Immunolocalization of Glutathione-Peroxidase (GSH-PO) in Human Adrenal Gland
—Studies on Adrenocortical Adenomas associated with Primary Aldosteronism and Cushing’s Syndrome—

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Immunocytochemical localization of glutathione-peroxidase (GSH-PO) in human adrenal glands was studied on adrenocortical adenomas associated with primary aldosteronism and Cushing's syndrome. Normal adrenal glands were obtained from non-adenomatous regions of the same surgical specimens. In the normal adrenal glands, GSH-PO was immunohistochemically localized in the zonae fasciculata and reticularis of the adrenal cortex. In immunoelectron microscopic investigations, GSH-PO was localized not only in cytoplasm (cytosol GSH-PO) but also in mitochondria (mitochondrial GSH-PO). Mitochondrial GSH-PO was mainly observed in lipid-depleted inner fasciculata cells. Cytosol GSH-PO was mainly localized in lipid-laden or lipofuscin granule-laden zona reticularis cells. In the adrenocortical adenoma cells associated with primary aldosteronism, GSH-PO was weak or negative. In immunoelectron microscopic investigations, GSH-PO was localized in cytoplasm near the lipid droplets. Mitochondrial GSH-PO was hardly seen. Based on our findings, cytosol GSH-PO may play an important role in protective effects against cell injury by lipid peroxides induced in the process of the steroid hormone synthesis or the cellular aging process, and mitochondrial GSH-PO was strongly suggested to be one of the most important enzymes for steroidogenesis, especially cortisol synthesis. Furthermore, the lipid peroxidation rate in the process of aldosterone synthesis may be less than that during cortisol synthesis. In the adrenocortical adenoma cells associated with Cushing's syndrome, GSH-PO was localized mainly in lipid-depleted compact cells. Intracellular localization of GSH-PO was observed only in cytoplasm near the well-developed smooth endoplasmic reticulum or round mitochondria. This suggests that the intramitochondrial lipid peroxidation rate is less in the adrenocortical adenoma cells than in the normal adrenocortical cells. Furthermore, intramitochondrially derived free radicals might be diffuse across the mitochondrial membrane, and cytosol GSH-PO may be enhanced as a result. These findings may correspond to the functional significance of the adrenocortical adenoma cells.

(Key Words: Glutathione-peroxidase (GSH-PO), Adrenocortical adenoma, Primary aldosteronism, Cushing's syndrome, Human)

INTRODUCTION

In our previous reports, it was demonstrated that immunocytochemical localization of glutathione-peroxidase (GSH-PO) which effectively reduces lipid peroxides, in the rat adrenocortical cells was observed not only in cytosol (cytosol GSH-PO) but in mitochondria (mitochondrial GSH-PO) and mitochondrial GSH-PO was increased by ACTH administration, immunocytochemically and biochemically (15, 17). Therefore, it was strongly suggested that mitochondrial GSH-PO may be induced by ACTH stimulation (15, 17), and we proposed the possible role of rat adrenocortical GSH-PO as follows: [A] reduction of cholesterol ester hydroperoxides induced by cell injury (hypophysectomy, cytosol GSH-PO) and [B] reduction of sterol hydroperoxides such as steroid intermediate to their metabolizable

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form for steroidogenesis (15, 17).

Recently, our laboratory success in obtaining highly purified GSH-PO of human red blood cells has made it possible for us to prepare large quantities of specific antibody against this enzyme.

In this study, we investigated intracellular localization of GSH-PO in human adrenocortical cells as well as human adrenocortical adenomas associated with primary aldosteronism and Cushing's syndrome in order to confirm the relationship between GSH-PO and adrenocortical lipid metabolism, including steroidogenesis.

MATERIALS AND METHODS

Materials

Seven adrenocortical adenomas were obtained from four patients with primary aldosteronism (two males and two females: 34 to 49 years of age) and three patients with Cushing's syndrome (three females: 19 to 50 years of age). Normal adrenal glands were obtained from the non-adenomatous regions of the same surgical specimens.

Immunohistochemical staining of adrenal glands

1) GSH-PO

The adrenal glands were fixed in periodate-lysine-4% paraformaldehyde solution (13) for 4 to 6 h at 4°C under constant agitation. The fixed tissues were then washed in 0.01 M phosphate-buffered saline (PBS) containing from 10% to eventually 20% sucrose 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 were then stained by Nakane's direct peroxidase-labeled antibody method using rabbit anti-human GSH-PO polyclonal antibody IgG Fab fragment (18).

For light microscopic observations of GSH-PO, 6 μm-thick frozen sections were incubated with antibody labeled with horseradish peroxidase (HRPO, Sigma Chemical Co., St. Louis, MO) for 1 h. After the incubation was completed, the sections were treated in Graham-Karnovsky's reaction medium (5), 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 GSH-PO, 6 μm-thick frozen sections were incubated with HRPO-labeled antibody for 6 h. After the incubation was completed, the sections were incubated for 30 min in Graham-Karnovsky's reaction medium (5), from which the substrate hydrogen peroxide was omitted, and then they were incubated in the complete reaction medium for 5 min. The sections were post-fixed in 2% OsO₄ in 0.1 M phosphate buffer, pH 7.4, for 90 min, dehydrated in graded ethanol series, and then embedded in Quetol 812 by an inverted gelatin capsule method. Ultrathin sections were prepared with a 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 in both light and electron microscopic examinations instead of anti-GSH-PO IgG Fab fragment labeled with HRPO.

RESULTS

A. Immunohistochemical observations

1. Normal adrenal gland

In the normal adrenal gland, GSH-PO was localized immunohistochemically, mainly in the interior of the cortex, i.e., inner fasciculata and zona reticularis (Fig. 1). In these cells, reaction products were observed diffusely in the cytoplasm (Fig. 2). In zona reticularis cells, lipofuscin granules were frequently observed in the cytoplasm. Immunohistochemically, GSH-PO was localized in the cytoplasm of the lipofuscin-laden cells (Fig. 2) and occasionally in the cytoplasm in the zona glomerulosa cells, but not in the medulla. The control serum (NRS) was negative for immunohistochemical localization of GSH-PO in the adrenocortical cells (Fig. 3).

2. Adrenocortical adenomas associated with primary aldosteronism

The adrenocortical adenoma cells mainly consisted of lipid-laden clear cells. Immunohistochemically, GSH-PO was weak or negative in the adrenocortical adenoma cells (Fig. 4).
Fig. 1 Immunohistochemical localization of GSH-PO in human adrenal gland. GSH-PO is seen in the interior of the cortex (arrows). G: Zona glomerulosa, F: Zona fasciculata, R: Zona reticularis, Peroxidase-labeled antibody method, × 60

Fig. 2 Intracellular localization of GSH-PO in adrenocortical cells under higher magnification. GSH-PO is evenly distributed in the cytoplasm (arrows). F: Zona fasciculata, R: Zona reticularis, Peroxidase-labeled antibody method, × 250

Fig. 3 In normal rabbit serum, no immunohistochemical reaction is observed in the adrenal cortex. G: Zona glomerulosa F: Zona fasciculata, R: Zona reticularis. Peroxidase-labeled antibody method, × 60

Fig. 4 Immunohistochemical localization of GSH-PO in adrenocortical adenoma cells associated with primary aldosteronism. GSH-PO is weak or negative in adenoma cells (arrows). Peroxidase-labeled antibody method, × 60
3. Adrenocortical adenomas associated with Cushing's syndrome

The adrenocortical adenoma cells consisted of lipid-laden clear cells and lipid-depleted compact cells. Immunohistochemically, GSH-PO was mainly localized in lipid depleted-compact cells and the intensity of staining was stronger than that in the normal adrenocortical cells (Figs. 5, 6).

B. Immunocytochemical observations

1. Normal adrenal gland

In the normal adrenal gland, GSH-PO was localized immunocytochemically in cytoplasm near the electron dense lipid droplets or mitochondria (cytosol GSH-PO) and also in mitochondria with vesicular cristae (mitochondrial GSH-PO). Mitochondrial GSH-PO was mainly localized in lipid droplet depleted inner fasciculata cells (Fig. 7). Cytosol GSH-PO was mainly localized in electron dense lipid laden or
lipofuscin granule laden zona reticularis cells (Fig. 8). Occasionally, cytosol GSH-PO, but not mitochondrial GSH-PO, was localized in zona glomerulosa cells (Fig. 9).

2. Adrenocortical adenomas associated with primary aldosteronism

Ultrastructurally, a large amount of lipid droplets was observed in the cytoplasm of the present adenoma cells. Immunocytochemically, GSH-PO was localized in cytoplasm near the lipid droplets or mitochondria (cytosol GSH-PO) (Fig. 10). However, the number of GSH-PO positive adenoma cells was remarkably decreased. Mitochondrial GSH-PO was

Fig. 7 Immunoelectron microscopic localization of GSH-PO in the adrenocortical cells of the human adrenal gland. GSH-PO is seen in the mitochondria. Peroxidase-labeled antibody method, × 18,000

Fig. 8 Immunoelectron microscopic localization of GSH-PO in the adrenocortical cells of the human adrenal gland. GSH-PO is seen in cytoplasmic matrix (arrows) near the lipid droplets or lipofuscin granules. Peroxidase-labeled antibody method, × 10,000
Fig. 9 Immunoelectron microscopic localization of GSH-PO in adrenocortical cells of the human adrenal gland. In zona glomerulosa cells, GSH-PO is seen in the cytoplasmic matrix (arrows). N: Nucleus, Peroxidase-labeled antibody method, × 10,000.

Fig. 10 Immunoelectron microscopic localization of GSH-PO in adrenocortical adenoma cells associated with primary aldosteronism. GSH-PO is seen in cytoplasmic matrix (arrows) near the lipid droplets or mitochondria.
L: Lipid droplet, N: Nucleus, Peroxidase-labeled antibody method, × 8,000.

Fig. 11 Immunoelectron microscopic localization of GSH-PO in adrenocortical adenoma cells associated with Cushing’s syndrome. GSH-PO is seen in cytoplasmic matrix (arrows) near the mitochondria.
M: Mitochondria, Peroxidase-labeled antibody method, × 15,000.
not seen.

3. Adrenocortical adenoma associated with Cushing's syndrome

Ultrastructurally, the adrenal cells consisted of two types: lipid droplet accumulated cells and lipid droplet depleted cells. Furthermore, a large amount of round mitochondria with tubulo-vesicular cristae was characteristically observed in both adrenocortical adenoma cells. Immunocytochemically, GSH-PO was observed in cytoplasm near the well developed smooth endoplasmic reticulum or round mitochondria (cytosol GSH-PO) (Fig. 11). However, intramitochondrial localization of GSH-PO was not seen.

DISCUSSION

In the present study, we clearly demonstrated the immunocytochemical localization of GSH-PO in normal human adrenocortical cells as well as human adrenocortical adenoma cells associated with primary aldosteronism and Cushing's syndrome. We found two types of GSH-PO, i.e., cytosol GSH-PO and mitochondrial GSH-PO. It was very interesting that cytosol GSH-PO is closely related to the lipofuscin granules, which are generally accepted to be complexes of lipids and proteins with a composition and characteristics indicating that they are derived from lipid peroxidation of polyunsaturated fatty acid of the subcellular membrane (3, 7). Lipid peroxidation is well known as a random free-radical reaction and membranes of mitochondria or endoplasmic reticulum, which are known to contain relatively large amounts of polyunsaturated fatty acids, are very susceptible to lipid peroxidation and concurrent damage (2-4, 12, 20).

On the other hand, contents of superoxide dismutase (SOD), vitamin E and glutathione-peroxidase (GSH-PO) are very high in the adrenal cortex (10), which may reflect the high oxygenase activities in the adrenal cortex. In contrast to SOD and vitamin E which are scavengers of singlet oxygen or free radicals, GSH-PO has been found to effectively reduce lipid peroxides (1).

Our findings and the above facts suggest that cytosol GSH-PO may play an important role in protective effects against cell injury by lipid peroxides induced in the process of steroid hormone synthesis or the cellular aging process. In fact, Wyllie et al. reported that most cell death in the adrenal cortex occurs in the zona reticularis (22) and this zone was also found to have the highest content of pigmented lipids as age pigment.

However, mitochondrial GSH-PO was localized in lipid-depleted compact cells of the inner part of the cortex. It is generally accepted that the zona fasciculata synthesizes cortisol as a final product, and it is also well known as an ACTH dependent zone. We previously reported that the activity of mitochondrial GSH-PO and GSH-PO-positive mitochondria in the rat adrenocortical cells was increased by ACTH administration (17). Thus, lipid peroxidation and subsequent lipid peroxide formation in the mitochondria might occur following administration of ACTH, i.e., enhanced steroidogenesis. In steroidogenesis, the mitochondrial steroid hydroxylase reaction is catalyzed by adrenodoxin, adrenodoxin reductase, cytochrome P-450 11B (steroid 11β-hydroxylase, P-450 11B) and cytochrome P-450 scc (cholesterol side chain cleavage, P-450 scc), which are located on the inner membrane, the intramitochondrial cristae (9, 14). P-450 scc is known as the first step in the conversion of cholesterol to pregnenolone and this step is activated by ACTH stimulation (9). In addition, Smith and Lieberman (21) suggested the appearance of sterol hydroperoxides as an intermediate in the conversion of pregnenolone.

In the present study, mitochondrial GSH-PO was localized in the zona fasciculata cells but not zonae glomerulosa and reticularis cells. P-450 11β activity has also been mainly restricted to the zona fasciculata (9, 14).

The present findings and these facts, strongly suggest that mitochondrial GSH-PO may be one of the most important enzymes for steroidogenesis, especially cortisol synthesis, including P-450 11β.

In the adrenocortical adenoma cells associated with primary aldosteronism, GSH-PO was immunohistochemically weak or negative in lipid-laden adenoma cells. These accumulated lipid droplets have been recognized as aldosterone or steroid hormone precursors, including cholesterol esters (16). In our previous report, accumulated cholesterol esters located in the interior of the cortex of the hypophysec-
tomized rat appear to be highly susceptible to subsequent lipid peroxidation into cholesterol ester hydroperoxides (15). Indeed, GSH-PO was strongly localized in this zone immunohistochemically (15, 17). In contrast, GSH-PO was weak or negative immunohistochemically in zona glomerulosa cells in both human and rat adrenal cortex.

Based on these data, the lipid peroxidation rate in the process of the aldosterone synthesis may be less than that in cortisol synthesis. It may also be assumed from our immunocytochemical studies that primary aldosteronism is derived from zona glomerulosa cells.

In adrenocortical adenoma cells associated with Cushing's syndrome, GSH-PO was localized mainly in lipid-depleted compact cells immunohistochemically. Intracellular localization of GSH-PO was observed only in cytoplasm near the well-developed smooth endoplasmic reticulum or round mitochondria filled with tubulo-vesicular cristae. However, no intramitochondrial GSH-PO was seen. It is generally accepted that adrenocortical adenomas associated with Cushing's syndrome secrete excess amounts of cortisol (11, 19). In such cases, serum ACTH levels have been found to be very low (6, 8). We proposed that, in normal human adrenocortical compact cells, intramitochondrial lipid peroxidation may be induced by cortisol synthesis, including P-450 11β. However, adenoma compact cells differ from normal adrenocortical compact cells, suggesting that the intramitochondrial lipid peroxidation rate is less in adrenocortical adenoma cells than in normal adrenocortical cells. Indeed, ultrastructurally, adenoma compact cells showing exceedingly well developed smooth endoplasmic reticulum and mitochondria and few lipid droplets might be considered as actively secreting elements, in which utilization in synthesis of steroid hormones such as cortisol exceeds its intracellular storage. Thus, steroid hormone precursors may be immediately employed in hormone synthesis and subsequent secretion. Furthermore, intramitochondrially derived free radicals might diffuse across the mitochondrial membrane. As a result, cytosol GSH-PO may be enhanced. These findings appear to correspond to the functional significance of adrenocortical adenoma cells.

The correlation between steroidogenesis and GSH-PO, including lipid peroxidation of cytosol and mitochondria in adrenocortical adenoma associated with Cushing’s syndrome, require further investigation.

REFERENCES
16) Murakoshi M, Osamura Y, Watanabe K, Izumi S, Komatsu N, Nomoto Y, Sakai H: Enzyme...


Arch Path 86: 419-432, 1968.

