INTRAMITOCHONDRIAL LOCALIZATION OF GLUTATHIONE-PEROXIDASE (GSH-PO) IN THE RAT ADRENOCORTICAL CELLS

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Immunocytochemical localization of glutathione-peroxidase (GSH-PO) in the rat adrenocortical cells was observed not only in cytosol (CYTOSOL GSH-PO) but in mitochondria (MITOCHONDRIAL GSH-PO) by the direct peroxidase labeled antibody method using anti-GSH-PO IgG Fab' fragment. In the untreated rat adrenocortical cells, CYTOSOL GSH-PO was observed mainly in the outer part of the zona fasciculata and zona reticularis. MITOCHONDRIAL GSH-PO was observed mainly in the inner part of zona fasciculata. Biochemical GSH-PO activity in the cytosol and mitochondrial fractions obtained from whole adrenal gland was comparable to the immunocytochemical results. In the hypophysectomized rat adrenocortical cells, markedly atrophic inner part of zona fasciculata showed only CYTOSOL GSH-PO, and biochemical activity of the cytosol GSH-PO was markedly increased. It has been reported by us that this atrophic zone contains accumulated cholesterol esters. By ACTH administration to the hypophysectomized rats, markedly hypertrophic outer part of zona fasciculata showed increased immunocytochemical localization of MITOCHONDRIAL GSH-PO. And biochemically, mitochondrial GSH-PO activity in this group was higher than that of the hypophysectomized group.

These results suggest that the CYTOSOL GSH-PO has its role in the reduction of cholesterol ester hydroperoxides and that the MITOCHONDRIAL GSH-PO in the reduction of sterol hydroperoxides produced during steroidogenesis.

In our previous report, immunohistochemical localization of glutathione-peroxidase (GSH-PO) was demonstrated in the rat adrenal cortex (10). It has been suggested that GSH-PO might have a very close relationship to the adrenocortical lipid metabolisms including steroidogenesis. As steroidogenesis occurs in the mitochondria as well as in the cytosol, it has been anticipated that GSH-PO should be present in the adrenocortical mitochondria. In order to confirm the possibility and further to clarify the role of GSH-PO in mitochondria, we studied the biochemical activity of the isolated fractions, i.e., cytosol and mitochondria, and immunocytochemical localization of GSH-PO by direct peroxidase labeled antibody method using IgG Fab' fragment which is known to enable the ultra-

structural localization of the substances in all organelles within the cells (4, 8, 9, 11).

MATERIALS AND METHODS

Animals and their tissue preparation

Male Wistar rats weighing 200 to 250 g were divided into three experimental groups. Group 1 consisted of the untreated controls. In group 2, rats were sacrificed 1 week after hypophysectomy. In group 3, rats were administered intramuscularly 0.5 mg of ACTH (Cortrosyn Z: N. V. Organon, Netherland) for 1 week after hypophysectomy. For the biochemical and immunohisto-cytochemical studies, the adrenal glands were obtained in a fresh state after decapitation.

1. *Biochemical studies*

(A) Preparation of cytosol and mitochondrial fractions;

Fresh adrenal glands were homogenized with 1 ml of mannitol/sucrose solution containing 0.21 M mannitol (Sigma Co.), 0.07 M sucrose, 0.1 mM EDTA, 0.7 mM 2-mercaptoethanol and 5 mM Tris/HCl (pH 7.5) by Potter type homogenizer. The homogenates were centrifuged at 700 g for 10 min and supernatants were recentrifuged at 8,500 g for 15 min. The supernatants and pellets were then carefully removed. The pellets were resuspended in 50 mM Tris/HCl containing 0.1 mM EDTA and 0.7 mM 2-mercaptoethanol, and recentrifuged at 8,500 g for 15 min. Finally, mitochondrial fraction (8,500 g pellets) and cytosol (8,500 g supernatants) were obtained.

(B) GSH-PO activity of cytosol and mitochondrial fractions;

100 μ l of obtained fractions were pre-incubated with 2.3 ml of reaction medium containing 0.1 M Tris/borate buffer (pH 8.5), 3 mM EDTA/Tris, 0.12 mM NADPH (Oriental Yeast Co.), 0.25 mM reduced glutathione (Sigma Co.) and 2.5 μ g of glutathione reductase (Sigma Co.) for 2 min at 37°C. The reaction was then started by the addition of 0.1 ml of cumen hydroperoxide (Matheson Coleman and Bell Manufacturing Chemist, U.S.A.). The decrease of the absorption at 340 nm was measured after 5 min incubation using a Hitachi spectrophotometer model 210. One unit of enzyme oxidized 1 μ mol NADPH to NADP/min at 37°C.

2. *Immunohisto-cytochemical studies*

For immunohisto-cytochemical studies of GSH-PO, the adrenal glands were fixed in periodate-lysine-4% paraformaldehyde solution (7) for 18 to 20 hr at 4°C. After the fixation, the adrenal glands were washed with 0.01 M phosphate buffered saline containing 20% sucrose solution overnight at 4°C. Then the 6 μ m frozen sections were prepared by cryostat and were attached on albumin-coated glass slides. Immunohisto-cytochemical localization of GSH-PO was done by Nakane's direct peroxidase labeled antibody method using anti-GSH-PO IgG Fab' fragment (12).

(A) Immunohistochemical staining;

For light microscopic observations of GSH-PO, 6 μ m frozen sections were incubated with antibody labeled with horseradish peroxidase (HRPO) for 60 min. After the incubation was completed, the sections were incubated in Graham-Karnovsky's reaction medium which contained 3,3'-diaminobenzidine (DAB) (2) and 0.05% H_2O_2 for 10 min. The sections were then counterstained with 1%

methyl green.

(B) Immunocytochemical staining;

For electron microscopic observations of GSH-PO, 6 μ m frozen sections were incubated with HRPO labeled antibody for 6 hr. After the incubation, the sections were fixed in 2% glutaraldehyde solution in 0.1 M sodium phosphate buffer (SPB) pH 7.4, for 20 min. Then the sections were pre-incubated for 30 min in DAB solution without H_2O_2 . After this pre-incubation, the sections were incubated in DAB solution containing 0.05% H_2O_2 for 5 min. Subsequently, the sections were post-fixed with 2% OsO_4 in 0.1 M SPB for 60 min, dehydrated in graded alcohols and embedded in Epon 812 by inverted gelatin capsule method. Ultrathin sections were obtained by LKB ultra-microtome and were investigated with a JEOL 100-C electron microscope without lead citrate and uranyl acetate staining.

As an immunologic negative control, normal rabbit serum (NRS) IgG Fab' fragment labeled with HRPO was applied on both light and electron microscopic observations instead of anti-GSH-PO antibody IgG Fab' fragment labeled with HRPO.

RESULTS

Biochemical studies

(1) GSH-PO activity of the isolated cytosol and mitochondrial fractions

GSH-PO activity of the cytosol and mitochondrial fractions are shown in Table 1. Regarding the ratio of the activity of the cytosol GSH-PO to mitochondrial GSH-PO, the normal untreated group showed about equal GSH-PO activity in the cytosol and mitochondrial fractions. In the hypophysectomized group, the cytosol GSH-PO activity was higher than that in the other experimental groups. By ACTH administration to the hypophysectomized group, the mitochondrial GSH-PO activity was stronger than that in the hypophysectomized group, but the cytosol GSH-PO activity was weaker than that in the hypophysectomized group.

Immunohisto-cytochemical studies

(1) Immunohistochemical studies

In the normal untreated group, GSH-PO was immunohistochemically localized in zonae fasciculata and reticularis in the rat adrenal cortex (Fig. 1). The control serum (NRS) was negative for immunohistochemical localization of GSH-PO in the adrenal cortex (Fig. 2). In the hypophysectomized group, the adrenal cortex was markedly atrophic. Immunohistochemically, GSH-PO was localized in this

TABLE 1. *GSH-PO activities of cytosol and mitochondrial fractions in the rat adrenal gland*

Condition	Fractions		Ratio of GSH-PO	
	Cytosol (u/mg protein)	Mitochondria (u/mg protein)	Mitochondria/ Cytosol	Cytosol/ Mitochondria
Normal untreated	0.277 $\pm$ 0.005	0.284 $\pm$ 0.011	1.02	0.98
Hypophysectomy	0.275 $\pm$ 0.011	0.099 $\pm$ 0.005	0.04	27.78
Hypophysectomy + ACTH administration	0.229 $\pm$ 0.004	0.121 $\pm$ 0.009	0.53	1.90

FIG. 7. Immunocytochemical localization of GSH-PO in normal untreated rat adrenocortical cells. Inner fasciculata cell GSH-PO is observed in mitochondria. Bar = 1 μ m, $\times$ 16,000

atrophic cortical zones, i.e., inner part of zona fasciculata (Fig. 3). By ACTH administration to the hypophysectomized group, the adrenal cortex was markedly hypertrophic. The most hypertrophic zone was observed in outer part of zona fasciculata. Immunohistochemically, GSH-PO was localized in these hypertrophic zona fasciculata cells (Fig. 4).

FIG. 1. Immunohistochemical localization of GSH-PO in normal untreated rat adrenal gland. GSH-PO is observed in zonae fasciculata (F) and reticularis (R) in the adrenal cortex. Peroxidase-labeled antibody method. $\times$ 85

FIG. 2. By control serum (NRS), no immunohistochemical reaction is observed in the adrenal gland. F: Zona fasciculata, R: Zona reticularis, Peroxidase-labeled antibody method. $\times$ 85

FIG. 3. Immunohistochemical localization of GSH-PO in hypophysectomized rat adrenal gland. The adrenal cortex is markedly atrophic and most atrophic cortical zone is observed in inner part of zona fasciculata and zona reticularis. GSH-PO is observed in these atrophic cortical zones (arrows). M: Medulla, Peroxidase-labeled antibody method. $\times$ 150

FIG. 4. By ACTH administration to the hypophysectomized rat, the adrenal cortex is markedly hypertrophic and most hypertrophic cortical area is observed in outer part of zona fasciculata. Immunohistochemically, GSH-PO is observed in this hypertrophic zone cells. Peroxidase-labeled antibody method. $\times$ 150

FIG. 5. Immunocytochemical localization of GSH-PO in normal untreated rat adrenocortical cells. GSH-PO is observed in cytosol around the electron dense lipid droplets (L) or mitochondria with vesicular cristae (M) (arrows). [outer fasciculata cell] Bar = 1 μ m, $\times$ 20,000

FIG. 6. Immunocytochemical localization of GSH-PO in normal untreated rat adrenocortical cells. GSH-PO is observed in cytosol around the mitochondria with tubulo-vesicular cristae (M) or electron dense lipid droplet (L) (arrows). [zona reticularis cell] Bar = 1 μ m, $\times$ 21,000

FIG. 8. Immunocytochemical localization of GSH-PO in hypophysectomized rat adrenocortical cells. GSH-PO is observed in cytosol around the mitochondria (M), accumulated lipid droplets (L) or degenerated organelles (arrows). Bar = 1 μ m, $\times 15,000$

FIG. 9. By ACTH administration to the hypophysectomized rat, immunocytochemical localization of GSH-PO is observed in the mitochondria (M) and number of GSH-PO positive mitochondria is increased remarkably. Bar = 1 μ m, $\times$ 20,000

(2) Immunocytochemical studies

In the normal untreated group, intracellular localization of GSH-PO was observed not only in cytosol (cytosol GSH-PO) around the mitochondria or electron dense lipid droplets but also in mitochondria (mitochondrial GSH-PO) in the rat adrenocortical cells. The cytosol GSH-PO was mainly observed in electron dense lipid laden cells, that is, the outer part of zona fasciculata and zona reticularis cells (Figs. 5, 6). On the other hand, mitochondrial GSH-PO was mainly observed in lipid depleted cells, that is, the inner part of zona fasciculata cells (Fig. 7). In the hypophysectomized group, intracellular localization of GSH-PO was observed only in cytosol around the accumulated lipid droplets or degenerated organellae (Fig. 8). While, no mitochondrial GSH-PO was seen in the adrenocortical cells of this group. By ACTH administration to the hypophysectomized group, intracellular localization of GSH-PO was observed in the mitochondria. The numbers of GSH-PO positive mitochondria were increased remarkably compared to those in the normal untreated group (Fig. 9).

DISCUSSION

In our present study, it was demonstrated that immunocytochemical localization of GSH-PO in the rat adrenocortical cells were observed not only in cytosol (cytosol GSH-PO) but in mitochondria (mitochondrial GSH-PO) by direct peroxidase labeled antibody method using anti-GSH-PO IgG Fab' fragment. Within the rat adrenocortical cells, immunocytochemically, cytosol GSH-PO was localized around the lipid droplets or mitochondria and mitochondrial GSH-PO was observed around the inner membrane, that is, intramitochondrial cristae. In addition, we found that immunocytochemical localization of GSH-PO exhibited special zonal distribution under the present experimental conditions.

In the normal untreated rat adrenocortical cells, cytosol GSH-PO was observed mainly in lipid laden clear cells and mitochondrial GSH-PO was mainly observed in lipid depleted compact cells. Biochemically, GSH-PO activity in the cytosol fraction was similar to that of mitochondrial fraction. Hypophysectomy induced the adrenocortical atrophy, especially the inner part of the zona fasciculata. These atrophic adrenocortical clear cells showed lipid accumulation including cholesterol esters. In this condition, immunocytochemically, GSH-PO was localized in the cytosol but no mitochondrial GSH-PO was seen. In fact, biochemical activity of GSH-PO in the mitochondrial fraction was markedly decreased. The possible role of cytosol GSH-PO was described in the previous report (10). It was further speculated that accumulated cholesterol esters may easily cause subsequent lipid peroxidation into cholesterol ester hydroperoxides. Therefore, we propose that the role of cytosol GSH-PO might be reduction of excess amount of accumulated cholesterol ester hydroperoxides.

By ACTH administration to the hypophysectomized rat, the adrenal cortex showed markedly hypertrophic, especially outer part of zona fasciculata. Immunocytochemically, GSH-PO in this condition was observed in mitochondria. And the number of GSH-PO positive mitochondria were increased compared to those in the normal untreated group. This fact suggest that mitochondrial GSH-PO was induced by ACTH administration. It is well known that ACTH is an activator

for steroidogenesis by the conversion of cholesterol into pregnenolone (3). This conversion occurs in the mitochondria and this reaction is catalyzed by adrenodoxin, adrenodoxin reductase and cytochrome P-450 scc (1). Recently, Mitani *et al.* reported that intramitochondrial localization of the above enzymes was demonstrated by peroxidase labeled antibody method applied on the bovine adrenocortical cells (8, 9). In addition, Izumi *et al.* reported that the number of adrenodoxin and adrenodoxin reductase positive mitochondria was increased with ACTH administered conditions applied on rats by the same technique (4). In our previous report, sterol hydroperoxides were detected in the mitochondrial fraction and it was suggested that adrenal mitochondrial GSH-PO would play at least in part, an important role in steroidogenesis (10). Our biochemical and immunocytochemical results indicate that the possible role of mitochondrial GSH-PO in the rat adrenocortical cells is to catalyze the reduction of the sterol hydroperoxides such as steroid intermediate to its metabolizable form for steroidogenesis (5, 6). Selective staining of GSH-PO for the adrenocortical mitochondria suggests various functional activities among the mitochondria.

In summary, the possible role of adrenocortical GSH-PO is proposed as follows, [A] reduction of cholesterol ester hydroperoxides induced by cell injury (hypophysectomy, CYTOSOL GSH-PO) [B] reduction of sterol hydroperoxides produced during steroidogenesis (ACTH administration, MITOCHONDRIAL GSH-PO).

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