REACTION SITES BY CERIUM-CYTOCHEMISTRY FOR THE HYDROGEN-PEROXIDE GENERATING ENZYMES IN THE MONGOLIAN GERBIL PAROTID GLAND

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In order to cytochemically demonstrate the endogenous glandular source of hydrogen-peroxide (H$_2$O$_2$), which is a component of the salivary peroxidase antimicrobial system, we applied the cerium method to the Mongolian gerbil parotid gland. When NADH and NADPH were used as substrates of the H$_2$O$_2$ generating enzyme, reaction products were observed in association with the plasma membrane and pinocytotic vesicles of the myoepithelial cell. No reaction product was formed in the absence of these substrates. The reaction was inhibited by the addition to the complete medium of catalase (H$_2$O$_2$ scavenger) or of p-benzoquinone (O$_2$ scavenger), or by heating prior to incubation. These results suggest that NADH- and NADPH-dependent H$_2$O$_2$ generating oxidase may be present in the myoepithelial cell. In addition, electron-dense precipitates, formed by the reaction of cerois ions, were also observed on the outer surface of the luminal plasma membrane of acinar and intercalated duct cells, and in the cristae and outer compartment of mitochondria mainly of duct cells. However, these reaction products were formed whether the substrates were contained in the cerium medium or not. Since the reaction was also weakened by catalase or p-benzoquinone, or by preheating, it is likely that some endogenous H$_2$O$_2$-generating factors, independent of the exogenous substrates, may exist in these sites.

Hydrogen peroxide (H$_2$O$_2$) is an active oxygen species which is toxic for living cells. However, it is known to be useful to several biophylaxis, such as the antibacterial system of myeloperoxidase-halide-H$_2$O$_2$ (17), or to the biosynthesis of thyroid hormones (9) or prostaglandins (21). In the oral cavity, it has been shown that the salivary peroxidase system, consisting of peroxidase enzyme, H$_2$O$_2$ and thiocyanate ion (SCN$^-$), contributes to the defense against toxic accumulation of H$_2$O$_2$ and to the antibacterial activity of saliva (6, 26, 27). That is to say, salivary peroxidase detoxifies H$_2$O$_2$ in the presence of SCN$^-$ by converting it into hypoiodocyanate ion (OSCNo$^-$), dioxygen and water. In addition, OSCNo$^-$ inhibits the growth and metabolism of oral pathogens.

It has been reported that H$_2$O$_2$ in the salivary peroxidase system is derived from leukocytes or oral bacteria primarily streptococci (6, 26). However, Pruitt et al. (22) suggested, by showing the presence of OSCN$^-$ in healthy human parotid saliva devoid of bacteria and leukocytes, that there is an endogenous glandular source of H$_2$O$_2$.

Cytochemical detection of H$_2$O$_2$ generation in living cells has been demonstrated, mainly on the plasma membrane of polymorphonuclear leukocyte (20), since Briggs et al. (5) devised the cerium method in which cerois ions trap H$_2$O$_2$ generated by oxidases. Cytochemical demonstration of H$_2$O$_2$ generation on the plasma membrane or in the peroxisome has also been reported by the cerium method in some epithelial tissues containing catalase or peroxidase, e.g. the thyroid gland (19), the liver (18, 30), the small intestine (14), the uterus (15) and the kidney (2). It has been suggested that several H$_2$O$_2$ generating oxidases may be involved in H$_2$O$_2$ generation in these epithelial tissues, such as NAD(P)H oxidase on the plasma membrane (14, 15, 18, 19) or D-amino acid oxidase in peroxisome (30).

In the rat parotid gland, Hand (13) reported the presence of peroxisome by catalase cytochemistry. However, there was up to this point no report cytochemically demonstrating the H$_2$O$_2$ generation system in the salivary gland. Therefore, in the present study, we applied the cerium method in order to cytochemically investigate the generating site of H$_2$O$_2$ in the salivary gland.
MATERIALS AND METHODS

Animals
Adult Mongolian gerbils (Meriones unguiculatus) weighing 60-80 g were used in this investigation. The animals were bred by the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Fixation and Cytochemistry
After the animals had been lightly anesthetised with ether, the parotid gland was excised. Small pieces of the tissues were immediately fixed for 1 hr in a cold 1% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4. The fixed tissue blocks were washed several times in the buffer and stored overnight. The blocks were then sliced into 50 μm thick sections with a microslicer (Dosaka EM, Kyoto, Japan). For the demonstration of H₂O₂ generating sites, the cerium method based on the method of Briggs et al. (5) was performed. The tissue sections were pre-incubated for 30 min at 37°C in 0.05 M Tris-maleate buffer, pH 7.5, containing 10 mM 3-amino-1,2,4-triazole and 2 mM CeCl₃, and incubated for 1 hr at 37°C in the same medium containing the following substrates for H₂O₂ generating oxidase: 0.8 mM NADH (No. N-8129, Sigma Chemical Co., St. Louis, MO) for NADH oxidase; 0.8 mM NADPH (No. N-7505, Sigma) for NADPH oxidase; 50 mM D-proline (Nacalai Tesque. Inc., Kyoto, Japan) for D-amino acid oxidase; and 50 mM D.L-β-hydroxybutyrate (Nacalai Tesque. Inc.) for L-β-hydroxyacid oxidase. Controls and inhibitory tests were carried out as follows: (1) incubation in a substrate-free medium; (2) addition to the complete medium of 10 mg/ml catalase (bovine liver, C-40, Sigma Chemical Co.), a scavenger of H₂O₂; (3) addition to the complete medium of 0.1 mM p-benzouquinone (Sigma Chemical Co.), a scavenger of O₂⁻; (4) addition to the complete medium of 1 mM chlorpromazine hydrochloride (Sigma Chemical Co.), an inhibitor of O₂⁻ generation; (5) addition to the complete medium of 1 mM o-phenanthroline monohydrate (Merk), a chelating agent; (6) heating for 2 min at 65–80°C in a 0.05 M Tris-maleate buffer, pH 7.5, prior to the incubation. After incubation, the tissue sections were rinsed in several changes of 0.05 M cacodylate buffer, pH 6.0, to remove cerium hydroxide.

Postfixation and Processing
Following incubation, the tissue sections were washed once in 0.05 M cacodylate buffer, pH 7.4, and postfixed in 1% osmium tetroxide in the same buffer for 1 hr at 4°C. After dehydration in an alcohol series, the sections were embedded in Epon-Araldite resin. The ultrathin sections, unstained or stained briefly with uranyl acetate, were examined using a Hitachi H-800 electron microscope.

RESULTS

Reaction sites and substrate dependence
When NADH and NADPH were used as the substrates, electron-dense deposits were observed at the following sites; (1) in the myoepithelial cell, the plasma membrane facing the acinar cell and the pinocytotic vesicles (Figs. 1-3), (2) the acinar and intercellular-canalicu lar plasma membrane of acinus and intercalated duct (Figs. 1-3), (2) the luminar and intercellular-canalicul ar plasma membrane of acinus and intercalated duct (Figs. 1, 2, 4) and (3) the membran e of the myoepithelial cell process (Arrow) in the substrate-free cerium medium (Fig. 6).

Figs. 1-5. Electron-micrographs of sections incubated in a cerium with NADH as substrate.

Fig. 1. Acinar cells (A) and an associated myoepithelial cell process (Arrow). Dense deposits can be observed on the plasma membrane of the myoepithelial cell and of the acinar lumen and intercellular canaliculi. Only the reaction at the myoepithelial cell is specific to NADH. ×8,600

Fig. 2. Transitional portion between an acinus (A) and an intercalated duct (ID). NADH-dependent reaction products are continuously present along the plasma membrane of the myoepithelial cell (Me). Deposits on the luminar plasma membrane of the intercalated duct (Arrowheads) are less than those on the acinus (Arrow). ×10,300

Fig. 3. A myoepithelial cell process (Me) and part of an adjacent capillary (Ca). NADH-dependent reaction products are present along the outer surface of the myoepithelial cell membrane facing the acinar cells, and in the pinocytotic vesicles. A small amount of deposit is also observable both on the basal plasma membrane of acinar cells facing the myoepithelial cell and on the plasma membrane and pinocytotic vesicles of the endothelial cell. ×24,000

Fig. 4. A part of an intercellular canalicul us. Dense deposits are continuously present along the outer surface of the plasma membrane. ×16,700 Inset: High magnification of the outlined area. ×78,000

Fig. 5. A part of a striated duct. Electron dense bodies are visible mainly in contact with the periphery of mitochondria. A small amount of deposit is also occasionally found on the cristae of mitochondria. These reaction products are independent of NADH. ×8,100 Inset: High magnification of the outlined area. ×19,600

Fig. 6. Electron-micrograph showing parts of the acini (A) incubated in a substrate-free cerium medium. Dense deposits can be observed on the plasma membrane of the lumen and intercellular canaliculi. There was no reaction on the plasma membrane of the myoepithelial cell process (Arrow). ×7,800
mitochondria mainly in the ductal cell (Fig. 5). Reaction products associated with the myoepithelial cell and the luminar and intercellular-canalicular plasma membrane were located evenly on the outer surface of the plasma membrane (Figs. 3, 4). On the other hand, the deposits associated with mitochondria were discretely present as amorphous bodies in the cristae and in the outer compartment (Fig. 5).

As regards the intensity of these reactions, there were significant differences from one reaction site to another as follows (Table 1): (1) reactions associated with the myoepithelial cell were obviously clear, but a little weaker than that of the luminar plasma membrane of the acinus; (2) reactions on the luminar and intercellular-canalicular plasma membrane were very intense in the acini, weak in the intercalated ducts and obscure in the striated ducts (Figs. 1, 2, 5); (3) reactions in the mitochondria were intense in the striated ductal cells, but obscure in the acinar and intercalated ductal cells.

The lateral plasma membrane and the other organelles of the acinar and ductal cells appeared negative for reaction product; but, a small amount was occasionally observed also on the basal plasma membrane which faces the myoepithelial cell, on the basal surface of the myoepithelial cell, and on the plasma membrane and pinocytotic vesicles of the endothelial cell of the capillary adjacent to the glandular cells (Fig. 3).

In the control specimens incubated in the medium from which the substrates were omitted, no reaction products associated with the myoepithelial cell were present, but deposits on the luminar plasma membrane and mitochondria were still observed (Table 1, Fig. 6). Therefore, only the reactions associated with the myoepithelial cell were dependent on NADH and NADPH, while the others were independent of these substrates.

When D-proline and D,L-α-hydroxybutyrate were used as the substrates, the reaction patterns were the same as those observed after incubation in the substrate-free medium. That is to say, the reaction products were present on the luminar and intercellular-canalicular plasma membrane and in the mitochondria, and no specific reaction for the substrates was found.

**Inhibitory tests** (Table 1)

All the reaction products were almost extinguished by the addition of catalase (Figs. 7, 8) or by heating prior to incubation, and were diminished by the addition of p-benzoquinone. When o-phenanthroline was added, although the reaction products of the myoepithelial cell were extinguished and those of the luminar and intercellular-canalicular plasma membrane were slightly diminished, those of the mitochondria could still be observed. By contrast, when chlorpromazine was added, while no reaction products appeared on the luminar and intercellular-canalicular plasma membrane and very little was visible in the mitochondria, it was still observable at the myoepithelial cell.

**DISCUSSION**

The present study indicated three kinds of reac-

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**Table 1. Reaction sites and their reactivities* by incubation in a NAD(P)H-cerium medium and the effects of some inhibitory tests in the Mongolian gerbil parotid gland**

<table>
<thead>
<tr>
<th>Reaction sites</th>
<th>MEC</th>
<th>Luminar membrane of</th>
<th>Mitochondria of</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AC</td>
<td>IDC</td>
</tr>
<tr>
<td>Experiments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>standard medium (SM)</td>
<td>+</td>
<td>+~#+</td>
<td>±~+</td>
</tr>
<tr>
<td>substrate free</td>
<td>−</td>
<td>++#</td>
<td>±~+</td>
</tr>
<tr>
<td>SM+Catalase</td>
<td>−</td>
<td>−~±</td>
<td>−</td>
</tr>
<tr>
<td>SM+p-BQ</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SM+CP</td>
<td>±~+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SM+o-PT</td>
<td>−</td>
<td>±~+</td>
<td>±</td>
</tr>
<tr>
<td>heating (80°C)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Reactivity is expressed by the amount of deposits: heavy, ++; visible, +; little or sparse, ±; invisible, −.
FIGS. 7, 8. Electron-micrographs showing a portion of an acinus (Fig. 7, × 4,600) and of a striated duct (Fig. 8, × 6,000) incubated in a NADH-cerium medium containing 10 mg/ml catalase as a scavenger of hydrogen peroxide. No reaction products are present.

Reactions with CeCl₃ which might involve the sites of the H₂O₂ generation in the Mongolian gerbil parotid gland. They are as follows: (a) NADH- and NADPH-dependent reactions on the plasma membrane of the myoepithelial cell, (b) substrates-independent reactions on the luminar and intercellular-canalicular plasma membrane of the acinus and of the intercalated duct, and (c) substrates-independent reactions within and on the mitochondria mainly in the ductal cells. Since catalase-positive peroxisomes are known to be present in the rat salivary glands (13), we also attempted to demonstrate the H₂O₂-generating peroxisome enzymes, i.e., D-amino acid oxidase and L-α-hydroxyacid oxidase. However, no reaction-positive organelle was found. The significance of each reaction is discussed in more detail below.

**NADH- and NADPH- dependent reactions on the plasma membrane of the myoepithelial cell**

It has been shown that NAD (P)H oxidase localizing on the plasma membrane of polymorphonuclear leukocytes or of thyroid follicular cells generates O₂⁻ as the primary metabolite, and O₂⁻ is then dismutated to H₂O₂ (25). In the present study, reaction on the outer plasma membrane and pinocytotic vesicles of the myoepithelial cell was dependent on NADH and NADPH as substrates, and was inhibited by catalase, p-benzoquinone, a scavenger of H₂O₂ and O₂⁻ respectively, or pre-heating. These results suggest that a similar H₂O₂ generation system via O₂⁻ formation by NAD(P)H oxidase may be present in association with the myoepithelial cell. Reaction was also prevented by o-phenanthroline, which is a chelating agent of copper or iron, so it may be suggested that this enzyme could be a metal-containing oxidase. It has been reported that o-phenanthroline inhibits the activities of NADH oxidase (28) and of xanthine oxidase (1).

Until now, there has been no report demonstrating the H₂O₂ generation system or the localization of NAD(P)H oxidase in the myoepithelial cell. In addition, there has been no knowledge about the functional relation between NAD(P)H oxidase and the myoepithelial cell.

NAD(P)H oxidase has been cytochemically demonstrated to localize on the plasma membranes of several epithelial cells containing catalase or peroxidase, e.g. the thyroid follicular cell (19), the epithelial cell of the endometrium (15), the hepatocyte, and the
absorptive cell of the duodeno-jejunum (14). It has been suggested that it is involved in the biosynthesis of thyroid hormone (9) and of estradiol (16), and also in antimicrobial actions, and in the control of transport system.

The localization of NAD(P)H oxidase associated with the myoepithelial cell in the present study was similar to that in previous reports on several phosphatases, *i.e.*, alkaline-phosphatase (10), adenylate cyclase (12), Mg$^{2+}$-ATPase (4) and 5'-nucleotidase (31). As NAD(P)H oxidase were also found on the capillary adjacent to the myoepithelial cell, it may be incorporated together with these phosphatases in some transport system between myoepithelial cells, secretory cells and capillaries.

**Substrate-independent reactions on the luminar and intercellular-canaliculur plasma membrane and associated with the mitochondria**

Although two kinds of substrate-independent reactions were observed on the luminar and intercellular-canaliculur plasma membrane and associated with the mitochondria, judging from the fact that these reactions were inhibited by catalase, p-benzoquinone, pre-heating or chlorpromazine, it seems likely that both reactions point to the presence of some endogenous H$_2$O$_2$ generation system. Chlorpromazine has been reported to inhibit plasma membrane O$_2^-$ generation by granulocyte (8) and also the activity of mitochondrial monoamine oxidase (3).

It has been also shown in several papers on cerium methods that non-specific deposits are present in the cristae and outer membranes of the mitochondria (2, 18). Christie and Stoward (7) suggested that the non-specific deposits formed in the mitochondria of myocardium had been produced by reaction with endogenous H$_2$O$_2$ which had been generated by a metal-containing thiol enzyme. Some similar H$_2$O$_2$-generating enzymes, such as monoamine oxidase of the outer mitochondrial membrane (11), may also be involved in the mitochondrial reactions which the present study has examined.

Cerous ions react with phosphate ions to form insoluble deposits of cerous phosphate. Ever since Veenhuis *et al.* (29) adapted this reaction to phospahatase cytochemistry, cerous ions have been used as a capturing agent for cytochemical study of several phosphatases. Yamashina *et al.* (31) applied this method to show the localization of 5'-nucleotidase in rat salivary glands, reporting that it was located on the outer surface of plasma membranes of secretory and myoepithelial cells. The localizations in their report were very similar to those in the present study, except that the reactions of luminar plasma membranes are substrate-dependent. Further research will be required in order to solve the question of why such a difference in substrate-dependency occurs when the cerium method is applied to H$_2$O$_2$-generating oxidase as opposed to when it is applied to phosphatase.

As described above, three kinds of reaction with CeCl$_3$ were observed in the Mongolian gerbil parotid gland. From the present study it was not possible to determine whether they are involved in the salivary peroxidase system. In previous reports by our laboratory (23, 24) we have shown that peroxidase could be cytochemically localized in the acinar cells of the Mongolian gerbil parotid and submandibular salivary glands. Judging from the secretory course of peroxidase, *i.e.*, it is synthesized in the acinar cells and is secreted by exocytosis into the lumina, it may be suggested that the luminar plasma membranes of acini may be the sites responsible for H$_2$O$_2$ generation in the salivary peroxidase system. The fact that reaction of the luminar plasma membranes was more intense in the acini than in the intercalated duct may show some relations to the localization of peroxidase. Further studies will be needed in order to demonstrate for certain the exact glandular source of H$_2$O$_2$ in the salivary peroxidase system.

**REFERENCES**

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