Surgical Stress Increases Renal Glutathione Content via Increased Glucocorticoid, and Resistance to Subsequent Oxidative Injury in the Rat: Significant Link Between Endocrine Response and Cell Defense System under the Stress

Makiko Ogasawara, Kaoru Nomura, Noriyuki Shibata*, Makoto Ujihara, Makio Kobayashi*, and Hiroshi Demura

Department of Medicine, Institute of Clinical Endocrinology,*Department of Pathology, Tokyo Women’s Medical University, Tokyo 162-8666, Japan

Abstract. Systemic and nonspecific stress response effects on the cellular defense mechanism were studied in the male rat kidney. Two days after laparotomy-induced surgical stress, rats showed increased serum corticosterone and renal cortical reduced glutathione (GSH). Rats were then injected s.c. with mercuric chloride (HgCl₂) to oxidatively injure renal tubuli. Increased serum creatinine levels indicated that laparotomy pretreatment lessened renal damage. To study the effects of the activated pituitary-adrenal axis on renal cortical GSH content and vulnerability to subsequent oxidative injury, rats were injected s.c. with ACTH on two consecutive days. ACTH administration increased both corticosterone and aldosterone. These rats showed increased, dose-dependent renal cortical GSH content, i.e., controls (n=7): 1.25 ± 0.23 µmol/g tissue, daily dose of 10 µg/100 gBW (n=7): 1.53 ± 0.24 µmol/g tissue, and daily dose of 40 µg/100 gBW (n=7): 2.31 ± 0.23 µmol/g tissue. Rats receiving daily doses of 40 µg of ACTH/100 gBW acquired resistance to oxidative injury, indicated by serum creatinine levels: controls (n=6), 22 ± 4 µmol/L; HgCl₂ (n=6), 145 ± 88 µmol/L; ACTH and HgCl₂ (n=6), 37 ± 11 µmol/L. Morphological evidence indicated that ACTH pretreatment in HgCl₂-injected rats prevented renal tissue from inflammatory cell infiltration but not from tubular degeneration. Cellular GSH content of LLC-PK1 cells, porcine renal-tubule-derived culture cells, increased significantly in incubation with dexamethasone or aldosterone, suggesting that adrenal steroids directly stimulate renal cell GSH. We demonstrated that stress or ACTH administration activates the defense mechanism in the kidney via increased GSH. This stress-activatable defense system may therefore indicate a connection between endocrine stress response and the cellular defense mechanism.

Key words: Stress response, Antioxidant, Glutathione, ACTH, Glucocorticoid, Kidney

plasma GSH levels in the rat [2]. Findings suggest that GSH could be synthesized by stress or its mediators. Although the classical theory holds that the systemic and nonspecific stress response enables systems to resist subsequent stress [3], the effect of such stress on the cellular defense mechanism remains unknown.

In this study, we studied how laparotomy affects tissue GSH content and resistance to oxidative stress in rats to clarify whether systemic and nonspecific stress increased the representative cellular antioxidant (GSH) and increased resistance to harmful stress. The rat kidney was targeted because of its weak antioxidant defense system [4] and vulnerability to oxidative stress. Oxidative stress and injury have been experimentally produced in animals by using an ischemia-reperfusion model [5], and by the administration of heavy metals [6]. Mercury is known to react with and deplete GSH and free sulfhydryl groups in renal tubuli. The decrease in GSH and free sulfhydryl groups may lead to oxidative stress, damaging tissue [7]. HgCl₂ causes acute tubular necrosis through oxidative injury [6, 8, 9]. A single s.c. HgCl₂ injection was used because HgCl₂ was accumulated specifically in the kidneys and caused oxidative injury to the kidneys less stressfully, for example, than ischemic reperfusion or irradiation.

**Materials and Methods**

**Animals and treatment**

Male Wistar rats, purchased from Nihon Ikagaku Doubutsu Shizai Laboratory, Tokyo, Japan, were maintained and treated at 9 weeks of age based on NIH guidelines and an institutional review board for the care and treatment of laboratory animals (permission Nos. 97–17 and 98–21). Mercuric chloride HgCl₂ was injected s.c. at a dose of 1.5 mg/kg of body weight (BW). This dose caused oxidative injury to renal proximal tubules and acute renal failure with increased serum creatinine and decreased endogeneous creatinine clearance [10]. A higher dose (2.25 mg/kgBW) was fatal to 50% of rats injected [10].

Surgical stress was induced by laparotomy, which included anesthesia with phentobarbital, a V shape incision at the abdominal wall, gut contact with tweezers, and immediate wound closing with clips. HgCl₂ was injected two days after surgical manipulation.

Synthetic ACTH (Cortrosin-Z, Daiichi Pharmaceutical Co. Ltd., Tokyo) was injected s.c. on two consecutive days at a daily dose of 10 or 40 µg/100 gBW. Saline was injected s.c. in controls. HgCl₂ was injected 24 h after the second ACTH or saline injection.

The rats were killed by decapitation. Rats injected with HgCl₂ were killed four days after the injection — the period required for renal cells to degenerate and regenerate simultaneously [8]. Trunk blood was collected to measure creatinine and steroids. Serum creatinine was measured by a colorimetric picric acid method employing Fuller’s earth [11]. Serum corticosterone was measured by a specific RIA for rat corticosterone (Diagnostic Products Corporation, Los Angeles, CA, USA); the detection limit was 16.45 nmol/L. Serum aldosterone was measured by a specific RIA (Dainabot Co., Tokyo).

Kidneys removed from the rats examined were fixed in 10% formalin and embedded in paraffin. Four-µm-thick kidney sections were stained with hematoxylin-eosin (H&E) and periodic acid-methenamine-silver (PAM). H&E-stained sections were provided for conventional histopathologic examination. PAM-stained sections were used for identifying the basement membrane.

**LLC-PK1 cell culture**

LLC-PK1 cells were cultured with medium 199 (Life Technologies, Inc., Grand Island, NY, USA) containing 3% fetal calf serum. Cell viability was measured by an aqueous tetrazolium/formazan assay and expressed as absorbance at A490 [12]. Cellular protein was measured by Lowry’s method with bovine serum albumin as a standard.

**GSH measurement**

Renal slices were obtained by slicing kidney transversely at the center and removing papilla. Renal cortical tissue and cultured cells were homogenated in 5% trichloroacetic acid with 0.01 M EDTA. GSH was extracted and measured by the method of Chung and Maines [13].

**Statistics**

Data were shown as the mean ± SD. Analysis of
Results

Laparotomy effects on renal cortical GSH content and on HgCl2-induced renal injury

Serum corticosterone levels increased significantly in 2-day-postlaparotomy rats, but serum aldosterone levels did not change (Table 1). Laparotomy therefore activated the pituitary-adrenal axis. Renal cortical GSH content was significantly higher in rats laparotomized two days earlier than in nonlaparotomized rats (Fig. 1). A comparison of vulnerability to HgCl2-induced oxidative stress in nonpretreated controls and 2-day-post-laparotomy rats showed that serum creatinine levels after HgCl2 injection were significantly lower in laparotomized rats than in controls (Fig. 2).

Effects of ACTH administration on renal cortical GSH content and on HgCl2-induced renal injury

Rats were injected s.c. with ACTH at a daily dose of 10 or 40 μg/100 gBW on two consecutive days, and serum levels of both corticosterone and aldosterone increased significantly (Table 1). In low-dose-injected rats, serum corticosterone levels did not increase, presumably because the serum was obtained 24 h after injection. ACTH increased renal cortical GSH content significantly and dose dependently (Fig. 3). Vulnerability to HgCl2-induced oxidative stress was then compared in rats with and without ACTH pretreatment. Rats were

<table>
<thead>
<tr>
<th>Stress effects</th>
<th>N</th>
<th>Corticosterone (nmol/L)</th>
<th>Aldosterone (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-day PL</td>
<td>6</td>
<td>241 ± 183</td>
<td>196 ± 52</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>132 ± 77</td>
<td>310 ± 331</td>
</tr>
<tr>
<td>2-days PL</td>
<td>7</td>
<td>343 ± 41**</td>
<td>214 ± 119</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>119 ± 60</td>
<td>340 ± 192</td>
</tr>
<tr>
<td>ACTH (10 μg/100 gBW)</td>
<td>7</td>
<td>90 ± 35</td>
<td>116 ± 97</td>
</tr>
<tr>
<td>ACTH (40 μg/100 gBW)</td>
<td>7</td>
<td>1247 ± 641*</td>
<td>1238 ± 850**</td>
</tr>
<tr>
<td>Controls</td>
<td>7</td>
<td>161 ± 120</td>
<td>178 ± 120</td>
</tr>
</tbody>
</table>

Table 1. Laparotomy and ACTH effects on serum corticosterone and aldosterone

* P<0.01, ** P<0.001 vs. controls.
injected with either saline (controls) or 40 µg of ACTH / 100 gBW on two consecutive days, and on the next day were injected with HgCl₂. After HgCl₂ injection, serum creatinine levels were significantly lower in rats pretreated with ACTH than in nonpretreated rats (Fig. 4). Renal damage and repair in HgCl₂-injected rats were studied morphologically (Fig. 5). Non-ACTH-pretreated HgCl₂-injected rats demonstrated proximal tubular cell degeneration, necrosis and regeneration, mainly in the lower half of the cortex together with the infiltration of inflammatory cells, including neutrophils, macrophages, and lymphocytes mainly in the corticomedullary junction (Fig. 5B and C). Inflammatory cells intermingled with detached epithelial cell debris infiltrated into tubular lumens through destroyed parts of the basement membrane.
(Fig. 5D), associated with hyaline-like cast formation in collecting tubuli. ACTH-pretreated HgCl₂-injected rats also had tubular cell changes similar to those in non-ACTH-pretreated HgCl₂-injected rats, but did not show signs of inflammatory cell infiltrates or hyaline-like casts (Fig. 5E). Features of ACTH-pretreated non-HgCl₂-injected rats resembled those of controls (data not shown).

The steroids effect on cellular GSH content in LLC-PK1 cells

Both dexamethasone and aldosterone increased cellular GSH content significantly without notable changes in either total protein content or cell viability (Fig. 6).

Discussion

To clarify the cytoprotective mechanism after the systemic and nonspecific stress response, we prepared an animal model in which rats laparotomized two days earlier became resistant to nephrotoxic HgCl₂. Renal GSH levels are reportedly critical to susceptibility to free-radical-mediated postischemic injury [5]. Our study showed that laparotomy stress increased both renal GSH content and resistance to oxidative insult. This finding suggests that renal GSH is induced by stress and renders the kidney resistant to HgCl₂-induced damage.

What factor mediated the stress response to increased renal cortical GSH content? The two principal components of the stress response are corticotropin-releasing hormone (CRH) and the locus ceruleus-norepinephrine/autonomic (sympathetic) nervous system [14]. CRH stimulates the pituitary adrenal axis. Laparotomized rats had significantly higher serum corticosterone levels than controls, indicating that the pituitary-adrenal axis was actually activated in laparotomized rats. ACTH increases renal cortical GSH content and decreases HgCl₂-induced renal damage. Although laparotomy stress may be regarded as causing an increase in aldosterone, the serum aldosterone concentration had not changed one or two days after laparotomy.

Because the kidney is not a target tissue of ACTH, ACTH increases renal GSH content by increasing circulating adrenal steroids. To study the direct link between the stress-activated pituitary adrenal axis and increased renal GSH content, we conducted an in vitro study with LLC-PK1 culture cells. In the study, cells were incubated with graded doses of dexamethasone or aldosterone for 24 h to increase cellular GSH. Both steroids significantly increased cell GSH content without any change in total protein content. Although the effects of laparotomy stress and ACTH administration were different in terms of aldosterone levels, we concluded that stress activated renal GSH synthesis was at least partially mediated by the activated pituitary-adrenal axis, and reduced ROSs-induced injury or responses. The present study does not clarify the mechanism by which adrenal steroids increase renal GSH content. Because γGCS is a late-limiting enzyme that synthesizes GSH as mentioned in the introduction, glucocorticoid may be considered to affect the activity of renal γGCS to increase GSH. The GSH level is also regulated by a redox cycle in which glutathione reductase (GR) and glutathione peroxidase (GPX) are involved. GR reduces the oxidized glutathione (GSSG) to reduced one (GSH), while GPX catalyzes GSH and H₂O₂ to form GSSG. The modification of the glutathione redox cycle was reported to affect the injurious effects of oxidative stress [15, 16] and inflammatory cytokines [17]. The nephrotoxic alkylating agent, S-(1, 2-dichlorovinyl)-L-cysteine (DCVC), causes oxidative stress and subsequently death of renal proximal tubular cells. The reactive metabolites of DCVC are considered to bind to GR and GPX, and to inhibit their activity. Therefore, the nephrotoxic effect of DCVC is caused by impairing the GSH redox cycle [15]. GR and GPX therefore seem to be involved in steroid effects on renal GSH levels, but this remains to be proven.

Oxidative stress reportedly activates a transcriptional factor, NF-κB, which then stimulates gene expression related to proinflammatory and immune responses such as interleukins, their receptors, and TNF [18]. These mediators may be involved in the HgCl₂-induced destruction of renal tubules and inflammatory cell infiltration. Glucocorticoids suppress NF-κB activity [19] and inflammatory and immune cytokine expression [20], as do antioxidants, including GSH [21]. The inhibition of inflammatory cell infiltration in ACTH-
pretreated rats may reflect this antiinflammatory glucocorticoid effect [22]. Metallothioneins (MTs) are low-molecular-weight proteins with a high cysteine content, and detoxify ROIs, even though their cellular content is far lower than GSH and their antioxidative roles are limited. MTs are induced by glucocorticoids [23] and lower mercury-induced renal toxicity [24]. Although we did not measure renal MT content in this study, it might increase after laparotomy or ACTH treatment and help renal cortical cells to resist to HgCl₂. Glucocorticoids work in this way to decrease HgCl₂-induced renal damage both by increasing renal cortical GSH content and possibly MTs, and presumably by suppressing inflammatory and immune responses. Although the kidney has a relatively weak antioxidant defense system [4], it is important to note that its defense mechanism can be strengthened or activated by preexposure to stress. Aldosterone was also found to be able to increase cellular GSH content in LLC-PK₁ renal epithelial cells. But because target cells for aldosterone (distal tubules) and for HgCl₂ (proximal tubules) were distinct, it seems unlikely that aldosterone has a protective effect in the in vivo model of renal damage caused by HgCl₂.

In conclusion, we have demonstrated that the stress-induced endocrine response activates the cellular defense mechanism in the kidney. GSH, the most abundant antioxidant within cells, was found to be increased by stress or glucocorticoids, increasing the cellular antioxidative activity.

Acknowledgments

We thank Ms. Megumi Takamatsu for her technical assistance, Dr. Shoji Nishiyama of Meiji Seika Kaisha, Ltd., for his consultation on the GSH assay, and Ms. June Streitmum-Setoguchi for editing the manuscript.
References


