Immunoelectron Microscopy Pinpoints Glutathione-Peroxidase (GSH-PO) in Neonatal Rat Adrenal Cortical Cells

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ABSTRACT. Localization of glutathione-peroxidase (GSH-PO) in adrenal cortical cells of neonatal rats were determined by immunocytochemical analysis. GSH-PO first appeared 14 days after birth. Intracellular localization of GSH-PO was mainly in cytosol (cytosol GSH-PO) but no intramitochondrial localization of GSH-PO was detected. Twenty-one days after birth, intramitochondrial localization of GSH-PO (mitochondrial GSH-PO) was present. Mitochondrial GSH-PO depends on ACTH stimulation; therefore, its presence in the adrenal cortical cells of neonatal rats may be the morphologic expression of the gradual acquisition of adult metabolic features during steroidogenesis. The intracellular GSH-PO staining pattern in adrenal cortical cells therefore should be a useful marker for steroidogenesis.

In adrenal cortical cells of the rats glutathione-peroxidase (GSH-PO), which effectively reduces lipid peroxides induced in some processes of steroidogenesis, is localized both in the cytosol (cytosol GSH-PO) and in the mitochondria (mitochondrial GSH-PO) (6–8). Both the cytosol GSH-PO and mitochondrial GSH-PO in these adrenal cortical cells are closely related to the functional state of the cells, especially of steroidogenesis (6–8).

To determine the functional significance of GSH-PO, including cytosol GSH-PO and mitochondrial GSH-PO, we studied the immunocytochemical localization of GSH-PO in adrenal cortical cells of neonatal rats.

MATERIALS AND METHODS

1, 3, 5, 7, 10, 12, 14, 16, 18, 21, and 28 days old male Wistar Imamichi rats were used. The rats were killed by decapitation, and their adrenal glands removed immediately. These glands were fixed in periodate lysine 4% paraformaldehyde solution (3) for 18 to 20 h at 4°C, then the fixed tissues were washed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing sucrose 10 to eventual 20% overnight at 4°C. Frozen sections (6 μm) were cut with a cryostat and placed on albumin-coated glass slides, after which they were washed in 0.01 M PBS then stained by Nakane’s direct peroxidase-labeled-antibody method using the anti-GSH-PO IgG Fab fragment (10).

For light microscopy, 6 μm frozen sections were incubated with the antibody labeled with horseradish peroxidase (HRPO) for 1 h. After incubation, the sections were incubated

Abbreviations used: GSH-PO, glutathione-peroxidase: ACTH, adrenocorticotropic hormone.
for 10 min in Graham-Karnovsky's reaction medium (2) which contained 3,3'-diaminobenzidine (DAB) and 0.005 % H$_2$O$_2$. The sections then were counter-stained for nuclei with 1 % methyl green.

For electron microscopy, 6 µm frozen sections were incubated with HRPO-labeled antibody for 6 h, then fixed for 20 min in 2 % glutaraldehyde in 0.1 M sodium phosphate buffer (SPB, pH 7.4). After incubation in DAB solution for 30 min, the sections were incubated for 5 min in DAB solution containing 0.005 % H$_2$O$_2$. After post-fixed for 90 min with 2 % OsO$_4$ in 0.1 M SPB, they were dehydrated in graded alcohols and embedded in Epon 812 by the inverted gelatin capsule method. Ultrathin sections were cut on an LKB ultra-microtome and were examined in a JEOL 100-C electron microscope without lead citrate and uranyl acetate staining.

Normal rabbit serum (NRS) IgG Fab labeled with HRPO instead of anti-GSH-PO IgG Fab labeled with HRPO was used as the immunologic negative control for both the light and electron microscopy observations.

RESULTS

Immunohistochemical localization of GSH-PO. In rat adrenal glands, GSH-PO first appeared on the 14th day after birth, mainly in the inner layer of the cortex, no

Fig. 1. Immunohistochemical localization of GSH-PO in the adrenal gland of a 14-day-old rat. GSH-PO is present in adrenal cortical cells in the inner layer of the cortex (arrow). No reaction products are present in the medulla (M). Peroxidase-labeled antibody method, ×400

Fig. 2. Immunohistochemical localization of GSH-PO in the adrenal gland of a 21-day-old rat. GSH-PO is present in the zonae fasciculata (F) and reticularis (R) of the cortex. M: Medulla, Peroxidase-labeled antibody method, ×400. (inset) Higher magnification of Fig. 2. The reaction products are present in the cytosol as granules. Peroxidase-labeled antibody method, ×1,500
Fig. 3. Immunocytochemical localization of GSH-PO in adrenal cortical cells of a 14-day-old rat. GSH-PO is present in the cytosol near round mitochondria with vesicular cristae (M). L: Lipid droplets, Bar—1 μm, ×20,000

Fig. 4. Immunocytochemical localization of GSH-PO in adrenal cortical cells of a 21-day-old rat. GSH-PO is present in the mitochondria (arrows). Bar—1 μm, ×23,000
reaction products were present in the medulla (Fig. 1). Thereafter, the number of GSH-PO positive adrenal cortical cells increased steadily. On the 21th day after birth, immunohistochemical staining of GSH-PO was clearly detected in the zonae fasciculata and reticularis of the cortex (Fig. 2). This zonal distribution of GSH-PO in the adrenal cortex is similar to that of adult rats (9).

**Immunocytochemical localization of GSH-PO.** In adrenal cortical cells of 14-day-old rats, GSH-PO was localized in the cytosol (cytosol GSH-PO) near round mitochondria with vesicular cristae (Fig. 3), no reaction products were present in the other cellular components including the mitochondria and endoplasmic reticulum. This cytosol GSH-PO in the adrenal cortical cells increased steadily thereafter. Intramitochondrial localization of GSH-PO (mitochondrial GSH-PO) was present in the adrenal cortical cells of 21-day-old rats (Fig. 4). Within the mitochondria, the reaction products were present only in the matrix. In mitochondrial GSH-PO-positive cells, cytosol GSH-PO was rare. Thus, the intracellular localization of GSH-PO in a single adrenal cortical cell differed from one to another, some were cytosol GSH-PO and others were mitochondrial GSH-PO.

**DISCUSSION**

The horseradish peroxidase (HRPO)-labeled antibody method has been used to determine the localization of intramitochondrial proteins, such as adrenodoxin, adrenodoxin reductase, cytochrome P450_{11\alpha}, cytochrome P450_{scc} and GSH-PO (4-8), in 6 μm frozen sections prepared from properly fixed tissue and HRPO-labeled Fab fragments. This technique shows the ultrastructural localization of these substances in all the organelles of the cell, including the mitochondria (4-8).

Previously, we demonstrated using immunocytochemical techniques that the GSH-PO in the adrenal cortical cells of adult rats is localized both in the cytosol (cytosol GSH-PO) and mitochondria (mitochondrial GSH-PO) (6-8). The number of GSH-PO-positive mitochondria markedly increased when ACTH was administered to normal or hypophysectomized rats (6). From this, we postulated that the mitochondrial GSH-PO in adrenal cortical cells may be ACTH dependent (6).

We here have reported that the GSH-PO in the adrenal cortical cells of neonatal rats is present both in the cytosol (cytosol GSH-PO) and mitochondria (mitochondrial GSH-PO). We also found that cytosol GSH-PO appeared when the rats were 14 days old and mitochondrial GSH-PO when they were 21 days old. The intracellular localization of GSH-PO differed from one adrenal cortical cell to another, some had cytosol GSH-PO and others mitochondrial GSH-PO. This heterogenous staining for GSH-PO in adrenal cortical cells has been reported in adult rats by us elsewhere (6).

Cytosol GSH-PO in adult rats is found mainly in lipid-laden clear cells and is intensily stained under low steroidogenic conditions including hypophysectomy (6). In our present study, cytosol GSH-PO appeared in the adrenal cortical cells of neonatal rats when they were 14 days old. No mitochondrial GSH-PO was detected at this age, therefore, the adrenal cortical mitochondria of 14-day-old rats must not be able to produce steroidogenesis. Takeuchi et al. (11) reported that during the second week of life the serum corticosterone contents in rats begin to increases, reaching adult levels about 25 days after birth. In addition, Butte et al. (1) showed that the functional activity of the pituitary-adrenal axis increases from day 11 to
near normal adult activity day 21 after birth. From our findings and these facts, adrenal cortical steroidogenesis before the 21st day after birth appears to be functionally immature or imperfect. We also speculate that the lipid peroxidation induced in some processes of steroidogenesis takes place in the cytosol or microsome, but not in mitochondria at an early age.

In our study reported here, mitochondrial GSH-PO appeared in the adrenal cortical cells of 21-day-old neonatal rats. As stated above mitochondrial GSH-PO depends on ACTH stimulation; i.e., active steroidogenesis (6). Therefore, the mitochondrial GSH-PO in the adrenal cortical cells of neonatal rats may be a morphologic expression of the gradual acquisition of adult features of steroidogenesis. In addition, the selective staining of GSH-PO for the adrenal cortical mitochondria was observed. A similar heterogeneity for mitochondria has been noted for adrenodoxin, adrenodoxin reductase, cytochrome P45011,9 and cytochrome P450 scc (4, 5). The ratio of stained to unstained mitochondria with respect to adrenodoxin, adrenodoxin reductase and GSH-PO varied with the physiological condition of the animals (4, 6). These results suggest that there may be functional heterogeneity among mitochondria within a cell, and that the physiological state dictates the degree of heterogeneity.

We conclude that the immunocytotoxicchemical localization of GSH-PO in the adrenal cortical cells of neonatal rats well reflects the functional state of the cells. Therefore, the intracellular GSH-PO staining pattern in adrenal cortical cells should be a useful marker for steroidogenesis.

REFERENCES


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