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Mouse HSF1 Disruption Perturbs Redox State and Increases Mitochondrial Oxidative Stress in Kidney

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ABSTRACT

Increased synthesis of heat shock proteins (Hsps), mainly regulated by heat shock factor 1 (*Hsf1*), protects the heart against oxidative stress under pathophysiological conditions such as ischemia/reperfusion. To investigate whether Hsps might exert a similar protective effect under physiological conditions in the kidney, we first evaluated the HSF1-dependent expression of several Hsps, including Hsp25, αB-crystallin (αBC), Hsp70, and Hsp90. Unlike either αBC or Hsp70, protein expression of Hsp25 and Hsp90 was decreased 26% and 50%, respectively, in *Hsf1* knockout compared with the wild-type mice. The effects of Hsp down-regulation on renal cellular redox status are presently unknown. Indeed, HSF1 deficiency caused a 37% decrease in renal cellular GSH/GSSG ratio, a marker of redox status, and a 40% increase in the rate of mitochondrial superoxide generation in *Hsf1* knockout compared with wild-type mice. HSF1 disruption also increased mitochondrial permeability transition pore opening and induced greater mitochondrial membrane potential change (48% increase versus wild type). Thus, the present study demonstrates that *Hsf1*-dependent transcription of selective Hsps is required for normal renal homeostasis, which protects renal cells against oxidative stress under physiological conditions. The source of mitochondrial superoxide generation is discussed. *Antioxid. Redox Signal.* 7, 465–471.

INTRODUCTION

Reactive oxygen species (ROS), mainly generated by mitochondria, are by-products of all aerobic metabolisms (9). Yet ROS are capable of damaging macromolecules, such as lipids, DNA, and proteins, and have been implicated in the etiology of a variety of pathological processes, such as diabetes, atherosclerosis, Alzheimer's disease, and aging (9). In the kidney, ROS-initiated oxidative stress has been reported to mediate a wide range of renal dysfunction, such as acute renal failure (5, 18), obstructive nephropathy (11), rhabdomyolysis (27), hyperlipidemia (21), and glomerular damage to chronic renal failure requiring dialysis (8). Therefore, enhancements of endogenous antioxidant systems should attenuate or prevent the oxidative stress, thereby alleviating the subsequent renal damage. Besides transgenic manipulation of conventional antioxidant enzymes such as superoxide dismutase (SOD) (17), heat shock proteins (Hsps) (with chaperone-like properties) have also been demonstrated to be protective against oxidative damage (23) and thus provide another avenue for up-regulation of cellular defense systems.

Although Hsp functions in kidney have been studied intensively under stress conditions (6), the roles of Hsps as antioxidative species under physiological conditions have not been investigated. To this end, we used a mouse model in which heat shock factor 1 (HSF1) was disrupted. Our purpose was to evaluate the expression profile of several renal Hsps and to investigate the consequences of such Hsp expression in the absence of HSF1 in terms of renal oxidative stress, redox status, and mitochondrial function.

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MATERIALS AND METHODS

Chemicals

Glutathione [both reduced (GSH) and oxidized (GSSG)], glutathione reductase, and safranine were purchased from ICN (Costa Mesa, CA, U.S.A.). Bovine serum albumin (BSA), Coomassie Brilliant Blue R-250, glycine, and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-αB-crystallin (αBC) antibody against the distal 14 amino acid residues to the stop codon of the C-terminus was produced by immunizing rabbits in this laboratory. Bicinchoninic acid protein assay kit was obtained from Pierce Inc. (Rockford, IL, U.S.A.). Antibodies against hsp25, hsp70, and hsp90 were purchased from Stressgen (Victoria, BC, Canada), and 2′,7′-dichlorofluorescin (DCFH) was obtained from Molecular Probes (Eugene, OR, U.S.A.).

Animals

The production and characterization of *Hsf1* knockout mouse have been reported previously and will be described briefly here (15). The *Hsf1* $^{-/-}$ deletion encompasses ~80% of the DNA-binding domain and the three leucine zippers that comprise the oligomerization domain. *Hsf1* knockout mice used in this study were generated with the mixed genetic background C,129X1 (129X1/SvJ × BALB/c) (15, 24). Mice homozygous for the *Hsf1* knockout allele were maintained in our breeding colony along with control littermates that were age-matched.

Western blot analysis of Hsps

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of Hsps, the supernatant resulting from mitochondrial isolation was performed according to Laemmli (12). Proteins were transferred onto Immobilon-P membranes at 100 V (constant voltage, 1 h) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, U.S.A.). The blots were incubated with 50 ml of 5% nonfat dried milk (wt/vol) for at least 1 h, and then washed for 3 × 10 min with Tris-buffered saline (20 m*M* Tris, 500 m*M* NaCl, pH 7.5) containing 0.1% Tween-20 (TBST). Blots were incubated for 1 h at room temperature with corresponding primary antibodies (diluted in TBST containing 0.2% BSA). The primary antibody was removed, and the blots were washed three times (10 min each) with TBST. The blots were then incubated in horseradish peroxidase-labeled corresponding secondary antibodies (all diluted 1:50,000 in TBST containing 0.2% BSA) for 1 h at room temperature. After the blots were washed with TBST three times (10 min each), Hsps were visualized with an enhanced chemiluminescence detection kit.

Determination of glutathione (GSH) and glutathione disulfide (GSSG)

GSH and GSSG were measured according to the method described by Anderson (2). In brief, kidney was homogenized in 5% sulfosalicylic acid and the homogenates were centrifuged at 10,000 *g* for 10 min. The supernatant was collected and distributed into two aliquots (100 µl) for individual measurement of GSSG and total glutathione. To conjugate GSH, the first aliquot was treated with 2-vinylpyridine followed by neutralization to pH 6–7 with triethanolamine. GSSG concentrations were then determined using the enzymatic recycling assay involving color development at 412 nm of 0.6 m*M* 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 0.2 m*M* NADPH and 1.8 units/ml glutathione reductase. Total GSH and GSSG concentrations were determined in the second aliquot using the same assay without adding 2-vinylpyridine. GSSG standards were used for calibration.

Preparation of mouse kidney mitochondria and submitochondrial particles (SMPs)

Mouse kidney mitochondria were isolated by differential centrifugation of kidney homogenates as described previously (13). In brief, kidney was rinsed in cold mitochondrial isolation buffer (225 m*M* sucrose, 5 m*M* potassium phosphate buffer, pH 7.4, 5 m*M* $MgCl_2$, 20 m*M* KCl, 2 m*M* EGTA, and 20 m*M* triethanolamine HCl, pH 7.4) to remove blood and then minced with scissors. A 5% (wt/vol) homogenate was prepared and centrifuged at 600 *g* for 10 min at 4°C, and the resulting supernatant was centrifuged for 5 min under the same conditions. The supernatant was further centrifuged at 15,000 *g* for 5 min at 4°C, and the resulting pellet contained mitochondria. To prepare SMPs, mitochondria were resuspended in 30 m*M* sodium phosphate, pH 7.4, and sonicated four times for 30 s with 1-min intervals in a Branson 2200 instrument (Danbury, CT, U.S.A.). The sonicated sample was centrifuged at 8,250 *g* for 10 min to remove any unbroken mitochondria, and the supernatant was further centrifuged at 80,000 *g* for 40 min. The supernatant contained mitochondrial matrix, whereas the pellet contained mitochondrial SMPs (25).

Measurement of superoxide anion ($O_2^{-\bullet}$) generation

Mitochondrial SMPs, prepared as described above, were resuspended in 100 m*M* phosphate buffer, pH 7.4. The rate of $O_2^{-\bullet}$ generation by SMPs was measured as SOD-inhibitable reduction of acetylated ferricytochrome *c* (3). The reaction mixture contained 10 µ*M* acetylated ferricytochrome *c,* 6 µ*M* rotenone, 1.2 µ*M* antimycin A, 100 units/ml SOD (in the reference cuvette), and 30–100 µg of SMP protein in 100 m*M* potassium phosphate buffer, pH 7.4. The reaction was initiated by addition of 7.5 m*M* succinate, and the reduction of acetylated cytochrome *c* was followed at 550 nm (ϵ = 27,700 M^{-1} cm^{-1}).

Assay of mitochondrial permeability transition

Mitochondria used for the permeability transition assay were prepared by a differential centrifugation protocol (7). Frozen mouse kidney was homogenized by a Teflon homogenizer in a medium containing 180 m*M* KCl, 10 m*M* EDTA, 0.5% BSA, 10 m*M* HEPES, pH 7.4. To remove EDTA and BSA, mitochondrial pellets were washed twice with 180 m*M* KCl, 10 m*M* HEPES, pH 7.4. Mitochondrial permeability transition (swelling) was monitored as the changes at 540 nm with 250 µg of mitochondrial protein in a medium containing 250 m*M* sucrose, 10 m*M* Tris-MOPS, 0.05 m*M* EGTA, pH 7.4, 5 m*M* pyruvate, 5 m*M* malate, and 1 m*M* phosphate.

Determination of mitochondrial membrane potential

Kidney mitochondrial membrane potential was measured using safranine according to the method of Akerman and Wikstrom (1). Mitochondria, prepared as in mitochondrial permeability transition pore (MPTP) analysis, were solubilized in membrane potential buffer containing 200 μ*M* sucrose, 20 m*M* HEPES, 20 m*M* KCl, pH 7.2. The final solution for membrane potential measurement contains 0.5 mg of mitochondrial protein, 6 μ*M* rotenone, 380 μ*M* EDTA, and 9.6 μ*M* safranine. The mixture was incubated at room temperature for 2 min followed by addition of 1.5 m*M* ATP and 1.5 m*M* succinate. Absorbance at 520 nm before and after the addition of ATP and succinate was recorded and used to calculate the relatively induced mitochondrial membrane potential.

Determination of total ROS level

Total kidney homogenate ROS was measured by the fluorescence probe DCFH (10). Under our experimental conditions, 1 μ*M* DCFH (in dimethyl sulfoxide) was incubated with 1 ml of 50 μg of mitochondrial proteins, and fluorescence was measured following a 1-h incubation using a fluorometer equipped with a 96-well plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Whole-body hyperthermic challenge

Wild-type adult mice of the C,129-*Hsf1* background were anesthetized with 1.2% Avertin (Aldrich, WI, U.S.A.) (250 mg/kg of body weight i.p.), treated with a sublethal heat shock by increasing the rectal temperature to 42°C for 15 min using a 250 A infrared heat lamp (General Electric), and then returned to their cages for 24 h of recovery. Anesthetized control mice were kept under similar conditions without heat shock, and their body temperature was maintained at 37 ± 0.5°C. Mice in both experimental groups were administered 0.2 ml of 0.9% sodium chloride intraperitoneally to compensate for insensitive water loss during the procedure and recovery periods.

Other methods

Protein concentration was determined by the bicinchoninic acid assay using BSA as the standard reagent. Statistical analysis, where indicated, was performed by Welch's *t* test.

RESULTS

HSF1 deficiency selectively reduces expression of Hsps in the unstressed adult kidney

Although we have previously reported the essential requirement for HSF1 for heat stress-induced Hsp expression in multiple organs of *Hsf1* knockout mice (24), the effects of HSF1 deficiency in the kidney, under physiological conditions, have not been thoroughly investigated. We thus surveyed the protein expression patterns of several Hsps from *Hsf1* knockout mice (5–7 months) and age-matched controls of similar genetic background (C,129X1) by immunoblot analysis. Our experiments were performed using the supernatants resulting from mitochondrial isolation with antibodies specifically directed against αBC, Hsp25, Hsp70, and Hsp90 (Fig. 1A). Densitometric data revealed that, in the absence of HSF1, the Hsp25 level was decreased by 26% and that of Hsp90 by 50% (Fig. 1B). No differences were detectable for αBC and Hsp70 (Fig. 1B). These results suggest that Hsps, albeit an essential requirement for the heat shock response, are differentially regulated in the kidney in the absence of HSF1 activity at normal temperature (37°C).

Renal redox status is perturbed in absence of HSF1 regulation

In cultured cells, forced overexpression of small Hsps, such as Hsp25, has been reported to influence the cellular

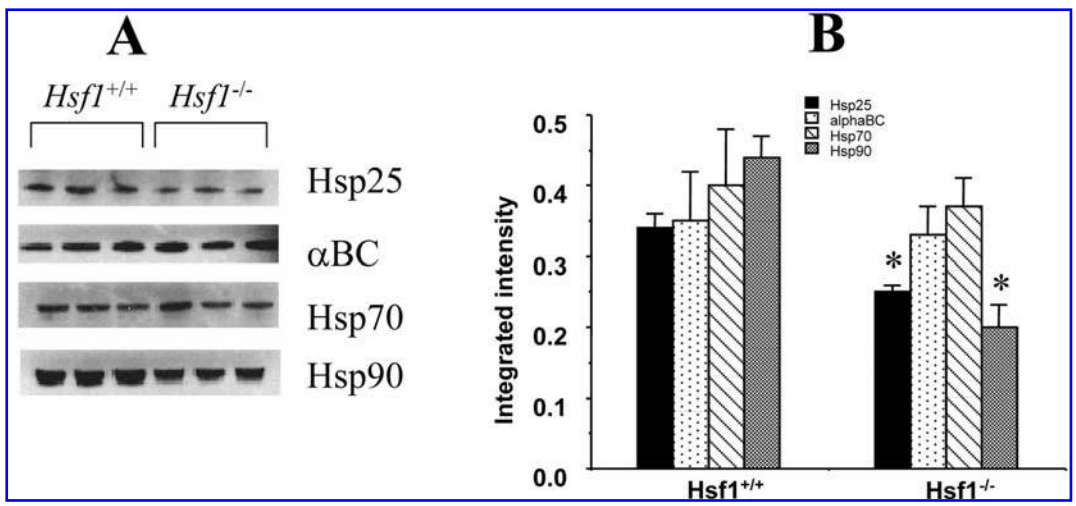

FIG. 1. HSP expression profile in the *Hsf1*$^{+/+}$ and *Hsf1*$^{-/-}$ kidney. HSF1 deficiency causes selective abnormalities in constitutive expression of Hsps. Immunoblots of the major classes of Hsps were determined in protein extracts isolated from the kidney of *Hsf1*$^{+/+}$ and *Hsf1*$^{-/-}$ animals. **(A)** Representative western blots for Hsp25, αBC, Hsp70, and chaperone Hsp90. **(B)** Densitometric data from three animals determined independently in age-matched animals. *$p < 0.05$ was considered significant using the alternate Welch's *t* test.

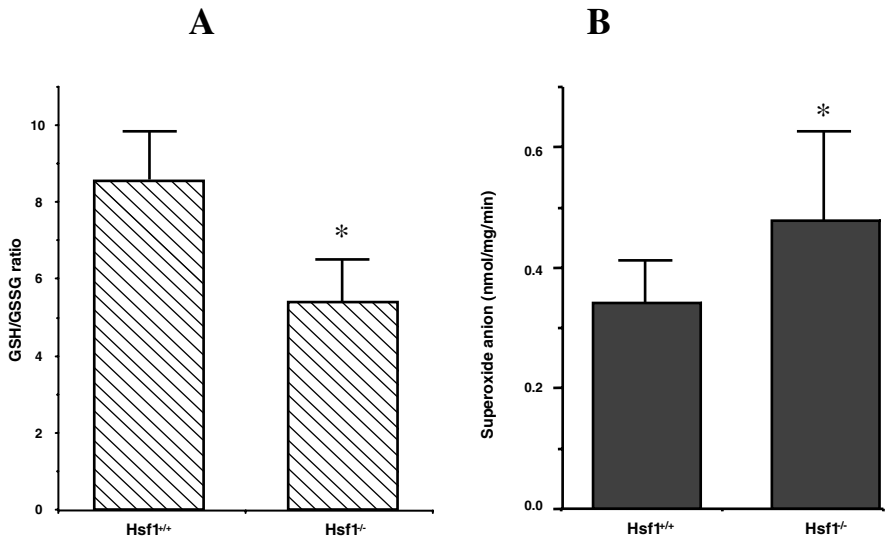

FIG. 2. **(A)** HSF1 disruption lowers the ratio of GSH/GSSG in kidney homogenate. GSH and GSSG levels were determined by a glutathione reductase-mediated recycling assay in the presence of NADPH and 5,5′-dithiobis(2-nitrobenzoic acid). **(B)** HSF1 disruption increases the rate of $O_2^{-\bullet}$ generation in mitochondria. SMPs were prepared from the kidney of 5–7-month-old wild-type mice and *Hsf1* knockout mice. $O_2^{-\bullet}$ generation was determined as SOD-inhibitable reduction of acetylated cytochrome *c*. Data are expressed as means ± SEM of three independent determinations made in separate animals. *$p < 0.05$ was considered significant using the alternate Welch's *t* test ($n = 3$).

detoxifying/antioxidative machinery by raising the intracellular concentration of GSH (4, 16, 20). To determine whether renal Hsp differential regulation in the absence of HSF1 has similar biochemical effects, we assessed the GSH/GSSG ratio in renal homogenates obtained from HSF1-deficient and wild-type animals. Our results show that the GSH/GSSG ratio is significantly decreased by 37% in the kidneys from HSF1-deficient mice compared with wild-type animals ($n = 3$/group) (Fig. 2A).

Level of mitochondria-generated $O_2^{-\bullet}$ is increased in absence of HSF1 regulation

The intracellular redox state is determined by the equilibrium between antioxidants and oxidants. Evidence for a decreased GSH/GSSG ratio in *Hsf1* knockout cells implies that prooxidants like ROS might be generated at a higher level. Therefore, to evaluate directly this consequence of HSF1 deficiency, we prepared renal SMPs and measured the rate of $O_2^{-\bullet}$ generation. Our results indicate that in the *Hsf1* knockout, the rate of $O_2^{-\bullet}$ production was 40% higher ($n = 3$, $p < 0.05$) than that in its wild-type counterpart (Fig. 2B).

HSF1 absence-induced Hsp deficiency causes mitochondrial dysfunction

In *in vitro* experimental systems, both a lower GSH/GSSG level and a higher rate of $O_2^{-\bullet}$ oxidation have been reported to cause mitochondrial dysfunction, as analyzed by measurement of MPTP opening and mitochondrial membrane potential. To determine if increased oxidative stress in the *Hsf1* knockout mouse has a direct effect on MPTP opening and mitochondrial membrane potential, we first monitored the swelling of isolated mitochondria by measuring light scattering at 540 nm. Figure 3A shows that, under our experimental conditions, the MPTP opens at a faster rate in the *Hsf1* knockout than in the wild type. We then measured mitochondrial membrane potential using safranine as an indicator. Figure 3B demonstrates that the induced change on mitochondrial membrane potential is greater in *Hsf1* knockout than in the wild-type animals. These results demonstrate that the HSF1-dependent pathway is a causal mechanism between oxidative stress and mitochondrial functional abnormalities and suggest a possible mechanism for its major role in cell survival and death pathways.

Total renal cellular ROS level is lower upon heat shock treatment in the presence of HSF1

It is known that renal Hsps are up-regulated after heat shock treatment to the whole animal (6). If Hsp deficiency in the absence of HSF1 results in a higher level of oxidative stress in the kidney, Hsp up-regulation in the presence of HSF1 (wild-type animals) should decrease the renal oxidative stress level. To test this possibility, wild-type animals were heat-shocked and total renal cellular ROS was measured using the fluorescence probe DCFH (10). When the diacetate form of the DCFH dye is added to tissue homogenate, ROS in the homogenates leads to the oxidation of DCFH, yielding the fluorescent product DCF (2′,7′-dichlorofluorescein) (10). Our results shown in Fig. 4 indicate that the ROS level in the treated animals is 28% lower compared with that in the nontreated HSF1 animals.

DISCUSSION

The present study demonstrates that, under physiological conditions, Hsps are differentially regulated in the absence of HSF1, suggesting that the HSF1 regulatory pathway exists to regulate both constitutive and stress-inducible Hsp. In the kidney, differential expression of Hsps in the absence of HSF1

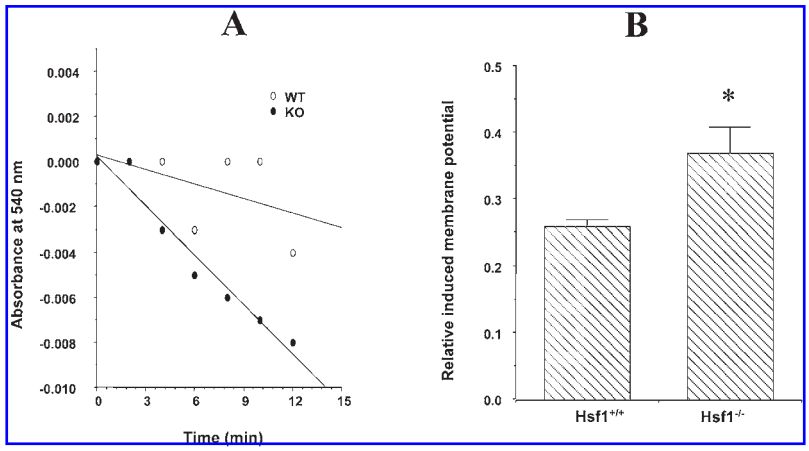

FIG. 3. (A) HSF1 deficiency increases renal MPTP opening. The swelling of isolated mitochondria, an index of MPTP opening, was monitored spectrophotometrically at 540 nm. Shown are representative tracings taken from one of three independent experiments performed on mitochondria isolated from *Hsf*$^{+/+}$ (WT) and *Hsf1*$^{-/-}$ (KO) animals. **(B)** Mitochondrial membrane potential determined by the use of safranine, as detailed in the text. *$p < 0.05$ was considered significant using the alternate Welch's *t* test ($n = 3$).

disrupts the balance of cellular redox status (as reflected by GSH/GSSG ratio), increases mitochondrial $O_2^{-\cdot}$ generation, and causes mitochondrial dysfunction, as evaluated by MPTP opening and mitochondrial membrane potential.

Recently, we have reported that Hsps were differentially regulated in the heart of HSF1-deficient mouse (26). A comparison between kidney and heart indicates that this regulation of Hsp expression is tissue-dependent in the absence of HSF1. In the kidney, for example, neither αBC nor Hsp70 expression is affected by HSF1 deficiency, whereas in the heart, these two Hsps are significantly down-regulated, suggesting that different Hsps might play more important roles in a tissue-specific manner. One consistent finding, however, is that Hsp25 is significantly down-regulated in both the kidney and heart of the HSF1-deficient mouse. Because Hsp25 is highly associated with the maintenance of cellular redox status (20), our study results suggest that Hsp25 plays a more basic role in both kidney and heart for cellular redox homeostasis.

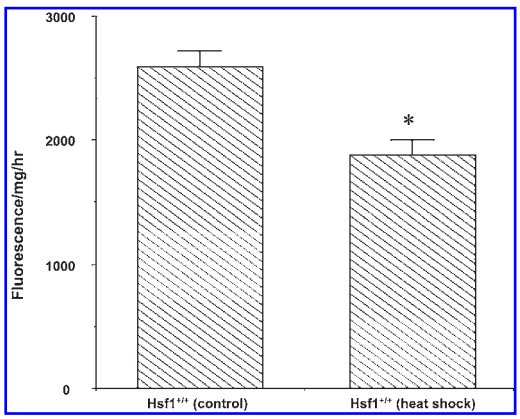

FIG. 4. Total renal cellular ROS level is lower upon heat shock treatment in the presence of HSF1. ROS were measured by the fluorescence probe DCFH. The reaction mixture, containing 50 μg of protein and 1 μ*M* DCFH, was incubated at room temperature for 60 min. Fluorescence was measured using a fluorometer equipped with a 96-well plate reader with excitation wavelength set at 485 nm and emission at 535 nm. *$p < 0.05$ was considered significant using the alternate Welch's *t* test ($n = 3$).

As demonstrated in this study, Hsp down-regulation caused by HSF1 deficiency has a negative effect on mitochondrial function under physiological conditions. Decreased Hsp expression of Hsp25 and Hsp90 causes a higher level of MPTP opening (Fig. 3A) and alters mitochondrial membrane potential (Fig. 3B). Such effects on mitochondrial function could have at least two consequences. Perturbation of mitochondrial electron transport chain, for example, could trigger electron leakage and thus subsequently increase mitochondrial $O_2^{-\cdot}$ generation (Fig. 2B). $O_2^{-\cdot}$ generation itself could further disrupt mitochondrial function, causing more $O_2^{-\cdot}$ generation, and thereby form a vicious cycle, termed so-called ROS-induced ROS production (28).

Induced mitochondrial dysfunction, a consequence of Hsp down-regulation, could be implicated in the cell death and survival pathway. Under physiological conditions in the absence of HSF1, however, our attempt to compare the released cytochrome *c* quantities between HSF1 knockout and wild type was not successful (data not shown). One possible reason could be that the difference in cytochrome *c* release from mitochondria between wild-type and HSF1-deficient animals is below the detection limit, as analyzed by western blot using anti-cytochrome *c* antibodies. Nonetheless, our result does not exclude the possibility that renal cells may undergo accelerated apoptosis by HSF1 deficiency under pathophysiological conditions.

Complex I has been established to be one of the main sites that generate $O_2^{-\cdot}$ (14). As $O_2^{-\cdot}$ generation is enhanced under pathophysiological conditions such as Leigh's disease and cardiomyopathy with cataracts (19), $O_2^{-\cdot}$ generation by complex I can also be increased by oxidative stress, particularly under the condition of a higher GSSG level (22). It has been reported that increased GSSG level leads to glutathionylation of certain complex I subunits (22), which could in turn result in more electron leakage, and thus more one-electron reduc-

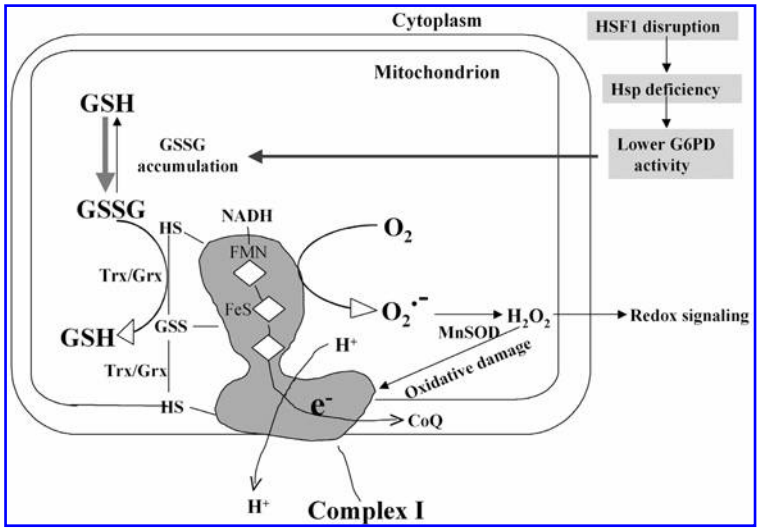

FIG. 5. A schematic model for the role of Hsps in complex I-mediated oxidative stress and mitochondrial dysfunction. The model was proposed based on studies by us and those by Taylor *et al.* (22). The expression of HSF1 disruption causes the down-regulations of Hsps such as Hsp25 and Hsp90, which attenuate the activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme responsible for GSSG reduction back to GSH (26). A lower G6PD activity can cause GSSG accumulation that is able to modify complex I subunits (22), leading to electron leakage and $O_2^{-\cdot}$ generation. Such $O_2^{-\cdot}$ can be converted to H_2O_2 by MnSOD; in turn, hydrogen peroxide (H_2O_2) can either further damage complex I proteins or diffuse out of mitochondria to serve as a signaling molecule. According to Taylor *et al.* (22), GSSG modification of complex I is a reversible process, due to the action of redox molecules such as thioredoxin (Trx) and glutaredoxin (Grx). Other types of oxidative modifications, such as nitration and carbonylation (if any), however, are not reversible (not shown in the model). Complex I-generated $O_2^{-\cdot}$ and $O_2^{-\cdot}$ derivative H_2O_2 can impair mitochondrial functions by altering MPTP opening and mitochondrial membrane potential, as demonstrated in this study.

tion of oxygen, as reflected by more $O_2^{-\cdot}$ generation. Although the specific modification status of complex I subunits was not analyzed directly, based on the evidence that the renal oxidative stress level is higher, we envision that in HSF1 knockout mice, a similar complex I modification profile, as reported by Taylor *et al.*, could exist that can enhance $O_2^{-\cdot}$ generation by complex I. Moreover, $O_2^{-\cdot}$ generated by complex I can have a self-infliction effect, that is, complex I itself can be oxidatively modified by its own $O_2^{-\cdot}$. Additionally, $O_2^{-\cdot}$ produced by complex I could serve as a signaling molecule that may be involved in cell death and survival pathways (22). Figure 5 is a proposed scheme centered on Hsps and redox-regulation complex I $O_2^{-\cdot}$ generation.

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ABBREVIATIONS

αBC, αB-crystallin; BSA, bovine serum albumin; DCFH, 2′,7′-dichlorofluorescin; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; HSF1, heat shock factor 1; HSP, heat shock protein; MPTP, mitochondrial permeability transition pore; $O_2^{-\cdot}$, superoxide anion; ROS, reactive oxygen species; SMP, submitochondrial particle; SOD, superoxide dismutase; TBST, Tris-buffered saline containing Tween-20.

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