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Enhanced Immunocytochemical Expression of Antioxidant Enzymes in Rat Submandibular Gland After Normobaric Oxygenation

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ABSTRACT

In order to clarify the role of antioxidant enzymes in the male rat submandibular gland against short-term normobaric oxygenation, we performed immunocytochemical staining of manganese-containing superoxide dismutase (Mn-SOD), copper- and zinc-containing SOD (Cu/Zn-SOD), catalase (CAT), glutathione peroxidase, and glutathione *S*-transferases (GST alpha, GST mu, and GST pi) between days 1 and 7 after normobaric oxygenation. Ultrastructural alterations and immunoreactivities for malondialdehyde (MDA), a lipid peroxidation-related molecule, of the acinar and ductal cells after the oxygenation were also investigated. Immunoreactivity for MDA was exhibited in the acinar cells throughout the experiment. On the other hand, immunoreactivity for the SODs, CAT, and GSTs was not altered, when compared to that of controls, but was significantly elevated in the granular, striated, and excretory ductal cells. Since an increase of lipid peroxidation as indicated by enhanced immunoreactivity for MDA was detected in the acinar and intercalated ductal cells, the results indicate that the enhanced antioxidant enzymes in the granular, striated, and excretory ductal cells play a crucial role in the self-defense system of the male rat submandibular gland against normobaric oxygenation. Anat Rec 268:371–380, 2002.

Key words: immunocytochemistry; oxygenation; superoxide dismutase; catalase; glutathione peroxidase; glutathione *S*-transferase; malondialdehyde; rat submandibular gland

Oxygen is a Yanus-faced molecule, which conditions life but exerts toxic effects. Reactive oxygen species (ROSs), which include hydroxy radicals, superoxide anion, hydrogen peroxide, and nitric oxide, are produced by oxygen exposure to normal cells. ROSs are very transient species due to their high chemical reactivities, which result in lipid peroxidation as well as in the oxidation of DNA and proteins (Haugaard, 1968; Matès et al., 1999).

The antioxidant enzyme system plays an important role in the cell defense against ROS-mediated cell damage. This system consists of three enzymes: superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.16), and peroxidase, of which glutathione peroxidase (GPx; EC 1.11.1.9) is the most common in mammalian cells (Matès, 2000). SOD is responsible for the elimination of cytotoxic active oxygen by catalyzing the dismutation of the superoxide radical to oxygen and hydrogen peroxide (Kurobe et al., 1990; Kurobe and Kato, 1991). There are a variety of

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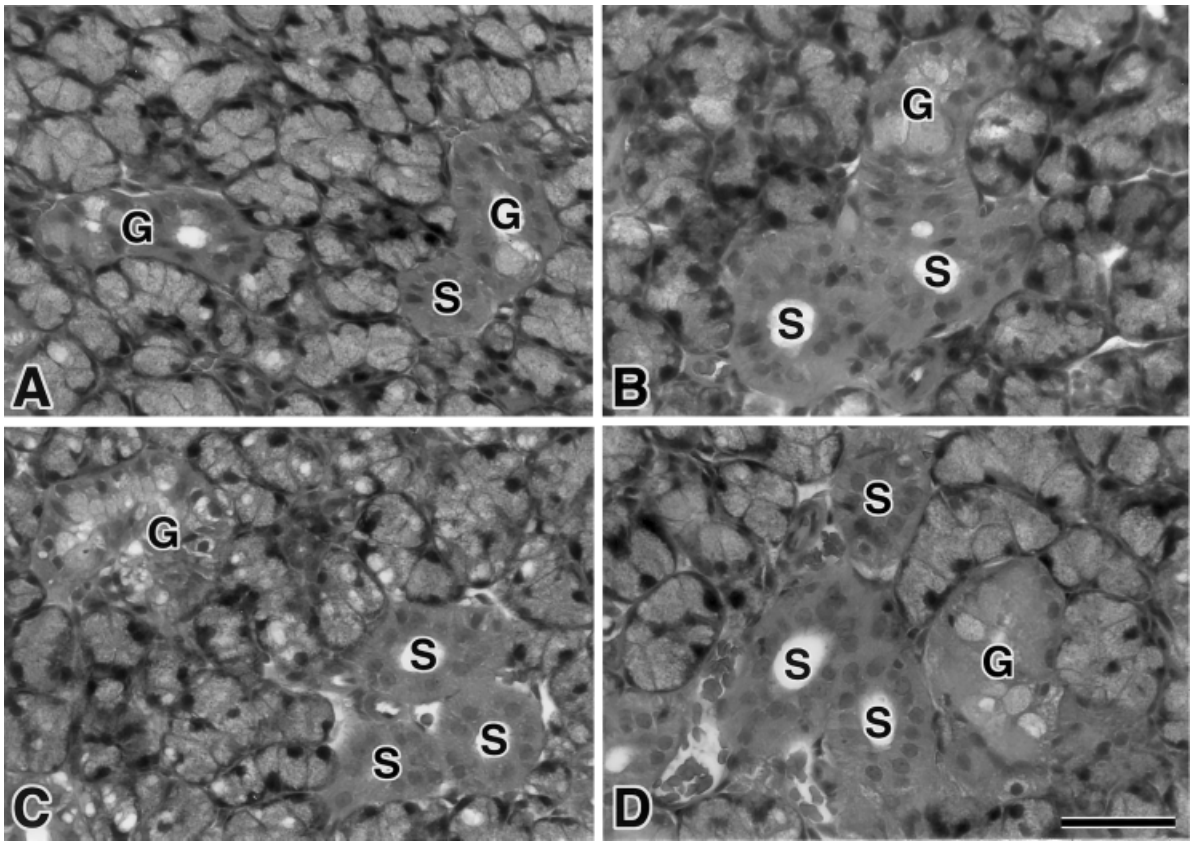

Fig. 1. Photomicrographs of rat submandibular gland after normobaric oxygenation at days 1 (**B**), 3 (**C**), and 7 (**D**), together with control (**A**), by HE-stained and paraffin-embedded sections. Abundant cytoplasmic vacuoles transiently appeared in acinar cells at day 3. G, granular duct; S, striated duct. Scale bar = 50 μm.

SOD isoenzymes in mammalian cells. Manganese-containing SOD (Mn-SOD) exists in mitochondria of various cells, while copper- and zinc-containing SOD (Cu/Zn-SOD) exists in the cytosol (Majima et al., 1998). CAT mainly exists in the peroxisome and reacts not only with hydrogen peroxide by activating its decomposition into water and oxygen, but also with hydrogen donors (Lledìas et al., 1998). GPx catalyzes a reduction in various hydroperoxides, including hydrogen peroxide through glutathione, thereby protecting mammalian cells against oxidative damage of the cytosol and mitochondoria (Flohè et al., 1973).

Glutathione *S*-transferase (GST; EC 2.5.1.18) is also a kind of antioxidant enzyme, since it catalyzes the decomposition of lipid hydroperoxides (Meyer et al., 1985; Ishikawa et al., 1986). GST constitutes a gene superfamily of xenobiotic-metabolizing enzymes that bind various ligands and catalyzes the nucleophilic addition of glutathione to diverse electrophilic substrates in a variety of cells (Jakoby, 1978; Salinas and Wong, 1999). Based on its biochemical characteristics, cytosolic GST is usually divided into four classes: alpha, mu, pi, and theta (Mannervik and Danielson, 1988; Meyer et al., 1991).

Previous immunohistochemical studies by others have shown the localization of antioxidant and xenobiotic-metabolizing enzymes in the ductal cells of normal mammalian salivary glands (Mn-SOD, rat (Yamamoto et al., 1999); Cu/Zn-SOD, rat (Thaete et al., 1985); CAT, mouse (Coleman and Hanker, 1978); and GSTs, human (Campbell et al., 1991)). However, these researchers did not refer the roles of these enzymes in the ductal cells. On the other

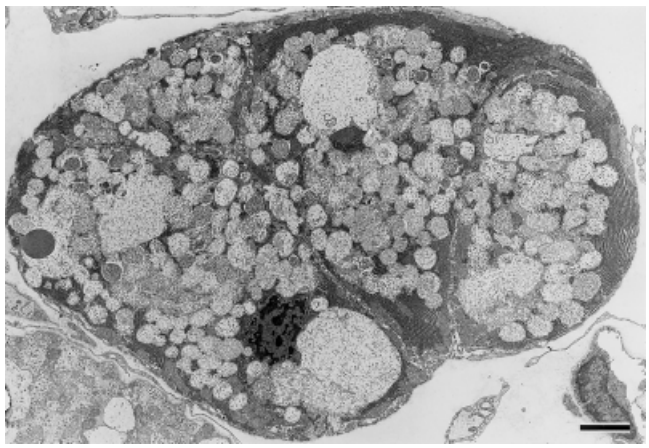

Fig. 2. Swelling of secretory granules and their fusions occurred in an acinar cell of rat submandibular gland after normobaric oxygenation at day 3. Scale bar = 2 μm.

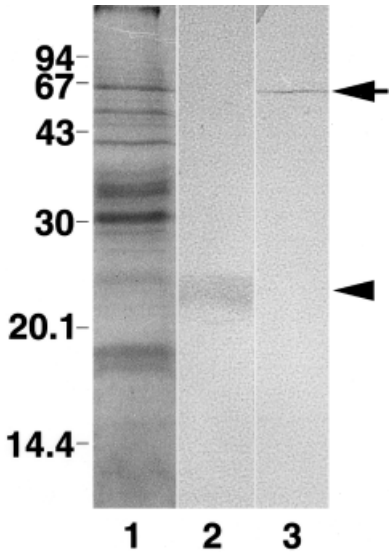

Fig. 3. SDS-PAGE (lane 1) and Western blotting (lane 2, anti-GPx; lane 3, anti-CAT) of soluble extracts from normal rat submandibular gland. Arrowhead indicates immunoreactivity for anti-GPx, and arrow indicates immunoreactivity for CAT. Positions of low-molecular-mass markers, expressed in kilodaltons, are indicated on the left side of the figure.

hand, Reddy Avula and Fernandes (1999) reported the data obtained from biochemical analyses of the above antioxidant enzymes in the mouse salivary gland under a low oxidative stress, but the expression of these antioxidant enzymes under a high oxidative stress loaded on this organ remains to be investigated. Oxygen therapy, including hyperbaric oxygenation, has been of clear benefit in many clinical settings. However, there are no available reports concerning the effects (i.e., damages) of high oxygen concentrations caused by this therapy on the salivary glands in experimental animals.

In the present study, in order to clarify the role of antioxidant enzymes (Mn-SOD, Cu/Zn-SOD, CAT, GPx, and GSTs) in the salivary gland against short-term normobaric oxygenation, we examined ultrastructural alterations and immunocytochemical expressions of the above enzymes in the adult male rat submandibular gland after this oxygenation. To identify cell damage by ROSs in the oxygenated submandibular gland, we also performed immunohistochemical detection of malondialdehyde (MDA), a marker of lipid peroxidation.

MATERIALS AND METHODS

Animals

Male Wistar rats aged 8 weeks and weighing 250 ± 30 g (Seac Yoshitomi, Fukuoka, Japan) were provided for the present study. The care and use of animals followed "The

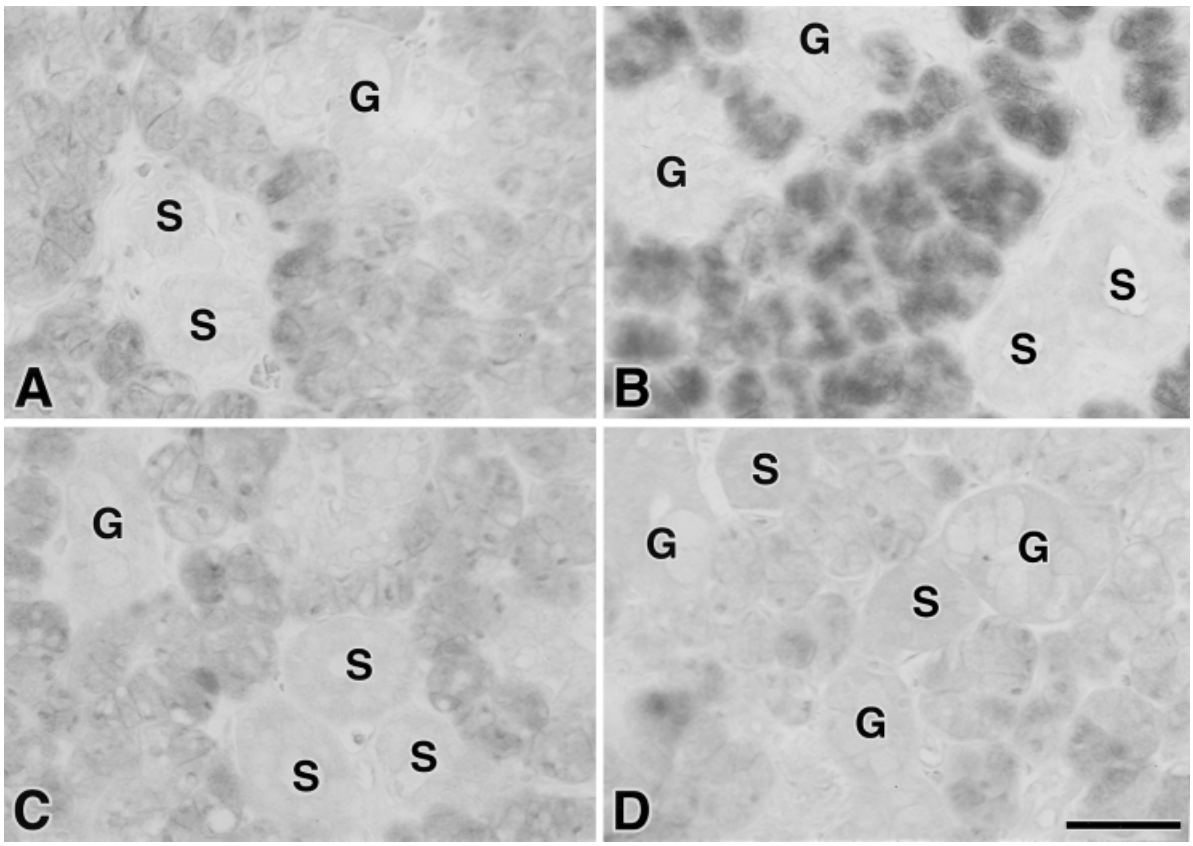

Fig. 4. Immunoreactivity for MDA in rat submandibular glands at days 1 (**B**), 3 (**C**), and 7 (**D**) after normobaric oxygenation. Immunoreactivity is enhanced in both acinar cells and intercalated ductal cells at day 1 only (B) when compared to those of the control (**A**). G, granular duct; S, striated duct. Scale bar = 50 μm.

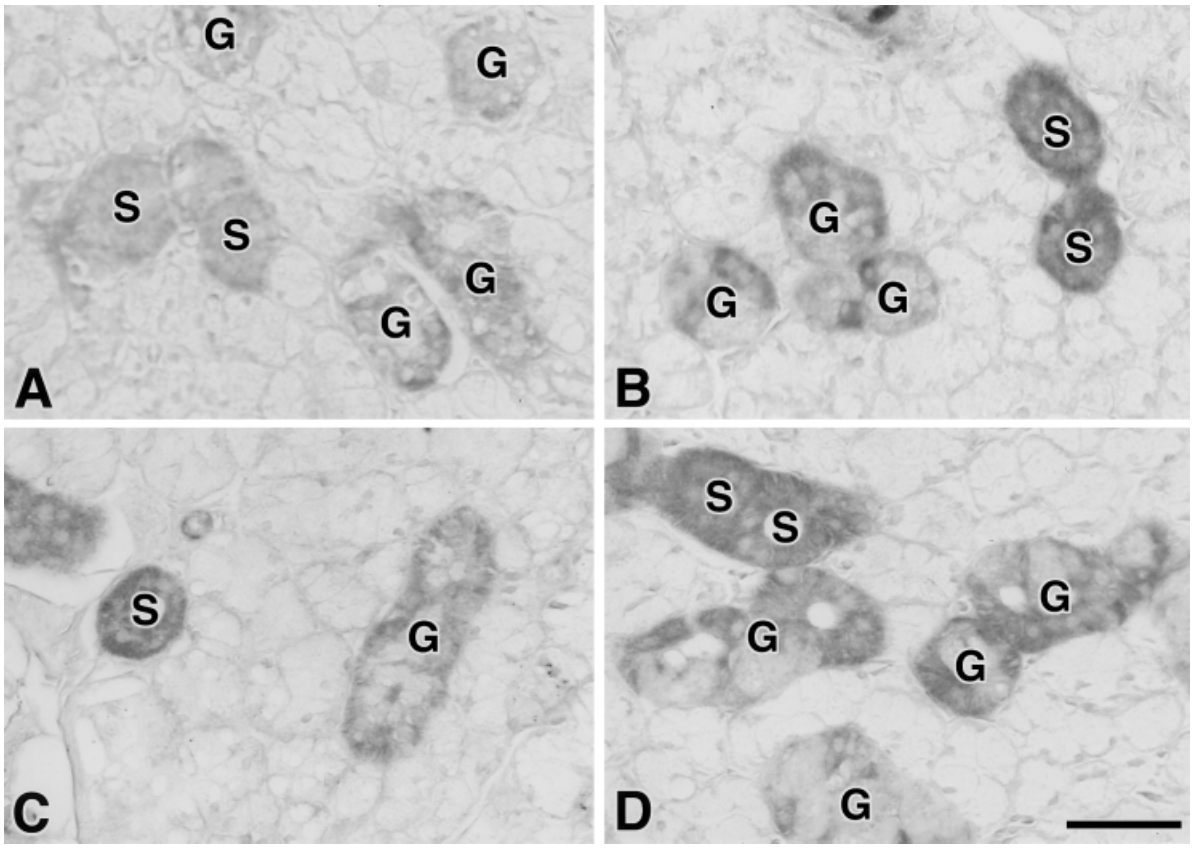

Fig. 5. Immunoreactivity for Mn-SOD in rat submandibular glands at days 1 (**B**), 3 (**C**), and 7 (**D**) after normobaric oxygenation. Immunoreactivity is markedly enhanced in both granular (G) and striated (S) ducts throughout the experiment when compared to those of the control (**A**). Scale bar = 50 μm.

Guiding Principles for the Care and Use of Animals," approved by our university in accordance with the principles of the Declaration of Helsinki.

Oxygenation

Rats were exposed to 100% oxygen gas in a High Pressure Chamber for Animal Experiment (Hanyuda, Tokyo, Japan) at normobaric pressure for 1 hr. The oxygen gas was continuously ventilated to prevent the retention of carbon dioxide (<0.1%) in the chamber, and the temperature maintained between 22 and 25°C. At days 1, 3, and 7, oxygenated (n = 10 at each period) and nonoxygenated (n = 8; normal air exposed in the same chamber) rats were deeply anesthetized with an intraperitoneal injection of 5 mg of pentobarbital per 100 g of body weight and utilized in the following experiments.

Light Microscopic and Immunocytochemical Samples

Animals were perfused with physiological saline from the left ventricle, followed by a solution of 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.2 for 5 min each. After perfusion, half of the isolated submandibular glands were immersed in a solution of 4% PFA in 0.1 M PB for 72 hr at 4°C. They were then rinsed with 0.1 M PB containing 10% sucrose and dehydrated through a graded ethanol series before being embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Serial sections of approximately 5 μm in thickness were prepared using a microtome and mounted on glass slides (MAS-coated Superfrost; Matsunami, Osaka, Japan). They were then air-dried at 4°C and stained either with Delafield's hematoxylin and eosin (HE) or immunostained as described below.

For detection of MDA, SODs, CAT, and GSTs, deparaffinized sections were irradiated with a microwave of 800 watts using MICROMED T/T microwave equipment (Milestone, Sorisole, Italy) for 20 min. Antigen-retrieved sections were then blocked with 0.1% hydrogen peroxide in methanol for 20 min to remove endogenous peroxidase and rinsed with phosphate-buffered saline (PBS). They were then incubated in a humid chamber for 16 hr at 4°C with one of the following: mouse anti-MDA monoclonal antibody (mAb) (NOF Company, Tokyo, Japan), rabbit anti-Mn-SOD polyclonal antibody (pAb) (StressGen, Victoria, Canada), rabbit anti-Cu/Zn-SOD pAb (StressGen, Victoria, Canada), mouse anti-CAT mAb (Sigma, St. Louis, MO), rabbit anti-GST alpha pAb, rabbit anti-GST mu pAb, or rabbit anti-GST pi pAb (Novocastra Laboratories, Newcastle upon Tyne, UK) at a dilution from 1:500 to 1:1,000 in PBS. After rinsing with PBS, the sections were

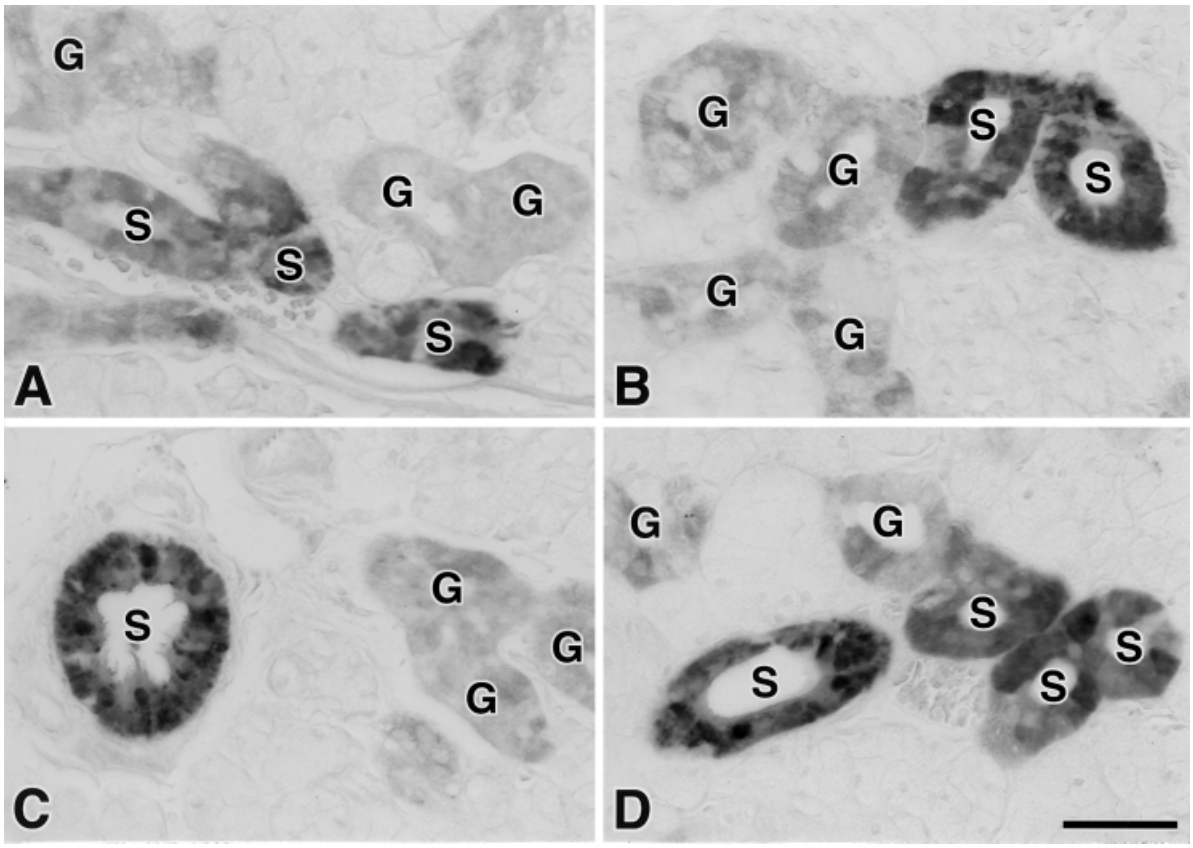

Fig. 6. Immunoreactivity for catalase in rat submandibular gland at days 1 (**B**), 3 (**C**), and 7 (**D**) after normobaric oxygenation. Immunoreactivity is markedly enhanced in the striated ducts (S) throughout the experiment when compared to those of the control (**A**). G, granular duct. Scale bar = 50 μm.

reacted with the indirect immunoperoxidase method (Histofine Simple Stain Rat MAX PO Multi Kit; Nichirei, Tokyo, Japan).

For detection of GPx, deparaffinized sections were blocked with 0.1% hydrogen peroxide in methanol for 20 min and rinsed with PBS before being incubated with 10% normal goat serum for a further 20 min. They were then incubated with the sheep anti-GPx pAb (Biogenesis, England, UK) diluted 1:2,400 in PBS in a humid chamber for 16 hr at 4°C. After rinsing in PBS, sections were reacted with the biotinylated donkey anti-sheep IgG pAb (Chemicon, Temecula, CA) at a dilution of 1:500 in PBS for 45 min. They were then rinsed with PBS and incubated with streptavidin-conjugated peroxidase (Dako, Carpinteria, CA) for 30 min.

The above peroxidase complexes were visualized by treatment with a freshly prepared solution of 0.1 mg/ml diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl (pH 7.6) containing 0.01% hydrogen peroxide for 7 min. Specificity of the above immunoreactivities was confirmed by replacing the primary antibodies with either normal rabbit sera or PBS.

Antibody Specification

Specificity of the antibodies for MDA (Yamada et al., 2001), Mn-SOD (Kurobe and Kato, 1991), Cu/Zn-SOD (Kurobe et al., 1990), and GSTs (Nishino et al., 2001) has already been demonstrated. Thus, in order to confirm specificity of the antibodies for CAT and GPx, we performed Western blot analyses in the rat submandibular glands according to the procedure described by Nishino et al. (2001).

Electron Microscopic Samples

The other halves of the submandibular glands were fixed in a mixture of 2% PFA and 2.5% glutaraldehyde in 0.1 M PB for 16 hr at 4°C. They then were postfixed with a solution of 1% osmium tetroxide in the same buffer for 2 hr at 4°C before being dehydrated in a graded series of acetone and embedded in epoxy resin. Ultrathin sections were prepared on an MT-X Ultramicrotome (RMC, Tucson, AZ) and stained with saturated uranyl acetate and lead citrate. They were then examined in a JEM 1210 electron microscope (JOEL, Tokyo, Japan).

RESULTS

In oxygenated rats, swelling of secretory granules and their fusions occurred in the acinar cells of the rat submandibular gland at day 3 (Figs. 1C and 2), but such swollen granules almost completely disappeared from the cells by day 7 (Fig. 1D).

Soluble extracts from the submandibular glands were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as shown in lane 1 of Figure

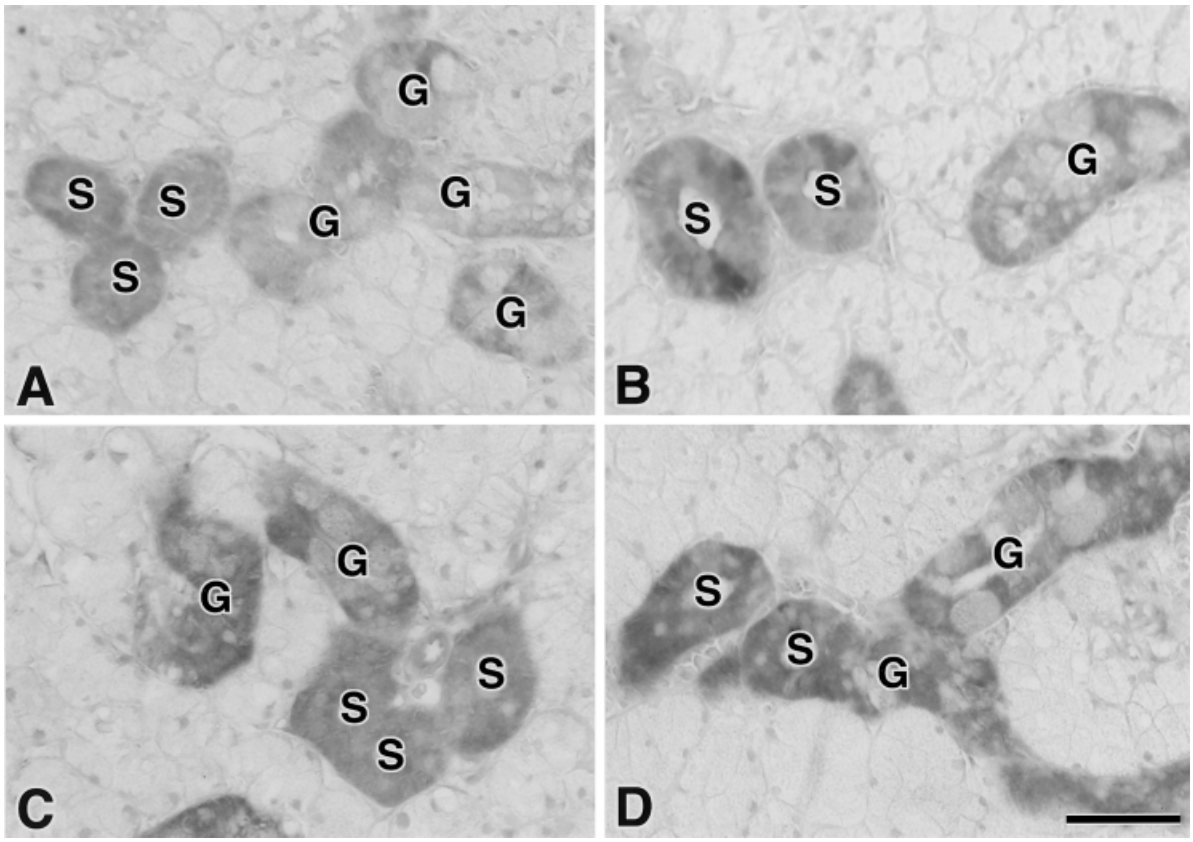

Fig. 7. Immunoreactivity of GST alpha in rat submandibular glands at days 1 (**B**), 3 (**C**), and 7 (**D**) after normobaric oxygenation. Immunoreactivity is slightly enhanced in granular (G) as well as in striated (S) ducts throughout the experiment when compared to those of the control (**A**). Scale bar = 50 μm.

3. In Western blot analyses, the anti-GPx antibody (lane 2) recognized a band of molecular mass of 22.3 kDa, while the anti-CAT antibody (lane 3) recognized a band of 60.0 kDa (Fig. 3).

Immunoreactivity for MDA was detected throughout the experiment in the acinar cells of both oxygenated and nonoxygenated rats, although the immunoreactive intensity was transiently enhanced in both acinar cells and intercalated ductal cells at day 1 after oxygenation (Fig. 4). Immunoreactivity for Mn-SOD was undetectable in the acinar cells, but it was generally found in the granular, striated, and excretory ductal cells of both oxygenated and nonoxygenated rats (Fig. 5). However, the present oxygenation characteristically induced an increase of the immunoreactive intensity in these ductal cells (Fig. 5B, C, and D). Immunoreactivity for Cu/Zn-SOD was seen in the granular, striated, and excretory ductal cells of both oxygenated and nonoxygenated rats. However, the immunoreactive intensity was transiently enhanced in the granular ductal cells only at day 1 after oxygenation.

Immunoreactivity for CAT was undetectable in the acinar cells of both oxygenated and nonoxygenated rats (Fig. 6). On the other hand, the immunoreactivity was occasionally seen in cells throughout the salivary ductal system of oxygenated and nonoxygenated rats, but it was especially enhanced in the striated and excretory ducts after oxygenation (Fig. 6). Immunoreactivity for GPx was undetectable in the acinar cells, whereas it was clearly detectable in the granular, striated, and excretory ductal cells of both oxygenated and nonoxygenated rats.

No immunoreactivities for three GST isoforms were detectable in the acinar cells of both oxygenated and nonoxygenated rats (Figs. 7–9). However, immunoreactivity for GST alpha was sparsely or weakly detectable in the striated and excretory ductal cells in nonoxygenated rats, and this was enhanced in cells comprising the salivary duct system after oxygenation (Fig. 7). Immunoreactivity for GST mu was seen in all ductal cells of nonoxygenated rats, and it was remarkably enhanced after oxygenation (Fig. 8). Immunoreactivity for GST pi was observed in all ductal cells of both oxygenated and nonoxygenated rats (Fig. 9). A slight increase of the immunoreactivity was only noticeable at day 1 after oxygenation (Fig. 9B).

The expression values based on subjective estimates of the above immunoreactivities for MDA and antioxidant enzymes (n = 8 each) are summarized in Table 1. Drastic changes in the number of immunoreactive ductal cells for antioxidant enzymes were not noticed in the present experiment. Immunoreactivities for the above enzymes were undetectable in the intercalated duct of oxygenated and nonoxygenated rats throughout the experiment. Control immunostaining using normal rabbit serum or PBS in adjacent sections showed no detectable positive immunoreactivities in all cases.

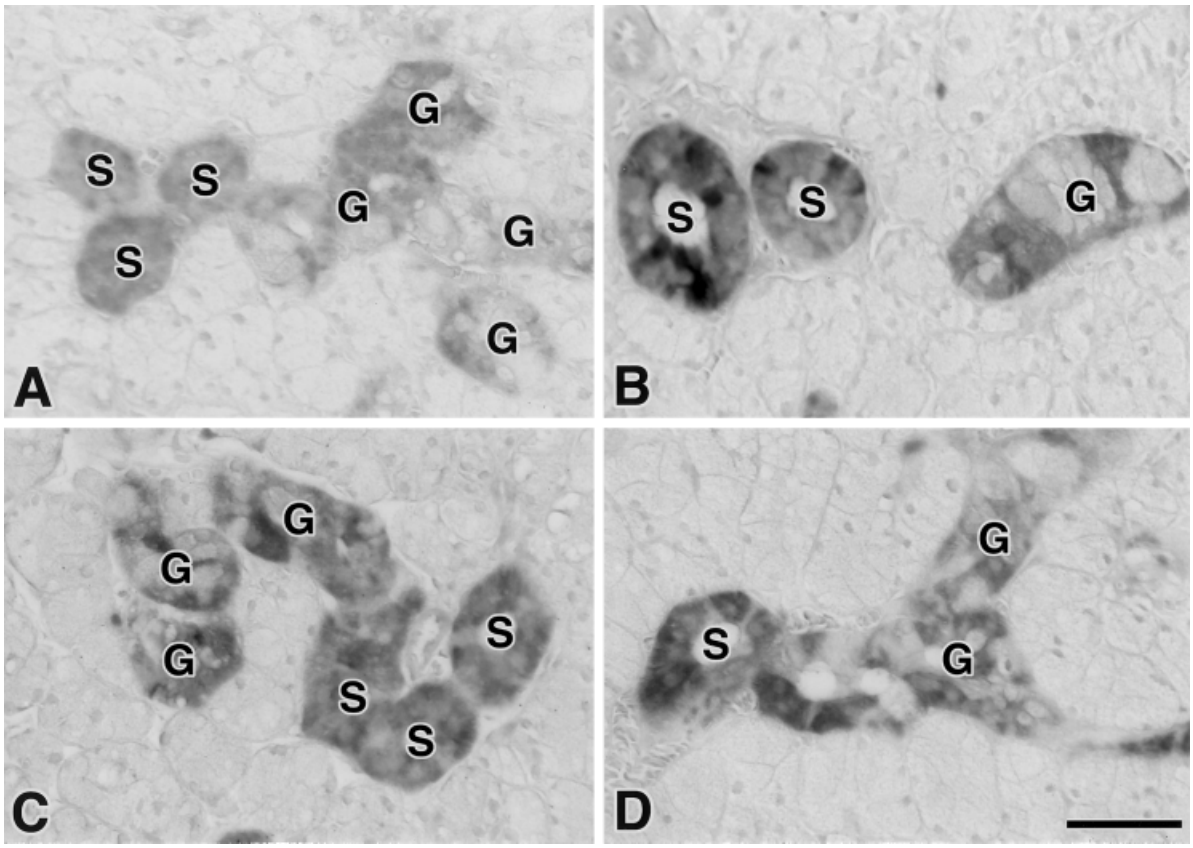

Fig. 8. Immunoreactivity of GST mu in rat submandibular glands at days 1 (**B**), 3 (**C**), and 7 (**D**) after normobaric oxygenation. Immunoreactivity is markedly enhanced in granular (G) as well as in striated (S) ducts throughout the experiment when compared to those of the control (**A**). Scale bar = 50 μm.

DISCUSSION

Western blot analyses in the present study revealed a single band of 22.3 kDa (anti-GPx antibody) and one of 60.0 kDa (anti-CAT antibody) in the rat submandibular gland. These molecular masses are identical to those of both enzyme subunits reported in other tissues of rats (Ho and Howard, 1992 (GPx); Furuta et al., 1986 (CAT)). Thus, our results indicated the usefulness of these antibodies for the immunocytochemical detection of both enzyme subunits in the rat submandibular gland.

MDA is the most abundant individual aldehyde arising from lipid peroxidation and is, therefore, a useful molecular marker for the occurrences of oxidative stress (Palinski et al., 1990). Recently, immunocytochemical detections of MDA were established and utilized for the identification of lipid peroxidative cells (Yamada et al., 2001). According to this method, our present results indicated that the main oxidative site in the rat submandibular gland is the acinar cell, since immunoreactivity for MDA was uniquely exhibited in such cells at day 1 after oxygenation. On the other hand, no immunoreactivities for MDA were observed in cells comprising the salivary duct system (excluding the intercalated duct) throughout the experiment. As described above, several researchers have determined that certain antioxidant enzymes are expressed in the normal salivary duct system but not in acinar cells. This was confirmed by the present immunocytochemistry of nonoxygenated rats. Thus, at present, we consider that ductal cells are able to defend themselves from ROSs using their own antioxidant enzyme activities, but acinar cells devoid of these enzymes do not have such an ability.

A previous study showed that cells comprising renal corpuscles of the normal rodent kidney lack immunoreactivity for antioxidant enzymes, whereas cells comprising the renal tubular system exhibit their intense immunoreactivities (Muse et al., 1994). These researchers insisted that the renal corpuscle cells devoid of these enzymes are more likely to be suffered from various inflammatory diseases by ROSs that are released from the blood cells. This explanation may also be applied to our results where, after oxygenation, the lipid peroxidation occurred only in acinar cells, followed by the severe morphological alterations of secretory granules such as swelling. However, the recovery process of such changes between days 3 and 7 after oxygenation remains uncertain. Peter et al. (1995) described that acinar cells of rat parotid and submandibular gland are more labile to ionizing radiation than ductal cells. This result is consistent with ours, indicating the relative paucity of antioxidant enzymes in acinar cells.

Both CAT and GPx are involved in the metabolic pathway from hydrogen peroxide to water (Flohè et al., 1973; Lledìas et al., 1998). Why expression of CAT was en-

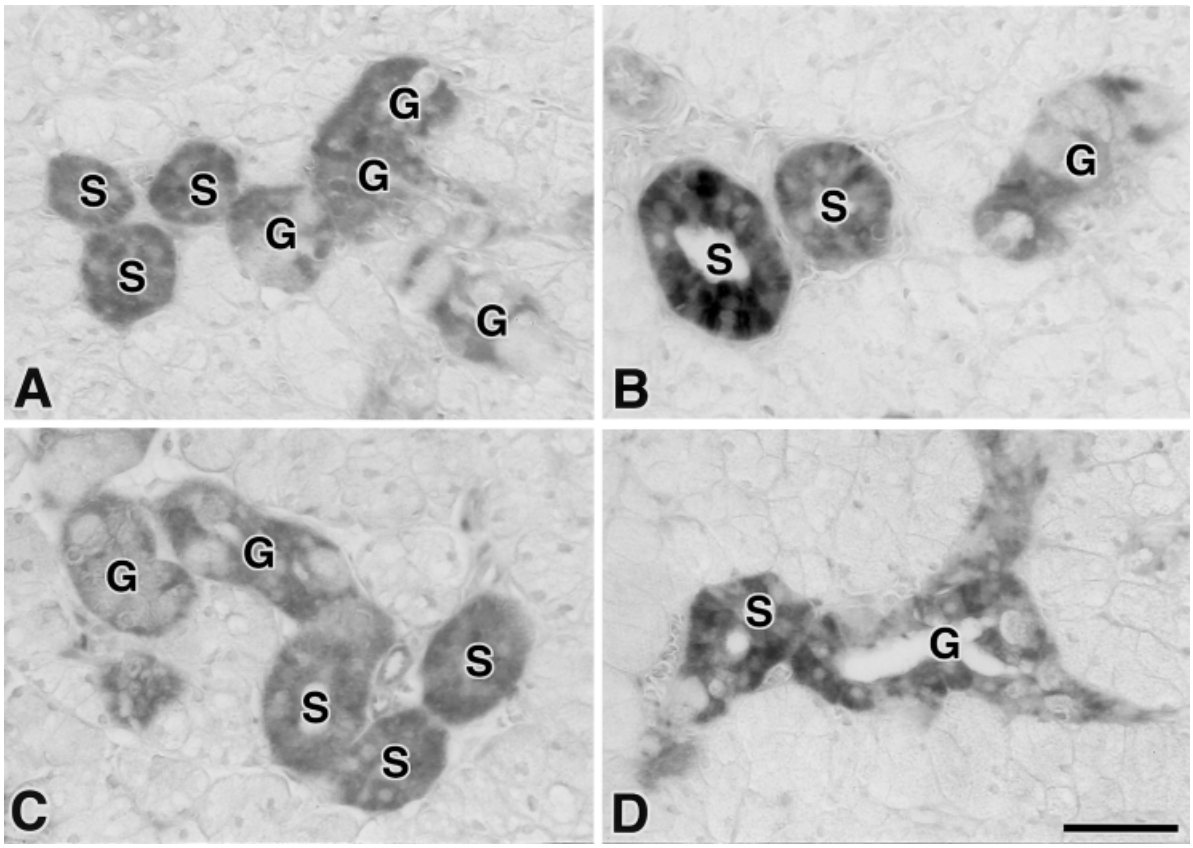

Fig. 9. Immunoreactivity of GST pi in rat submandibular glands at days 1 (**B**), 3 (**C**), and 7 (**D**) after normobaric oxygenation. Immunoreactivity is markedly enhanced in granular (G) as well as in striated (S) ducts at day 1 when compared to those of the control (**A**). Scale bar = 50 μm.

hanced only in the ductal cells after normobaric oxygenation is of much debate. Since our electron microscopic study did not reveal a significant increase in the number of peroxisomes, the enhanced immunoreactivity may be caused by an increase of cytoplasmic and/or mitocondrial CAT. In addition, Hand (1973) reported that acinar cells in the rat parotid and other exocrine glands possess an abundance of peroxisomes, and it is now widely accepted that these inclusions contain CAT. However, immunoreactivity for this enzyme was not detected in acinar cells of the rat submandibular gland in the present experiment. This result fundamentally argues for the data using mouse reported by Coleman and Hanker (1978). Therefore, why immunoreactivity for CAT lacks in peroxisomes in acinar cells of the submandibular gland of both experimental animals should be elucidated in further studies. It was reported that expressions of GST isoforms were enhanced in the mouse kidney proximal tubular cells under an oxidative stress condition (Fujita et al., 2001). Considered together, our results suggest that these three GST isoforms are also involved in the metabolizing of the oxidative stress-derived molecules.

The oral cavity is one of the important sites of defense against ROSs such as hydrogen peroxide, which is synthesized by oral bacteria, and this defense system is greatly influenced by the actions of salivary peroxidase (EC 1.11.1.7; Carlsson, 1987; Kiser et al., 1996). Since the antioxidant enzymes, examined by the present immunocytochemistry, are not included in the salivary secretions under normal conditions (Carlsson, 1987), it is reasonable to suppose that the enhanced expressions of these antioxidant enzymes in the ductal cells are the defense against ROSs after normobaric oxygenation.

Previous researches analyzed the oxygen toxicity of laboratory animals with long-term oxygenation (24 hr ~ several months) and reported severe cell damage, as reviewed by Balentine (1982). However, to elucidate the effects of oxygen therapy on the submandibular glands, the present study dealt only with short-term oxygenation. Our results indicate that the salivary glands are able to defend themselves against hyperoxia using their own antioxidant enzymes in spite of the transient acinar cell damage. However, whether such a self-defense mechanism by the antioxidant enzymes in acute hyperoxic conditions reflects the hormetic effect raised by Calabrese and Baldwin (2000) remains uncertain. Quissell et al. (1994) reported that the optimal oxygenation is necessary for the acinar cells of the rat submandibular gland in primary culture. This may mean that slight cell damage induced by oxygen leads to hormetic effects stimulating and activating these cells.

In summary, we demonstrated that normobaric oxygenation induced an enhancement of antioxidant enzyme activities in cells comprising the salivary duct system of the

TABLE 1. Subjective estimates of the density of immunoreactive cells for MDA, SODs, CAT, GPx, and GSTs in the rat submandibular glands after normobaric oxygenation

Antibody group	Acinar cells	Granular duct cells	Striated duct cells	Excretory duct cells
MDA				
Control	±	–	–	–
Day 1	+++	–	–	–
Day 3	±	–	–	–
Day 7	±	–	–	–
Mn-SOD				
Control	–	+	+	+
Day 1	–	+++	+++	+++
Day 3	–	++	++	++
Day 7	–	+++	+++	+++
Cu/Zn-SOD				
Control	+	++	++	++
Day 1	+	+++	++	++
Day 3	+	++	++	++
Day 7	+	++	++	++
CAT				
Control	–	±	++	++
Day 1	–	+	+++	+++
Day 3	–	+	+++	+++
Day 7	–	±	+++	+++
GPx				
Control	–	+	+	+
Day 1	–	+	+	+
Day 3	–	+	+	+
Day 7	–	+	+	+
GST alpha				
Control	–	±	+	+
Day 1	–	++	++	++
Day 3	–	++	++	++
Day 7	–	++	++	++
GST mu				
Control	–	+	+	+
Day 1	–	+++	+++	+++
Day 3	–	++	++	++
Day 7	–	++	++	+++
GST pi				
Control	–	+	+	+
Day 1	–	++	++	++
Day 3	–	+	+	+
Day 7	–	+	+	+

–, negative-immunoreaction (ir); ±, sparse-ir; +, weak-ir; ++, medium-ir; +++, strong-ir (n = 8).

male rat submandibular gland. These enzymes are thought to play crucial roles in the self-defense mechanism against oxidation in this organ.

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