IMMUNOLOCALIZATION OF GLUTATHIONE- REDUCTASE (GSSG-RD) IN THE RAT ADRENAL CORTEX — EFFECT OF HYPOPHYSECTOMY AND ACTH ADMINISTRATION —

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Abstract: Immunocytochemical localization of glutathione-reductase (GSSG-RD) in rat adrenal glands was studied in 1) normal untreated, 2) hypophysectomized, and 3) ACTH administered groups in order to clarify the biological significance of GSSG-RD in the adrenal cortical lipid metabolism including steroidogenesis. In the normal untreated group, GSSG-RD was immunohistochemically localized in all three zones of the adrenal cortex. In immunoelectron microscopic investigations, GSSG-RD was observed mainly in mitochondria. The hypophysectomized group showed atrophy of the zonae fasciculata and reticularis. GSSG-RD staining of the atrophic cortical zones was weakened. In immunoelectron microscopic investigations, GSSG-RD was observed not only in mitochondria but also in cytoplasmic matrix. On ACTH administration, the adrenal cortex became markedly hypertrophic, especially in the outer fasciculata. Immunohistochemically, GSSG-RD was mainly localized in the cells of those hypertrophic cortical zones. In immunoelectron microscopic investigations, GSSG-RD was observed not only in mitochondria but also in cytoplasmic matrix. These staining patterns were similar to those of glutathione-peroxidase (GSH-PO). GSH-PO reduces the lipid peroxides with concomitant oxidation of glutathione. Oxidized glutathione is reduced by GSSG-RD under the presence of NADPH. These two enzymes are known to be key enzymes of the lipid peroxides scavenging system. Based on our findings, it is strongly suggested that a very close relationship lies between the adrenal cortical GSSG-RD and steroidogenesis. (*J Toxicol Pathol* 8: 51~57, 1995)

Key words: Glutathione-reductase (GSSG-RD), Adrenal cortex, Hypophysectomy, ACTH, Mitochondria

Introduction

In a previous reports¹⁻⁴, it was shown that immunocytochemical localization of glutathione-peroxidase (GSH-PO) which effectively reduces the lipid peroxides, in the rat adrenal cortical cells was observed not only in cytoplasmic matrix but in mitochondria, and mitochondrial GSH-PO was

increased by ACTH administration, immunocytochemically and biochemically. Therefore, we strongly suggest that mitochondrial GSH-PO may be induced by ACTH administration¹⁻⁴. And the possible role of the rat adrenal cortical GSH-PO was proposed as follows; (A) reduction of cholesterol ester hydroperoxides induced by cell injury and (B) reduction of sterol hydroperoxides such as steroid intermediate to its metabolizable form for steroidogenesis¹⁻⁴.

Recently, our laboratory succeeded in obtaining highly purified glutathione-reductase (GSSG-RD) of rat liver and has made it possible for us to

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prepare large quantities of specific antibody against this enzyme⁵. GSSG-RD is considered to play an important role in the maintenance of glutathione in the reduced form. In the present study, in order to clarify the biological significance of GSSG-RD in the adrenal gland, attempts were made to localize the GSSG-RD immunocytochemically in the adrenal glands of normal, hypophysectomized and ACTH-administered rats.

Materials and Methods

Animals

Male Sprague-Dawley rats were purchased from Charles River Japan Inc. (Atsugi, Japan) at the age of seven weeks. The animals were kept in a barrier-maintained room, which was maintained at a temperature of $22 \pm 2^\circ\text{C}$ and humidity of $55 \pm 15\%$. The room was ventilated twenty one times per hour and provided with 12 hr of light (from 800 hr to 2,000 hr). The animals were housed individually in plastic cages (CLEA Japan, Inc.). Solid food (CE-2, CLEA Japan, Inc.) and tap water were available to all animals *ad libitum*. Then one week was allowed for the adjustment of the animals to laboratory conditions.

Experiments

Group 1 consisted of five untreated controls. In group 2, five rats were sacrificed 1 week after hypophysectomy. In group 3, five rats were treated intramuscularly 0.5 mg/head of ACTH (Cortrosyn Z, N.V. Organon, Netherlands) daily for 1 week after hypophysectomy. Each rat was killed by decapitation, and the adrenal gland was removed.

Immunocytochemical staining for adrenal gland

GSSG-RD

The adrenal glands were fixed in periodate-lysine-4% paraformaldehyde solution⁶ for 4 to 6 hr at 4°C under constant agitation. The fixed tissues were then washed in 0.01 M phosphate-buffered saline (PBS) containing sucrose from 10 to eventually 20% overnight at 4°C . Subsequently, 6 μm -thick frozen sections were prepared from the washed tissues in a cryostat, and were placed on

albumin-coated glass slides. The sections were washed in 0.01 M PBS and then were stained by Nakane's direct peroxidase-labeled antibody method using rabbit anti-rat GSSG-RD polyclonal antibody IgG Fab fragment⁷.

For light microscopic observations of GSSG-RD, 6 μm -thick frozen sections were incubated with the antibody labeled with horseradish peroxidase (HRPO, Sigma Chemical Co., St. Louis, MO) for 1 hr. After the incubation was completed, the sections were treated in Graham-Karnovsky's reaction medium⁸ which contained 20 mg% 3, 3'-diaminobenzidine (DAB, Wako Pure Chemical Industries, Osaka) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 5 to 10 min at room temperature. Then the sections were counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer, pH 4.2.

For electron microscopic observations of GSSG-RD, 6 μm -thick frozen sections were incubated with HRPO-labeled antibody for 6 hr. After the incubation was completed, the sections were incubated for 30 min in Graham-Karnovsky's reaction medium⁸ from which the substrate hydrogen peroxide was omitted, and then they were incubated in the fully equipped reaction medium for 5 min. The sections were post-fixed in 2% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 90 min, dehydrated in graded ethanol series, and embedded in Quetol 812 by an inverted gelatin capsule method. Ultrathin sections were prepared with an LKB ultra-microtome and were observed under a JEOL 1200EX electron microscope.

As an immunologic negative control, normal rabbit serum (NRS) IgG Fab fragment labeled with HRPO was applied on both light and electron microscopic investigations instead of anti-GSSG-RD IgG Fab fragment labeled with HRPO.

Results

Group 1 (Intact control)

GSSG-RD was localized in the cortical cells in all three zones of the adrenal cortex (Fig. 1A). No reaction products were seen in the medulla. The control serum (NRS) was negative for immunohistochemical localization of GSSG-RD in

Fig. 1. Immunohistochemical localization of GSSG-RD in control (A), hypophysectomized (C) and ACTH-treated (D) rat adrenal glands. GSSG-RD is localized in cortical cells of all zones of the adrenal cortex (A). In atrophic cortical zones of hypophysectomized rats (C), GSSG-RD staining is weaker than that of A. By ACTH administration (D), GSSG-RD is mainly localized in the adrenal cortical cells of outer fasciculata. B is control test (NRS). Peroxidase-labeled antibody method, $\times 60$ (A, B, D), $\times 150$ (C).

Fig. 2. Immunoelectron microscopic localization of GSSG-RD in control rat adrenal cortical cells. Intramitochondrial localization of GSSG-RD is clearly seen. Peroxidase-labeled antibody method, $\times 10,000$.

A

B

Fig. 3. Immunoelectron microscopic localization of GSSG-RD in hypophysectomized rat adrenal cortical cells. GSSG-RD is observed not only in mitochondria (A) but also in cytoplasmic matrix (B). Peroxidase-labeled antibody method, $\times 8,000$ (A), $\times 10,000$ (B).

A

B

Fig. 4. Immunoelectron microscopic localization of GSSG-RD in ACTH treated rat adrenal cortical cells. GSSG-RD is observed not only in mitochondria (A) but also in cytoplasmic matrix (B). Peroxidase-labeled antibody method, $\times 8,000$ (A), $\times 10,000$ (B).

the adrenal gland (Fig. 1B). In immunoelectron microscopic investigations, GSSG-RD was observed mainly in mitochondria in the adrenal cortical cells (Fig. 2).

Group 2 (Hypophysectomy)

The adrenal gland became markedly atrophic. Atrophy was most conspicuous in the zonae fasciculata and reticularis. The cortical cells of this atrophic zones showed degenerative process such as decrease of cell size, deposition of lipofuscin, and lipid accumulation. Immunohistochemically, GSSG-RD was localized mainly in the cortical cells of the zona glomerulosa and outer fasciculata

(Fig. 1C). GSSG-RD staining of the atrophic cortical zones were weakened. In immunoelectron microscopic investigations, GSSG-RD was observed not only in mitochondria (Fig. 3A) but also in cytoplasmic matrix (Fig. 3B).

Group 3 (ACTH administration)

The adrenal gland became markedly hypertrophic. Hypertrophy was most conspicuous in the outer fasciculata. Immunohistochemically, GSSG-RD was mainly localized in the cells of those hypertrophic cortical zones (Fig. 1D). In immunoelectron microscopic investigations, GSSG-RD was observed not only in mitochondria

(Fig. 4A) but also in cytoplasmic matrix (Fig. 4B).

Discussion

GSH-PO reduces the lipid peroxides with concomitant oxidation of glutathione. Oxidized glutathione is reduced by GSSG-RD under the presence of NADPH. These two enzymes are known to be key enzymes of lipid peroxides scavenging system. It is generally accepted that lipid peroxidation is the reaction of oxidative deterioration of lipids and peroxidation involves the direct action of oxygen and lipid to form free radical intermediates and to produce semistable peroxides. Thus free radicals result in the lipid peroxidation and damage of cellular membrane with release of intracellular components, e.g., lysosomal enzymes, leading to further tissue damage and even in tumor promotion⁹⁻¹².

A large number of studies have been reported on the role fulfilled by GSH-PO in the protection of various tissues against lipid peroxidation, immunohistochemical localization of GSH-PO was demonstrated in the liver¹³⁻¹⁶ and in steroid hormone-producing tissues including the adrenal gland¹⁻⁴, testis¹⁷, and ovary¹⁸, GSH-PO was well stained in specific cells of the tissues examined, but the staining intensity was altered by functional state of the cells. Furthermore GSH-PO staining was modified by lipoperoxidative damage in tissues or cells. Therefore, we proposed that the pattern of GSH-PO staining should be a more sensitive and specific indicator of oxidative damage in tissues. Thus, the staining pattern of GSH-PO is thought to be a useful marker for lipid peroxidation in the cells.

In our previous reports¹⁻⁴, immunoelectron microscopic localization of GSH-PO was described in the rat adrenal cortical cells and a very close relationship was suggested between steroidogenesis and GSH-PO. In the present study, GSSG-RD was found in the adrenal cortical cells under the experimental condition. Furthermore, immunoelectron microscopic localization of GSSG-RD was observed not only in mitochondria but also in cytoplasmic matrix. This staining pattern was similar to that of GSH-PO. These findings strongly suggest that a very close relation-

ship lies between the adrenal cortical GSSG-RD and steroidogenesis.

According to Kitahara *et al.*¹⁹ who studied the activities of GSH-PO and GSSG-RD in preneoplastic foci and hyperplastic nodules of the liver in rats produced by the administration of diethyl-nitrosamine followed by N-2-fluorenylacetamide, GSH-PO activity was markedly decreased, whereas GSSG-RD activity was enhanced. In addition, according to Shinoda *et al.*²⁰ who studied immunohistochemical staining for GSH-PO and GSSG-RD in enzyme-altered hyperplastic foci and hepatoma of rats induced by 3'-methyl-4-dimethylaminoazobenzene, immunostaining for GSSG-RD was intense in the above materials, whereas GSH-PO staining was weak or negative. In this regard, they speculated that intense GSSG-RD staining in hyperplastic foci may reflect the active cell proliferation. In other words, the pattern of GSSG-RD staining is thought to be a good marker for preneoplastic and neoplastic cells. Further work in this area is now in progress in our laboratory.

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