Electron Microscopic Immunocytochemistry on the Colocalization of ATP Synthase and Cytochrome P-450 of Side Chain Cleavage Enzymes in Mitochondria of Rat and Bovine Adrenal Cortical Cells

Tomoko YOSHINAGA-HIRABAYASHI¹, Hideaki TSURI¹, Kazunori ISHIMURA² and Tadao HASHIMOTO³

Departments of Anatomy¹ and Physiological Chemistry³, Osaka University Medical School, Suita; and Department of Anatomy², Tokushima University School of Medicine, Tokushima, Japan

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Summary. To clarify whether ATP synthase and side chain cleavage enzymes (SCC) can coexist in a mitochondrion of the adrenal cortical cell, and whether the functional heterogeneity of mitochondria can occur in an adrenal cortical cell, the immunostainability of these two enzymes in mitochondria was compared by electron microscope using pre- and post-embedding methods.

In the pre-embedding method, the inner membrane of most mitochondria in the adrenal cortical cell was positively stained for SCC, 11 β -hydroxylase, and ATP synthase, but among these immunopositive mitochondria we often observed negatively immunoreacted ones in the same adrenal cortical cell. In the positively stained mitochondria, immunoreaction products were evenly localized along the inner mitochondrial membrane. We therefore think that these differences in the immunostainability are caused by technical artifacts, namely the inadequate penetration of the antibody.

In the post-embedding immunocytochemistry, gold particles showing the presence of the enzymes were observed on all mitochondria. Two different antibodies, anti-ATP synthase antibody and anti-cytochrome P-450_{scc} antibody, which were labelled with gold particles of varying diameter (5 nm, 10 nm each) could be observed on all the mitochondria of the bovine adrenal cortical cells. No heterogeneities as to the stainability for both ATP synthase and SCC were detected in mitochondria of the rat and bovine adrenal cortical cells.

These results indicate that in the rat and bovine adrenal cortical cells, both ATP synthase and SCC are evenly and simultaneously present on the inner membrane of all mitochondria. Hence, mitochondria in the adrenal cortical cell seem to be homogeneous in their steroid and ATP synthesizing abilities.

The steroid secretory cells possess several kinds of enzymes in their mitochondria which are essential to the steroid biosynthesis, such as side chain cleavage enzymes (SCC) and 11 β -hydroxylase.

We previously made a pre-embedding immunocytochemical study on the localization of cytochrome P-450 of SCC and cytochrome P-450 of 11 β -hydroxylase (ISHIMURA et al., 1985), and found that these were present on the inner mitochondrial membranes of bovine adrenal cortical cells. This study also revealed the occurrence of two types of mitochondria, immunopositive and immuno-negative, for these enzymes. This led us to believe that the negatively stained mitochondria might be brought about by an inadequate penetration of the antibody, because both immunopositive and negative mitochondria showed no difference in the structure of cristae, a characteristic reflecting the functional state of mitochondria. However, MITANI et al. (1982), who observed a similar phenomenon, attributed the difference in immunostainability of the mitochondria to some functional heterogeneity.

It is well known that the most important and fundamental role of mitochondria is the supply of ATP which is synthesized by ATP synthase (ATP synthesizing enzyme). This leads to the interesting problem of whether or not the mitochondria of the adrenal cortex have the functional heterogeneity as to the production of ATP and synthesis of steroid hormones.

The present study aims to clarify the distribution of ATP synthase and SCC in mitochondria of rat and

bovine adrenal cortical cells using both pre- and post-embedding immunocytochemical methods.

Fig. 1. a and b. Specificity of anti-ATP synthase antibody. **a.** Microsomal fraction of rat liver tissues is subjected to SDS-polyacrylamide gel electrophoresis. Proteins separated on gels are stained with Coomassie brilliant blue. **b.** Proteins separated under the same condition are transferred to nitrocellulose paper. Subsequently ATP synthase on the paper is labeled with 0.5% (v/v) anti-ATP synthase antibody and peroxidase conjugated goat anti-rabbit IgG. ATP synthase is demonstrated by the peroxidase reaction with DAB and H_2O_2 . The present immunoblotting examination shows that the antiserum we used specifically reacts with α , β , and γ subunits of ATP synthase.

MATERIALS AND METHODS

Young male Wistar rats were anesthetized and perfused *via* the left heart ventricle with 4% paraformaldehyde solution buffered at pH 7.4 with Millonig's phosphate, and adrenal glands and liver were excised. The organs were cut into small pieces and immersed in the same fixative for 12 h at 4°C.

Bovine adrenal glands were obtained at a local slaughterhouse. The glands were cut out within 20 min after death, cut into small pieces and immersed in the same fixative solution mentioned above.

For pre-embedding immunocytochemistry, after the fixation, the tissue samples were rinsed in phosphate buffered saline (PBS) solution containing 10%, 15% and 20% sucrose 3–6 h, respectively. Frozen sections cut in an Ames cryostat II (Miles) were mounted on albumin-coated glass slides and incubated in the first antibodies (rabbit antiserum against ATP synthase (1 : 500), anti-cytochrome P-450scc (1 : 100) (OXYgene, Dallas), anti-cytochrome P-450_{11 β} (1 : 100) (a gift from Dr. M. OKAMOTO) overnight at 4°C, treated with horse raddish peroxidase (HRP)-conjugated anti-rabbit IgG (1 : 100) for 4 h at room temperature, and then colored in a medium containing 0.005% 3–3' diaminobenzidine and 0.03% H_2O_2 for 5–10 min at room temperature. The sections were postfixied in 1% OsO_4 solution buffered at pH 7.4 with Millonig's phosphate for 30 min, stained with 2% uranyl acetate solution, dehydrated in a series of graded concentrations of ethanol, and embedded in Epon epoxy resin. Ultrathin sections cut in a Reichert Ultracut N were observed without staining in a JEOL 1200EX electron microscope.

For post-embedding immunocytochemistry, tissues cut into small pieces were immersed in 4% paraformaldehyde solution for 2 h at 4°C. After the fixation, the tissue samples were dehydrated in ethanol cooled to –20°C, and embedded in Lowicryl K4M

Fig. 2 a and b. Pre-embedding electron microscopic immunocytochemistry of SCC (**a**) and 11 β -hydroxylase (**b**) of bovine adrenal cortical cell. Immunoreaction products for the enzymes are localized on the inner membrane of some mitochondria (*M*). Weakly or negatively stained mitochondria (*) can be observed. a: $\times 40,200$, b: $\times 44,300$

Fig. 3 a and b. Electron microscopic immunocytochemistry of ATP synthase of rat adrenal cortical cell. **a.** In the pre-embedding method, immunoreacted products are observed on the inner membrane of mitochondria (*M*). A weakly reacted mitochondrion (*) can be observed. **b.** In the post-embedding method, gold particles (10 nm in diameter) are localized on all mitochondria (*M*) of varying shape. *N* nucleus, *L* lipid droplet. a: $\times 23,800$, b: $\times 68,000$

Fig. 4 a and b. Post-embedding immunocytochemistry with double staining. Large gold particles (10 nm) indicate the localization of cytochrome P-450scc (arrows), and small gold particles (5 nm) show the localization of ATP synthase (arrow heads). Both sizes of gold particles are localized on the all mitochondria (*M*). *SER* smooth endoplasmic reticulum. a: $\times 30,000$, b: $\times 90,000$

Figs. 2-4. Legends on the opposite page.

at -20°C . Ultrathin sections mounted on nickel grids were incubated in the first antiserum, anti-cytochrome P-450_{scc} (1:100) (OXYgene, Dallas) or anti-ATP synthase (1:500), for 4 h at room temperature, then treated with colloidal gold (10 nm in diameter) conjugated anti-rabbit IgG for 2 h at room temperature. For double immunostaining, one side of each thin section mounted nickel grid was treated with P-450_{scc} antiserum, then with the second antibody labeled with colloidal gold (10 nm in diameter). The other side of the grid was further treated with anti-ATP synthase antibody followed by incubation with the second antibody labeled with smaller colloidal gold (5 nm in diameter). They were then examined in a JOEL 1200 EX electron microscope.

The isolation and purification method of liver mitochondrial ATP synthase has been described elsewhere (HASHIMOTO et al., 1981). The specificity of anti-ATP synthase antibody was determined using the immunoblot method as follows. The mitochondrial fraction of rat liver was subjected to SDS-polyacrylamide gel electrophoresis according to the method of LAEMMLI (1970). Proteins separated on the gels were stained with Coomassie brilliant blue (Fig. 1a). Proteins separated under the same condition were blotted on a nitrocellulose paper as described by TOWBIN et al. (1979). Then ATP synthase on the paper was labelled with 0.5% (v/v) anti ATP synthase antibody and peroxidase-conjugated goat anti-rabbit IgG. Three subunits of ATP synthase (α , β and γ) were demonstrated by the peroxidase reaction with DAB and H_2O_2 (Fig. 1b).

RESULTS

Pre-embedding immunocytochemistry

In bovine and rat adrenal cortical cells processed with the pre-embedding method, some mitochondria were positive for immunostaining of ATP synthase, SCC, and 11β -hydroxylase, while others were negative. In the immunopositive mitochondria, reaction products were evenly localized along the matrix side of the inner membranes of mitochondria (Figs. 2a, b, 3a). Though each adrenal cortical cell had both positively and negatively stained mitochondria, no prominent ultrastructural differences were recognized between these mitochondria, which have not lamellar but tubulovesicular cristae. Other organelles such as smooth endoplasmic reticulum, Golgi apparatus and lipid droplets were entirely negative for the immunostaining. In control sections, the immunoreaction was completely negative.

The antibodies, anti-cytochrome P-450_{scc} and anti-cytochrome P-450_{11 β} , cross-reacted with bovine adrenal gland cells, but did not have any cross-reactivity with rat adrenal gland cells.

Post-embedding immunocytochemistry

Gold particles showing the presence of ATP synthase were localized on all mitochondria of rat adrenal cortical cells, regardless of the shape of mitochondria (Fig. 3b). In the double immunostaining, both large (10 nm) and small (5 nm) gold particles, showing the presence of side chain cleavage enzyme (SCC) and ATP synthase, respectively, were present on all the mitochondria of bovine adrenal cortical cells (Fig. 4a, b).

DISCUSSION

One of the important roles of mitochondria is the production of ATP. The synthesis of this compound, known to be catalyzed by ATP synthase is the ATP synthesizing enzyme. This enzyme is made up of two major parts: F_0 and F_1 . It is well known that the F_1 part is composed of five subunits, α , β , γ , δ and ϵ . In this study, we used an antiserum which specifically recognizes α , β , and γ subunits. Using this antiserum, we found that α , β , and γ subunits were evenly present along the inner mitochondrial membrane of some mitochondria by pre-embedding method, and also present on all the mitochondria of the adrenal cortical cells by the post-embedding method. In addition, both ATP synthase, which is a general enzyme to all the cells, and SCC, which is a special enzyme to steroid producing cells, are colocalized in all the mitochondria of the bovine adrenal cortical cells.

In our previous pre-embedding immunocytochemical study on the localization of cytochrome P-450_{scc} and P-450_{11 β} , we found two types of mitochondria in a bovine adrenal cortical cell, positively stained mitochondria and negatively stained ones (ISHIMURA et al., 1985). MITANI et al. (1982) attributed this different stainability to the functional heterogeneity of mitochondria. However, we believed the results could be brought about by differences in antibody penetration, because no ultrastructural differences between positively and negatively stained mitochondria were detected. In the frozen section, some mitochondria maintained an intact form and intact mitochondrial membrane, but others did not. The antibodies (anti-cytochrome P-450_{scc} antibody, anti-ATP synthase antibody, anti-cytochrome P-450_{11 β} antibody, and HRP-conjugated antibodies) have to pass two mitochondrial membranes (inner- and outer-mitochondrial

membranes) to reach the antigens on the matrix side of the mitochondria. The antibodies barely penetrate the intact mitochondrial membrane, but easily come into the mitochondrial matrix through the broken portion of membranes. The penetration ability of antibodies has considerable influence on the immunostainability, and seems to follow an all-or-nothing law. In the post-embedding method, ultra-thin sections are so thin that the cutting parts of the intracellular organs including mitochondria are exposed to the surface of the section. Each antibody can easily react to each antigen on the surface of the inner-mitochondrial membrane, so that all the mitochondria are positively stained for the antibodies. Several years ago, GUEZE et al. (1987) demonstrated with the post-embedding method that cytochrome P-450scc and P-450_{11β} were equally localized in the same mitochondria. This means that no heterogeneity is present in mitochondria of the adrenal cortical cells as to the steroid synthesizing ability. Biochemical studies have reported that most of mitochondrial enzymes such as cytochrome P-450scc, P-450_{11β}, and many subunits of ATP synthase are synthesized on the ribosomes in the cytoplasm and then carried into mitochondria (SCHATZ and MASON, 1974; ANDERSON et al., 1981). Hence, it is more likely that all mitochondria of steroid producing cells are homogenous as to biosynthesis of steroid hormones and ATP. The present results clearly show that mitochondria of the adrenal cortical cell are homogenous in their ability for steroid synthesis and ATP production.

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平林 朋子
565 吹田市山田丘 2-2
大阪大学医学部
第三解剖学教室

Dr. Tomoko HIRABAYASHI
Department of Anatomy
Osaka University Medical School
2-2 Yamadaoka, Suita
565 Japan