to those from control rats, we expected to see increases in expression of these carrier proteins. Despite this expectation, neither mRNA (Table 2) nor protein (Fig. 2) expression of either the DIC or OGC were elevated in renal mitochondria from NPX rats as compared to those in renal mitochondria from control rats.

Besides the GSH system, redox status in mitochondria is also regulated by two other enzymes, superoxide dismutase 2 (Sod2) and thioredoxin 2 (Trx2). Measurements of protein expression of Sod2 and Trx2 (Fig. 3), however, showed no significant differences between renal mitochondria of control and NPX rats.

3.4. Impact of compensatory hypertrophy on functional characteristics of renal mitochondria

Activities of several enzymes that are indicative of cellular energetics and redox status were measured in preparations of mitochondria and cytoplasm from renal cortex of control and NPX rats (Table 4). With the exception of small decreases in mitochondrial GPX and cytoplasmic GST in kidneys of NPX rats, there were no significant differences in activities of GSH-dependent detoxication enzymes between control and NPX rat kidneys. In contrast, activities of malic dehydrogenase, glutamate dehydrogenase and succinate: cytochrome c oxidoreductase were significantly higher in renal mitochondria from NPX rats as compared to those from control rats. These increases in key enzymes of mitochondrial intermediary metabolism without corresponding changes in mitochondrial GSH-dependent enzymes are consistent with there being changes in mitochondrial redox status in kidneys of NPX rats.

Succinate-stimulated (complex II) state 3 respiration was also higher in renal mitochondria from NPX rats than in those from control rats (Table 5). Similarly, the RCR value, which gives an indication of how well oxygen consumption is coupled to ADP phosphorylation, was significantly elevated with succinate (complex II) as respiratory substrate in renal mitochondria from NPX rats relative to that in renal mitochondria from control rats.

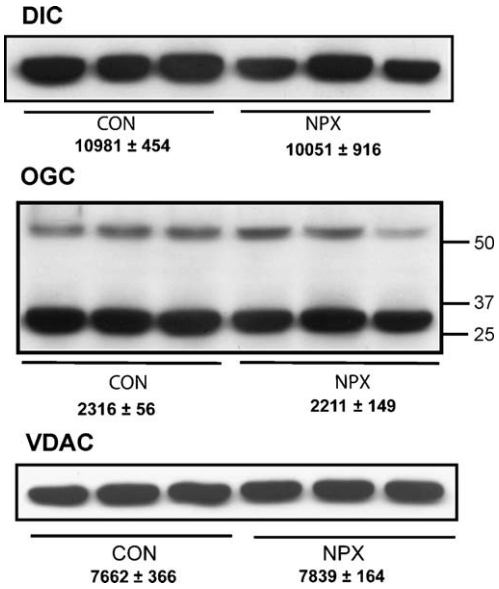

Fig. 2. Effect of compensatory renal growth on protein expression of renal mitochondrial GSH transporters.
Mitochondrial samples were isolated from rat kidney cortex of control (CON) and uninephrectomized (NPX) rats and subjected to immunoblotting with anti-DIC, OGC and VDAC. The VDAC protein served as a mitochondrial loading control. Note that the OGC protein was detected as both monomers (M_r = 32.5 kDa) and a dimer (M_r = 65 kDa). Blots were scanned and quantified by densitometry using GelEval 1.22 software. Data represent means ± SEM of measurements from renal mitochondrial samples from three CON and three NPX rats. No significant differences were detected between corresponding CON and NPX samples.

This significant acceleration of mitochondrial respiratory activity after compensatory renal cellular hypertrophy is consistent with the increased activities of mitochondrial dehydrogenases noted above and the general hypothesis that renal mitochondria from NPX rats are in a hypermetabolic state.

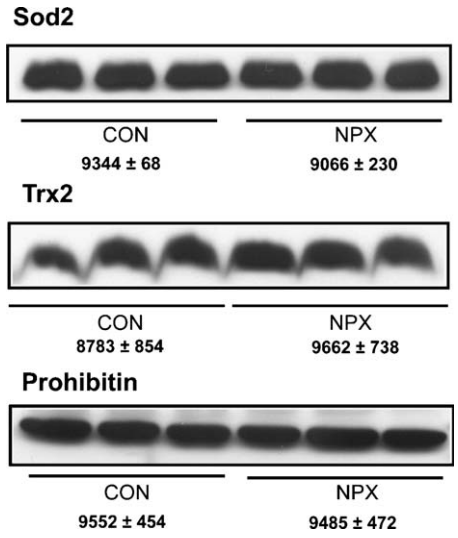

Fig. 3. Effect of compensatory renal growth on protein expression of renal mitochondrial Sod2 and Trx2.
Mitochondrial samples were isolated from rat kidney cortex of control (CON) and uninephrectomized (NPX) rats and subjected to immunoblotting with anti-Sod2, anti-Trx2, and anti-prohibitin. The prohibitin protein served as a mitochondrial loading control. Blots were scanned and quantified by densitometry using GelEval 1.22 software. Data represent means ± SEM of measurements from renal mitochondrial samples from three CON and three NPX rats. No significant differences were detected between corresponding CON and NPX samples.

Table 4
Effect of compensatory renal hypertrophy on GSH-dependent and mitochondrial enzymes. Enzyme activities were measured in cytoplasm or mitochondria isolated from kidney(s) of uninephrectomized (NPX) rats at 10 days post-surgery and age-matched control rats. Results are expressed as mU/mg protein and are means ± SEM of measurements from six rats from each group.

Enzyme	Control	NPX
GSSG reductase		
Mitochondria	16.5 ± 1.8	19.9 ± 0.9
Cytoplasm	21.1 ± 0.8	19.6 ± 0.8
GSH peroxidase		
Mitochondria	12.6 ± 0.5	9.69 ± 0.06*
Cytoplasm	11.7 ± 0.9	10.9 ± 0.8
GSH *S*-transferase		
Mitochondria	4.91 ± 0.97	2.71 ± 0.31
Cytoplasm	34.1 ± 0.7	25.6 ± 0.2*
Malic dehydrogenase		
Mitochondria	27.1 ± 1.7	38.3 ± 1.4*
Glutamate dehydrogenase		
Mitochondria	57.7 ± 4.2	77.5 ± 6.1*
Succinate: cytochrome c oxidoreductase		
Mitochondria	28.7 ± 1.1	56.7 ± 4.2*

Abbreviations: GGT, γ-glutamyltransferase.
* Significantly different ($P < 0.05$) from the corresponding value in control rats.

3.5. Assessment of basal and toxicant-induced oxidative stress in renal mitochondria

The hypermetabolic state of renal mitochondria from NPX rats, and the evidence of renal dysfunction, suggest that a higher state of oxidative stress exists that may contribute to higher susceptibility to chemically induced injury. To compare basal and toxicant-induced oxidative stress between renal mitochondria from control and NPX rats, we analyzed lipid peroxidation by using two assays (one assay based on MDA formation and one based on degradation of cis-parinaric acid) and measured aconitase activity as a marker for mitochondrial oxidative stress.

The results of the MDA assay show that there is no significant difference in renal mitochondrial lipid peroxidation between control and NPX rats under either basal conditions or when mitochondria are challenged by exposure to tBH (Fig. 4A). The naturally occurring polyunsaturated fatty acid cis-parinaric acid is used in eukaryotic cells as a very sensitive marker for the initial stages of lipid peroxidation [29]. When double bonds of cis-parinaric acid are broken in lipid peroxidation reactions, decay in fluorescence is used to indirectly monitor the degree of membrane lipid peroxidation. Under anaerobic conditions ($N_2 + Fe^{2+}$), there was no significant difference in either basal or tBH-induced mitochondrial lipid peroxidation between control and NPX rats (Fig. 4B).

Because mitochondrial aconitase activity is a sensitive indicator of redox homeostasis and its activity is uniquely sensitive to various physiological and pathological conditions [35], it is frequently viewed as a marker of oxidative stress in biological systems. The underlying mechanism is believed to be the facile inactivation of its iron–sulfur prosthetic group by toxicants such as peroxides [31,35,36]. As shown in Fig. 4C, renal aconitase activity was significantly lower in NPX rats relative to control rats even without any exogenous toxicant, suggesting that renal compensatory hypertrophy does cause an increase in underlying mitochondrial oxidative stress.

The progressive oxidative stress in animals with renal insufficiency can lead to oxidation of proteins, carbohydrates, nucleic acids, lipids and accumulation of harmful byproducts in various tissues [37,38]. As a further assessment of mitochondrial redox status, we analyzed protein adducts of two oxidative stress markers, 3-NT and HNE, by Western blot analysis in renal mitochondria. No differences were observed in blots for 3-NT modified proteins (data not shown). However, assessment of HNE-adducted proteins showed significantly higher staining in renal mitochondria from NPX rats, with the most prominent staining at 45 kDa and 52 kDa (Fig. 4D).

4. Discussion

4.1. Altered renal function and evidence for renal injury after compensatory renal hypertrophy

The major underlying hypothesis to explain the physiological and pathological changes that occur in remnant renal tissue after compensatory renal hypertrophy begins with the increased need for renal function requiring increased mitochondrial electron transport to generate ATP [2,9–11]. This increase in mitochondrial function produces more reactive oxygen species, which in turn leads to a higher basal state of oxidant stress as compared to mitochondria from normal kidneys. For the NPX model of reduced renal mass, we [16,22,32] proposed that this higher, basal level of oxidant stress has two consequences: first, it leads to a greater susceptibility of NPX rats *in vivo* or renal PT cells or renal mitochondria *in vitro* to a broad range of oxidants and other nephrotoxic chemicals; second, mitochondrial GSH status is altered after compensatory renal hypertrophy in a complex manner that partially, but not completely, enables adaptation of the PT cell to the hypermetabolic state.

The present study not only confirmed the increase in kidney weight and urine production that accompany the compensatory response, but also provided evidence at the *in vivo* level that this compensation is associated with some degree of renal damage. Several markers, which are all considered diagnostic for renal function, were uniformly altered in a manner consistent with mild renal injury. Evidence for renal injury at the level of the PT cell was also observed by the increases in U-NAG, U-GGT, and U-GSSG.

4.2. Evidence for a hypermetabolic state in compensatory renal hypertrophy

The basic concept of the hypermetabolic state in compensatory renal hypertrophy is that the remnant renal tissue must work harder to make up for the lost function. The principal means to accomplish this is through increased generation of ATP by the mitochondria, which is tightly regulated in the kidneys according to the need for metabolic energy [39]. Evidence for increased rates of mitochondrial metabolism were the significant increases in activities of malic, glutamate, and succinate dehydrogenases and State 3 rates of succinate-dependent respiration. Significantly

Table 5
Effects of compensatory renal hypertrophy on mitochondrial respiration. Mitochondrial succinate-dependent State 3 and State 4 respiration were measured in suspensions of isolated mitochondria from kidney(s) of control and NPX rats. Rates of oxygen consumption were determined in an Oxygraph with a Clark-type electrode using 3.3 mM succinate as respiratory substrate in the presence of 5 μM rotenone. Results are means ± SEM of measurements from six separate mitochondrial preparations from each group. RCR = respiratory control ratio = State 3 rate/State 4 rate.

Parameter	Control	NPX
State 4 (nmol O_2/min per mg protein)	18.6 ± 1.4	19.7 ± 1.0
State 3 (nmol O_2/min per mg protein)	27.0 ± 2.8	41.7 ± 1.5*
RCR	1.88 ± 0.02	3.16 ± 0.10*

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

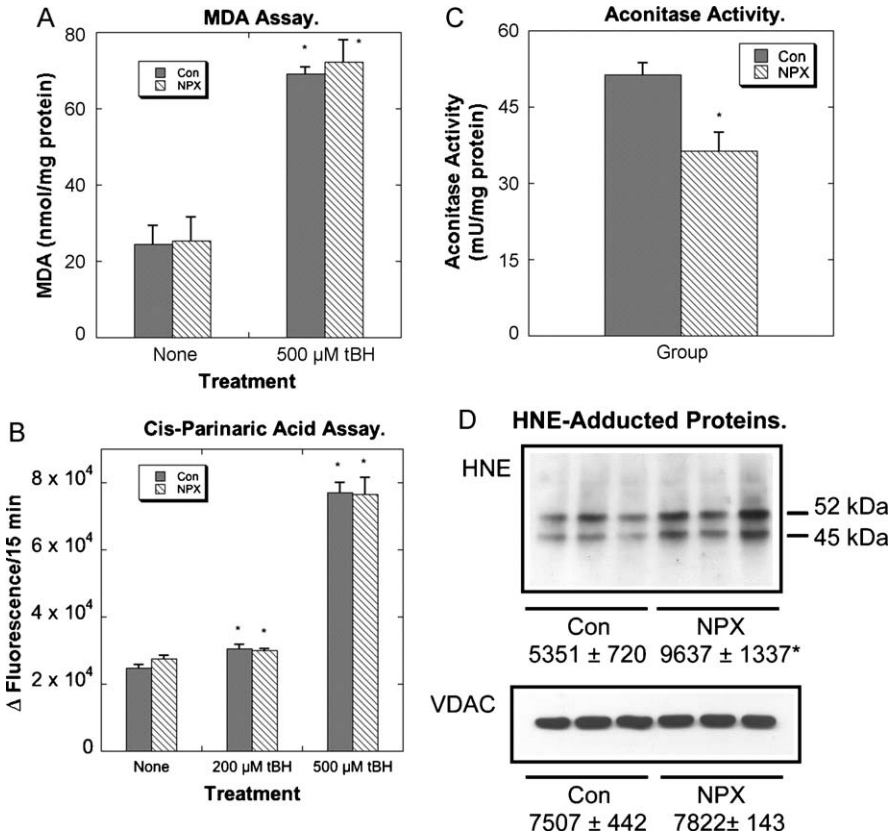

Fig. 4. Assessment of effects of compensatory renal growth on basal and toxicant-induced oxidative stress in renal mitochondria.
A. Basal and tBH-induced lipid peroxidation in renal mitochondria from CON and NPX rats was measured using malondialdehyde (MDA) formation, as determined with 1-methyl-2-phenylindole. Renal mitochondrial samples from CON and NPX rats were incubated with buffer or 500 μM tBH for 1 h. Results are means ± SEM of three separate samples from each group. *Significantly different ($P < 0.05$) from the corresponding untreated samples. B. Basal and tert-butyl hydroperoxide (tBH)-induced lipid peroxidation in renal mitochondria from control (CON) and uninephrectomized (NPX) rats was measured using fluorescent cis-parinaric acid. Renal mitochondrial samples were incubated with 6.4 μM cis-parinaric acid + 5 μM hemin and treated with either 200 μM or 500 μM tBH. Results are means ± SEM of three separate samples from each group. *Significantly different ($P < 0.05$) from the corresponding untreated samples. C. Mitochondrial aconitase activity in renal mitochondria from CON and NPX rats was measured as NADPH formation at A_{340}. Results are means ± SEM of three separate samples from each group. *Significantly different ($P < 0.05$) from the value in mitochondria from CON rats. D. Adducts of 4-hydroxy-2-nonenal (4-HNE) with mitochondrial proteins were determined by Western blot analysis. VDAC protein was used as a mitochondrial loading control. Blots were quantified by densitometry using GelEval 1.22 software. Data are presented means ± SEM of three separate samples from each group. *Significantly different ($P < 0.05$) compared to corresponding sample from CON rats.

higher protein expression of the $(Na^+ + K^+)$-ATPase in kidneys from NPX rats was also observed (cf. Fig. 1), which agrees with our previous observation of higher enzymatic activity in renal PT cells from NPX rats [32]. Because this protein is one of the primary consumers of ATP in the renal PT cell [39], its higher expression and activity in kidneys from NPX rats provide further support that compensatory renal hypertrophy generates a hypermetabolic state in the remnant renal cell due to increased energy demands.

4.3. Does compensatory renal hypertrophy result in oxidative stress?

The current studies pursued the question of oxidative stress by assessing both basal and toxicant-stimulated lipid peroxidation using two well established assays, formation of MDA and degradation of cis-parinaric acid. We also measured activity of mitochondrial aconitase, which, as noted above, is considered a highly sensitive indicator of oxidative stress due to the facile oxidation of its Fe–S cluster leading to inactivation [31,35,36].

Neither assessment of lipid peroxidation showed a significant difference between control and NPX kidneys, either in the absence or presence of toxicant. Expression of two important redox proteins in renal mitochondria, Sod2 and Trx2, were also unchanged after compensatory renal hypertrophy. An important consideration in evaluating such data, however, is the manner by which the measurements are normalized. In the present work, enzyme activities and levels of metabolites were normalized to total protein content, which was the only normalization parameter available and is the one most commonly used. Part of the compensatory hypertrophy response, however, is an increase in protein per cell [2–4,7]. We previously illustrated the impact of this by normalizing activities in renal PT cell primary cultures derived from either control or NPX rats to both protein and DNA content [32]. Whereas the amount of total protein per cell increases as a consequence of compensatory renal hypertrophy, the amount of DNA per cell does not change owing to the absence of a significant hyperplasia after a reduction in renal mass. Thus, lack of a difference between samples from control and NPX rats when normalized to protein equates to an increase, although one that is proportional to protein.

Further support for the conclusion that there is a degree of redox imbalance in renal mitochondria of NPX rats was the significant increase in the amount of HNE-modified mitochondrial protein (cf. Fig. 4). The increase in Western blot density was 1.7-fold and was predominantly detected in two protein bands of molecular weight 45 kDa and 52 kDa. Although the identity of these modified proteins is currently unknown, their detection and the extent of the differences suggest they may be important in determining the compensatory hypertrophy phenotype. Potential nitration of mitochondrial proteins was also assessed, but no

difference was found, suggesting that increases in reactive nitrogen species were not a component of the compensatory hypertrophy phenotype.

4.4. Mechanisms underlying the increased levels of cytoplasmic GSH

We previously showed that compensatory renal hypertrophy is associated with higher activity of γ-glutamylcysteine synthetase (GCS) [14,26,32]. While this could certainly be one mechanism for the increase in the cytoplasmic pool of GSH, increased transport across the BLM of plasma or renal periplasma GSH is also a potential mechanism. Thus, increase in activity and/or expression of plasma membrane carrier proteins responsible for GSH transport into the PT cell may also help explain some of the data.

Several candidate carrier proteins have been identified for uptake of GSH into the renal PT cell [40–42]. These include Oat1, Oat3, and the sodium-dicarboxylate 3 (NaC3; *Slc13a3*) carriers on the renal BLM. Additionally, both Mrp2 and Mrp5 can mediate efflux of GSH out of the renal PT cell across the BBM and BLM, respectively. The present study demonstrated significantly higher protein expression (~2-fold increases) for both Oat1 and Oat3 in renal homogenates from NPX rats. Whereas no difference was observed in Mrp5 expression, Mrp2 protein expression was modestly (~30%) higher in renal tissue from NPX rats. Thus, although there is higher efflux by Mrp2, the increases in uptake by Oat1 and Oat3 would seem to predominate, thereby leading to higher intracellular accumulation of GSH. These results are consistent with our previous finding of higher activity of both Oat1 and Oat3 in renal BLM vesicles from NPX rats [43].

Despite our expectation that mRNA levels of the Oat carriers would be higher in kidneys of NPX rats, we found no differences between kidneys of the two groups of rats. This indicates that the changes in the plasma membrane carriers that occur as a consequence of compensatory renal growth are post-transcriptional and/or post-translational, and probably involve a combination of effects, including increased mRNA translation, increased protein stability, and/or decreased protein degradation.

4.5. Mechanisms underlying the increased levels of mitochondrial GSH

Although most of the GSH detected when one measures total cellular content resides in the cytoplasm, changes in the status of GSH in the mitochondrial pool would also be expected to occur. Such changes in mitochondrial GSH content have important impacts on mitochondrial function, cellular energetics, and ultimately cell viability [44]. The present study demonstrated a significantly higher level of mitochondrial GSH in NPX rats. Because GSH synthesis in the renal PT cell only occurs in the cytoplasm [45], transport of GSH across the mitochondrial inner membrane would appear to be the sole source of GSH for the mitochondrial matrix. In the renal cortex and PT cell, the DIC and OGC are the two carriers we identified as being responsible for this transport process [33,34]. The functional importance of this GSH pool has been demonstrated in studies in which genetic manipulation of DIC or OGC expression has markedly altered redox status and susceptibility to oxidants and other nephrotoxicants [46–48].

Besides showing an increase in mitochondrial GSH content, we previously [22] found markedly increased rates of GSH uptake into renal mitochondria. While this suggested to us that expression of the DIC and/or the OGC might be up-regulated in compensatory renal hypertrophy, measurements of both mRNA and protein expression for the two mitochondrial carriers showed no differences between control and NPX rats.

To understand, then, what is responsible for the increased content of GSH in renal mitochondria from NPX rats, one has to consider the functions of the DIC and OGC in dicarboxylate transport and the observations that activities of several mitochondrial dehydrogenases and succinate-dependent respiration were significantly higher in renal mitochondria from NPX rats. The increased mitochondrial uptake of GSH in compensatory renal hypertrophy, therefore, is likely due to kinetic or mass action effects resulting from the hypermetabolic state. In other words, the higher supply of dicarboxylate substrate for the two carriers drives the uptake of GSH into the mitochondria.

4.6. Summary and conclusions

The present work has confirmed the existence of a hypermetabolic state in renal mitochondria from NPX rats. Although some parameters of oxidative stress were not increased relative to protein, detection of a higher amount of HNE-modified proteins and the greater extent of oxidant-induced inhibition of respiration [22,32] are consistent with a higher basal level of oxidative stress. Moreover, *in vivo* assessments of renal function demonstrated a modest degree of renal, and specifically proximal tubular, injury after uninephrectomy and compensatory renal growth. This is a significant finding with important clinical implications, because of the prevalence of reduced functional renal mass in aging and many diseases.

Based on previous and current assessments of mitochondrial GSH status and intermediary metabolism, we conclude that rather than being an adaptive increase due to oxidative stress, the increase in mitochondrial GSH content is due to the hypermetabolic state that results in increased fluxes of intermediary metabolites. Several of the intermediary metabolites are dicarboxylates or dicarboxylate precursors, so that activities of the two mitochondrial GSH transporters are increased due to kinetics or mass action. In contrast to this situation, the higher concentrations of GSH in renal cytoplasm appear to result from increased expression and activity of multiple plasma membrane transporters (i.e., Oat1 and Oat3) and of the rate-limiting enzyme for GSH synthesis, GCS.

Despite these increased levels of renal GSH, animals *in vivo* and renal cells and mitochondria *in vitro* are more susceptible to various forms of chemically induced toxicity. It would appear, therefore, that the higher levels of GSH are insufficient to maintain optimal redox homeostasis. The present results also suggest potential pharmacological targets for development of therapeutic approaches to improve renal function. These include the DIC and OGC, which regulate mitochondrial GSH content, and the yet-unidentified proteins that are modified by HNE. Studies are underway to modulate GSH status in ways that can restore optimal renal function and redox balance and diminish the susceptibility to chemically induced injury.

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