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Immunohistochemical localization of senescence marker protein-30 (SMP30) in the submandibular gland and ultrastructural changes of the granular duct cells in SMP30 knockout mice

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Summary. Senescence Marker Protein-30 (SMP30) is a calcium-regulating protein that decreases in an androgen-independent manner as aging occurs. An enzyme-labeled antibody technique has demonstrated that SMP30 localized to the ducts (granular, intercalated, and striated ducts) of mouse submandibular glands. Immunoelectronmicroscopy demonstrated that the granular duct cells were strongly positive for SMP30, but that pillar cells in the granular duct were negative for the protein. In SMP30-knockout (KO) mice, the granular ducts were smaller in diameter. Swelling of mitochondria in the granular duct cells was observed; however, this phenomenon was not observed in the pillar cells. After administration of α-isoproterenol, a beta-adrenergic stimulant, a large numbers of small secretory granules were present in the granular duct cells and an expansion of the rough endoplasmic reticulum in SMP30-wild type (WT) mice; in contrast, little change was observed in SMP30-KO mice. These results suggest that SMP30 may be closely related to a signal transduction pathway in the granular duct cells of submandibular glands.

Key words: SMP30, Submandibular gland, Granular duct, α-isoproterenol, Immunohistochemistry

Introduction

SMP30, a 30-kDa protein that was isolated from the liver, was named senescence marker protein because its expression levels decrease as mice age. During the process of investigating age-related changes in gene and protein expression, Fujita and Maruyama (1991) performed an analysis of rat liver soluble proteins by 2-dimensional electrophoresis. Two proteins, one with *pI* 4.9 and the other with *pI* 7.3, that decreased with aging were found. The protein with *pI* 4.9 was not controlled by sex hormones, and had decreased levels of expression in aged males and females. Fujita et al. (1992) named this protein senescence marker protein-30 (SMP30) after its molecular weight of 30 kDa. It was later shown that this protein is present in additional organs including the kidney, pancreas, skeletal muscle, and brain. It was subsequently demonstrated that human and mouse SMP30 have approximately 89% homology, and SMP30 homologs from other animal species are also highly conserved (Fujita et al., 1995, 1996). Experimental gene transfer into cultured cells demonstrated that SMP30 is actively involved in intracellular Ca^{2+} homeostasis by increasing calmodulin-dependent plasma membrane-Ca^{2+}-pump activity (Fujita et al., 1998). These observations suggest that SMP30 is involved the important biological function of intracellular Ca^{2+} homeostasis (Fujita et al., 1998).

Ca^{2+}-signaling in the salivary glands is closely involved in secretory function. Segawa et al. (2000) have suggested that aged salivary glands experience a decline in secretory activity at the cellular level; this is probably due to impairment of signaling processes downstream of receptor activation and second messenger production. To elucidate the relationship between SMP30 and salivary gland function, we investigated the localization of SMP30 in mouse submandibular glands using an anti-SMP30 antibody. We found that the secretory duct and, in particular, the granular duct cells, stained positive using this anti-SMP30 antibody, while the acinar cells were almost negative. In SMP30 knockout (KO) mice, swelling of mitochondria was observed in the granular duct cells. We also compared the effects of α-isoproterenol, a beta-adrenergic stimulant, on SMP30-KO and wild-type (WT) mice. In SMP30-WT mice, a large numbers of small secretory granules were present

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in the granular duct cells, and an expansion of rough endoplasmic reticulum (rER) was observed after administration of α-isoproterenol. However, significant changes were not observed in SMP30-KO mice. These findings suggest that SMP30 is involved in signal transduction related to the secretory function of the duct system in mouse submandibular glands.

Materials and methods

Localization of SMP30 in the normal mouse submandibular gland

For immunohistochemical staining, submandibular glands were removed from adult mice (C57BL/6, 13-week-old male; n=5 and 13-week-old female; n=5), fixed in 4% paraformaldehyde, and frozen sections were prepared. After blocking endogenous peroxidase activity with 3% aqueous hydrogen peroxide, frozen sections were incubated first with anti-SMP30 antibody (Fujita et al., 1995, 1996), and then with HRP-labeled anti-rabbit IgG secondary antibody (DAKO, Japan). Color was developed with a DAB/hydrogen peroxide solution, and the sections were observed under a light microscope. Sections treated with 1% bovine serum albumin instead of anti-SMP30 antibody served as controls.

For immunoelectronmicroscopy, small portions of the submandibular glands of the adult mice mentioned above were cut into small pieces and fixed with 4% paraformaldehyde. These tissue samples were dehydrated and embedded in LR white resin and ultrathin sections were prepared. The ultrathin sections were first incubated with the anti-SMP30 antibody (Fujita et al. 1995, 1996), and then with HRP-labeled anti-rabbit IgG secondary antibody (DAKO, Japan). Sections treated with 1% bovine serum albumin instead of anti-SMP30 antibody served as controls.

Engineering of the SMP30-KO mouse

Ishigami et al. (2002) introduced a null mutation in the SMP30 gene into the germ line of mice. Two 37-week-old female and five 19-week-old male SMP30-KO mice were produced from C57BL/6 mice (for details of SMP30-KO mouse see the reference).

Morphology of SMP30-KO mouse submandibular glands

For morphological analysis, the submandibular glands were removed from 37-week-old female SMP30-KO and -WT mice, two of each, cut into small pieces, fixed in 2.5% glutaraldehyde followed by 1% osmic acid, and embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy.

Effects of α-isoproterenol administration

Nineteen-week-old male SMP30-KO and -WT mice, three of each, were intraperitoneally administered 5 mg/kg of α-isoproterenol daily for 3 weeks. Following this treatment, the submandibular gland was removed from each animal. Tissue samples were fixed in 2.5% glutaraldehyde, postfixed in 1% osmic acid, and embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy. Two untreated SMP30-KO and -WT mice were used as controls.

Results

Localization of SMP30 in the normal mouse

The epithelial cells of the secretory ducts, particularly the granular and striated ducts, were stained strongly positive with the anti-SMP30 antibody (Fig. 1). Electron microscopy demonstrated that the intercalated ducts were also positive (Fig. 2). The granular cells,

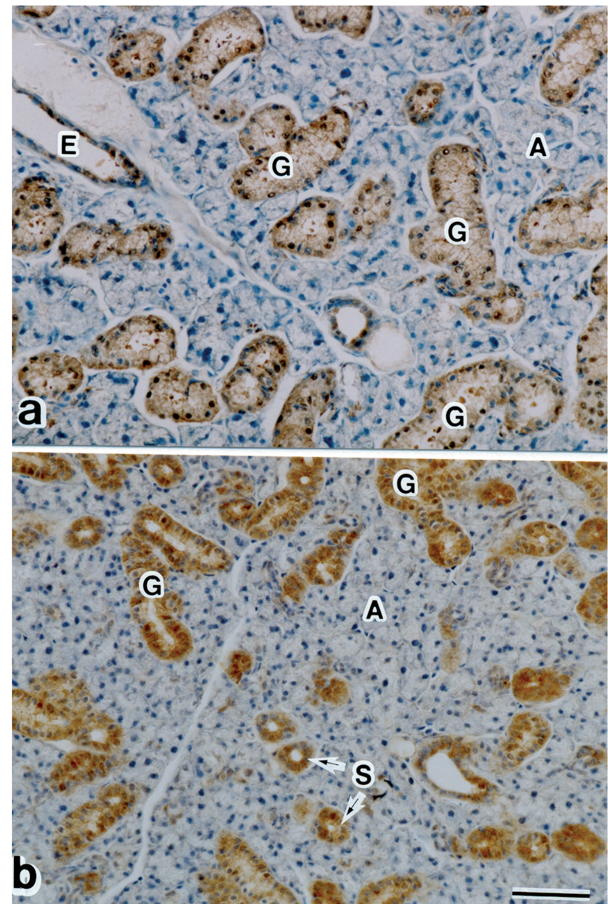

Fig. 1. Immunostaining with anti-SMP30 antibody. **a.** 13-week-old male. Granular and striated ducts stained positive with anti-SMP30 antibody. The excretory duct and acinar cells were almost negative. **b.** 13-week-old female. Granular and striated ducts stained positive. The granular ducts of the female were smaller than those of the male. G: granular ducts, S: striated ducts, E: excretory ducts, A: acinar cells. Bar: 100 μm.

which are the principal cells in the granular duct, stained positive with the anti-SMP30 antibody. However, the pillar cells in the granular duct were not stained (Fig. 3).

Morphology of the SMP30-KO mouse

Compared with SMP30-WT mice, the granular ducts of SMP30-KO mice were smaller in diameter. Cells in the epithelium of the secretory ducts, and particularly the granular cells in the granular ducts, showed swelling of mitochondria (Fig. 4). Unlike the granular cells, pillar cells present in the epithelium of granular ducts did not show swelling of mitochondria (Fig. 5).

Effects of α-isoproterenol administration

Three weeks of daily α-isoproterenol administration increased submandibular gland weight in both SMP30-WT and -KO mice (Fig. 6). Administration of α-isoproterenol caused acinar cell hypertrophy. Acinar cell hypertrophy was more prominent in SMP30-KO mice than WT mice (Fig. 7). In SMP30-WT mice, the secretory granules in the granular duct cells showed equally in size. After α-isoproterenol administration, secretory granules showed variability in size with the presence of small granules. Furthermore, an expansion of rER was observed in the granular cells after α-isoproterenol administration (Fig. 8). In contrast, the granular ducts were smaller and sparse in SMP30-KO mice (Fig. 7a, b). After α-isoproterenol administration to SMP30-KO mice, little change was observed: swelling of mitochondria still occurred in the granular cells. The increasing small secretory granules and expansion of rER were not observed (Fig. 9).

Discussion

In this study, we have demonstrated that SMP30 was strongly expressed in the epithelium of duct system, particularly in the granular cells in granular ducts. Acinar cells showed almost no SMP30 staining. These results suggest that SMP30 is closely related to a function of the ductal epithelial cells. In the SMP30-KO mice, there were no clear changes in the morphology of the acinar cells; however, marked mitochondrial changes

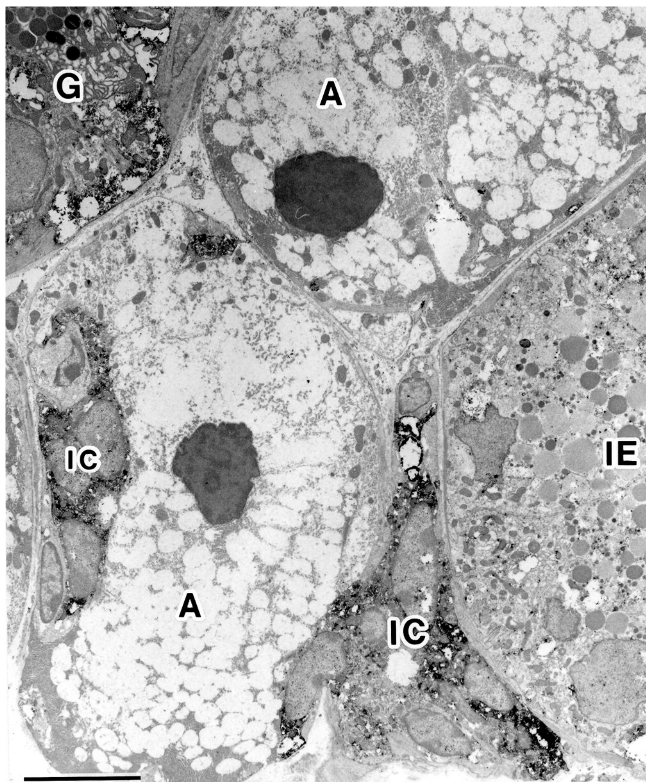

Fig. 2. Immunoelectron photomicrograph. 13-week-old male. Epithelial cells of intercalated ducts (IC) were stained positive, but acinar cells were negative. G: granular ducts, IE: intralobular excretory ducts, A: acinar cells. Bar: 10 µm.

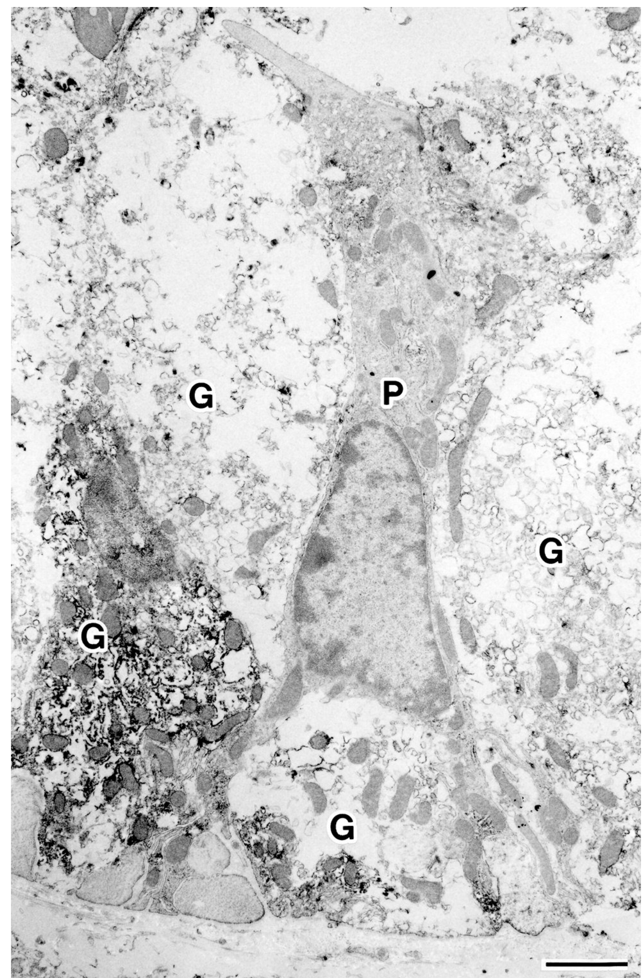

Fig. 3. Immunoelectron photomicrograph of the granular ducts. Granular cells (G) were positive, but pillar cells (P) were negative. Bar: 2 µm.

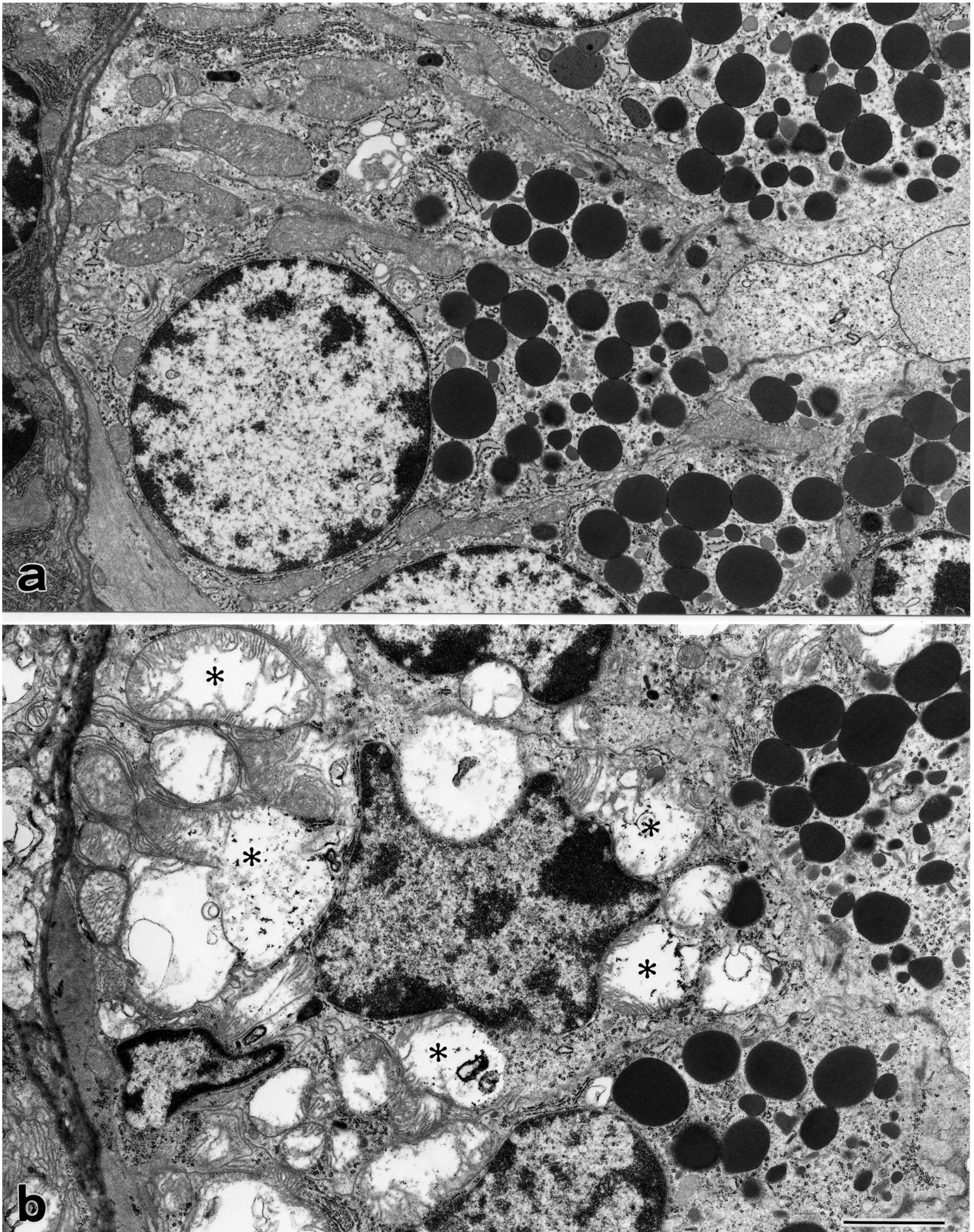

Fig. 4. Electron photomicrographs of the granular duct cells. **a.** SMP30-WT mouse (37-week-old female). No mitochondrial swelling was noted. **b.** SMP30-KO mouse (37-week-old female). Swollen mitochondria (*) were observed. Bar: 2 µm.

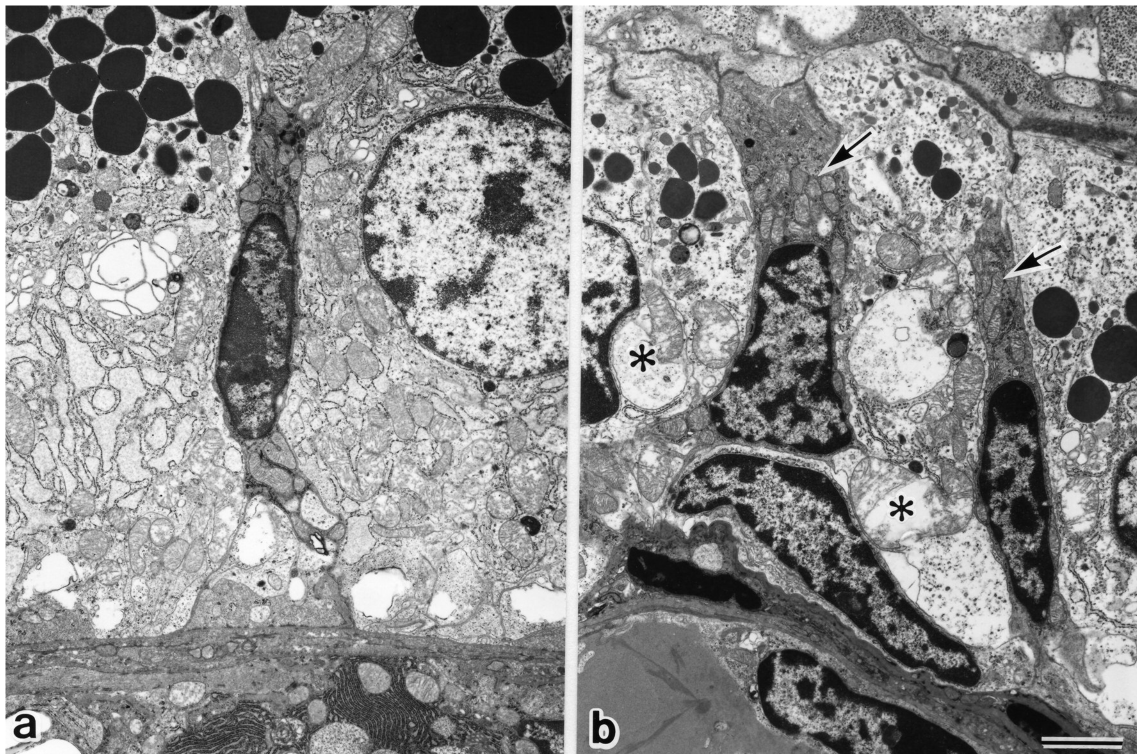

Fig. 5. Electron photomicrographs of the granular duct cells. **a.** SMP30-WT mouse (37-week-old female). Mitochondria in the granular and pillar cells showed a normal appearance. **b.** SMP30-KO mouse (37-week-old female). Swollen mitochondria (*) were observed. Unlike the granular cells, the pillar cells present in the granular duct epithelium did not show swelling of mitochondria (arrows). Bar: 2 μm.

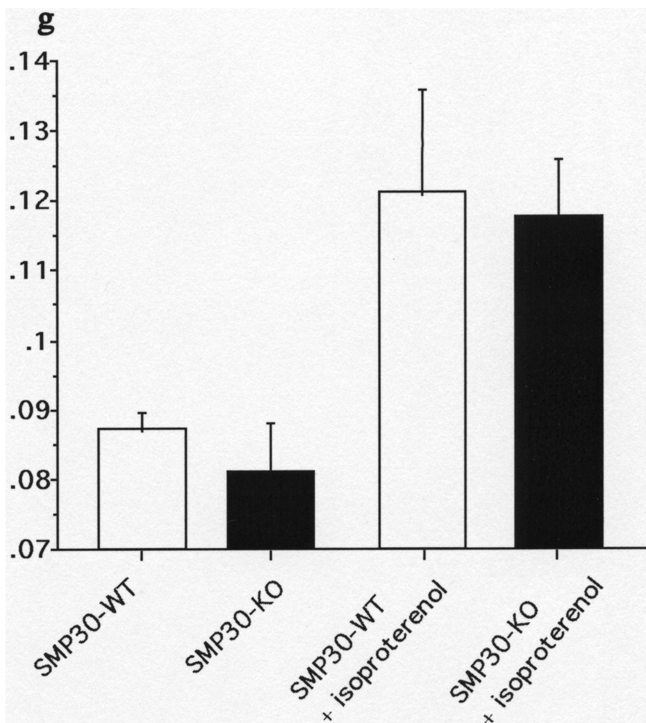

Fig. 6. Weight of the submandibular gland with or without α-isoproterenol administration.

were observed in the granular cells, particularly in the granular ducts. Recently, Xue et al. have reported that the Ca^{2+}-binding protein regucalcin, which is identical to SMP30, increases mitochondrial Ca^{2+}-ATPase activity, enhancing ATP-dependent Ca^{2+} uptake into mitochondria (Xue et al., 2000). Since digitonin, vanadate, and dithiothreitol inhibit the effects of regucalcin, regucalcin may increase mitochondrial Ca^{2+} pump activity by acting on an SH residue in the active site that is related to the phosphorylation of mitochondrial Ca^{2+}-ATPase (Xue et al., 2000). A close relationship exists between Ca^{2+}-ATPase activity and mitochondrial morphology. Swelling of mitochondria has been reported to occur in association with inhibition of Ca^{2+}-ATPase activity by the organophosphorus pesticide paraquat (Ueda et al., 1985). Swelling of mitochondria was observed in SMP30-KO mice, particularly in the granular cells. Morphological changes of the mitochondria may be related to loss of the Ca^{2+} regulating function of SMP30. In the submandibular gland, some Ca^{2+} regulating proteins are present. Expression of calmodulin has been immunohistochemically demonstrated in striated and intercalated duct epithelial cells. In contrast, the expression of calmodulin has not been demonstrated in the granular cells that occupy the majority of the granular duct epithelium (Hashimoto et al., 1992). Furthermore, calmodulin has been observed in pillar cells, which are present in small

numbers in the granular ducts (Hashimoto et al., 1992). Acinar cells are positive for S-100 beta, another Ca-binding protein (Lubert, 1991). We observed little mitochondrial swelling in pillar and acinar cells. Since only the granular cells are negative for the Ca-binding proteins including calmodulin, S-100 alpha, and S-100 beta (Hashimoto et al., 1992), the deficiency in SMP30 has a striking consequence in granular cells. Although further studies are needed to clarify the relationship between SMP30 and other Ca-binding proteins, it appears that SMP30 plays an important role in the granular duct cells.

In the salivary glands, Ca^{2+} plays an important role in the mechanism of salivary secretion as a second messenger for sympathetic and parasympathetic stimulation. It is well known that long-term treatment with ß-adrenergic stimulants, such as α-isoproterenol, causes hypertrophy in the parotid and submandibular glands (Brenner and Wulf, 1981; Henriksson, 1982). In normal animals, the administration of α-isoproterenol increases cAMP levels and activates the cAMP-dependent protein-phosphorylating enzyme. This enzyme phosphorylates phosphorylase kinase and promotes glycogenolysis (Lubert, 1991). Henriksson treated rats chronically with α-isoproterenol (Henriksson, 1982). Isoproterenol increased the submandibular gland weight and the acinar cell size, and caused a significant amylase release and accumulation of cAMP in the acinar cells (Kanagasuntheram and Randle, 1976). The Ca^{2+} signaling mediated by activation of beta-adrenoceptors was studied in a purified preparation of ducts from rat submandibular glands (Nezu et al.,

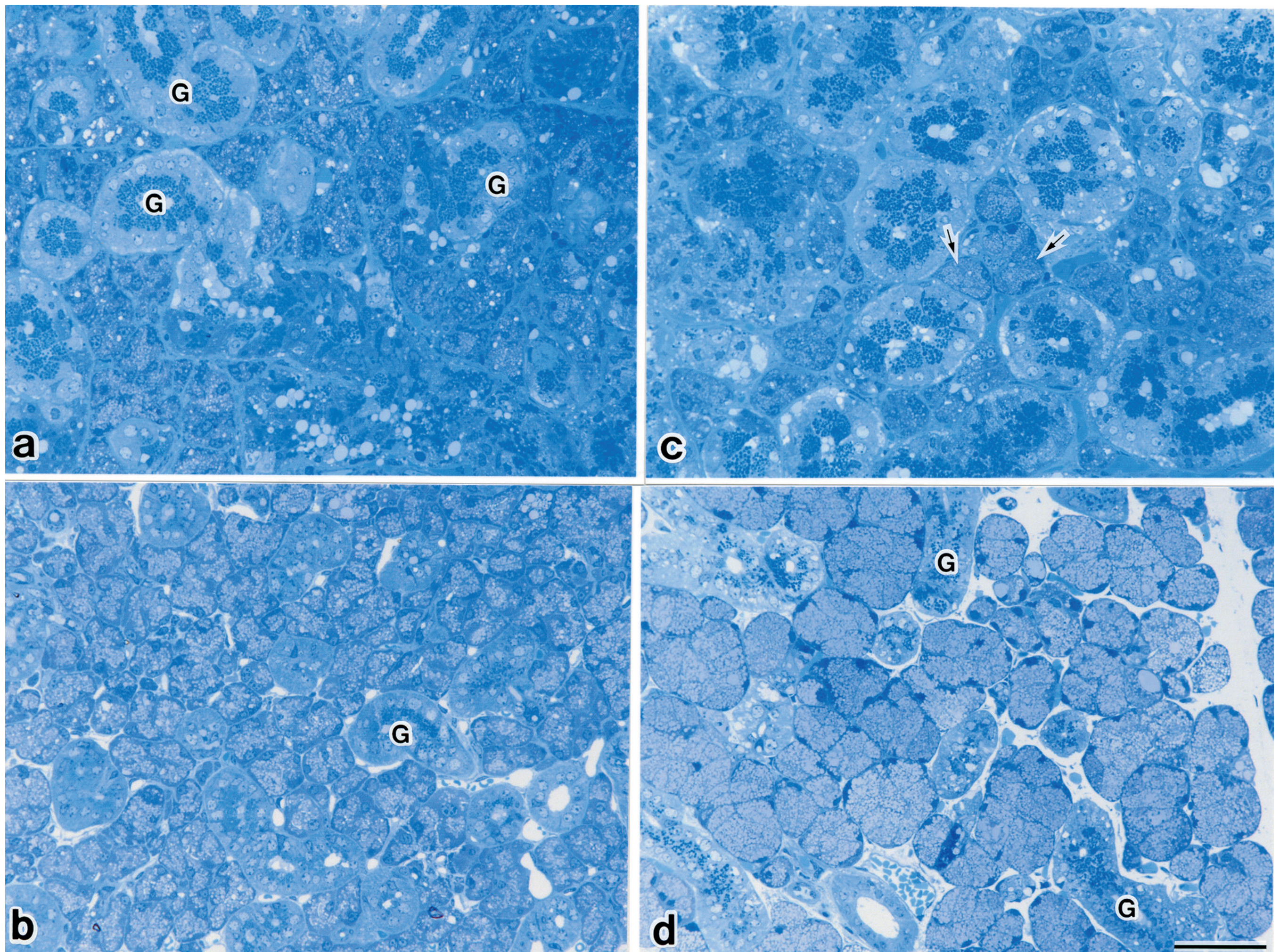

Fig. 7. Toluidine blue-stained semi-thin sections of the submandibular gland. **a, b.** No treatment. **a.** SMP30-WT mouse (19-week-old male). **b.** SMP30-KO mouse (19-week-old male). Not only was the diameter of the granular ducts (G) smaller, but also the granular ducts were sparse in the KO-mouse relative to the SMP30-WT mouse. **c, d.** After α-isoproterenol administration. **c.** SMP30-WT mouse (19-week-old male). After α-isoproterenol administration, arrows indicated the acinar cell hypertrophy. **d.** SMP30-KO mouse (19-week-old male). After α-isoproterenol administration, the acinar cell hypertrophy was clearly demonstrated. Bar: 50 µm.

2000). Isoproterenol treatment caused a small but significant increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). This α-isoproterenol-induced increase in $[Ca^{2+}]_i$ was completely inhibited by the beta-adrenoceptor antagonist propranolol, but not by the alpha-adrenoceptor antagonist phentolamine. The isoproterenol-induced increase in $[Ca^{2+}]_i$ in rat submandibular ducts is mediated by the activation of beta-adrenoceptors and subsequent accumulation of cAMP (Nezu et al., 2000). In this study, acinar cells from both SMP30-WT and -KO mice showed hypertrophy, indicating that the response of acinar cells to the adrenergic stimulant was not affected even in SMP30-KO mice. However, in SMP30-WT a large number of small secretory granules and expansion of rER were observed in the granular cells after administration of α-isoproterenol. The submandibular gland possesses a double autonomic innervation,

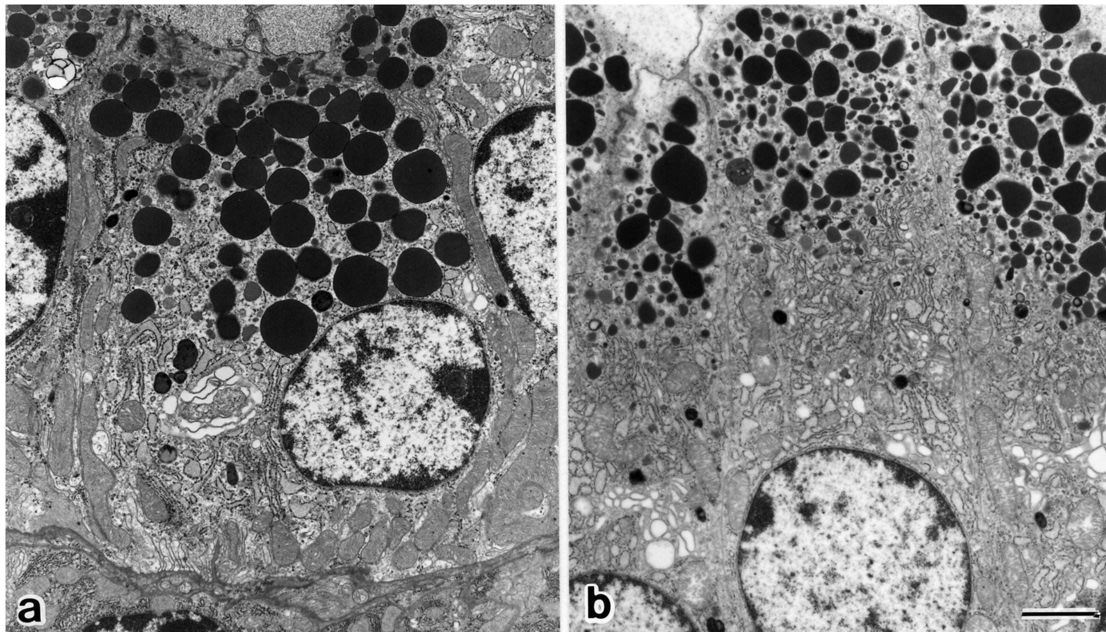

Fig. 8. Electron photomicrographs of SMP30-WT mouse submandibular glands with or without α-isoproterenol administration. **a.** SMP30-WT mouse (19-week-old male), no treatment. **b.** After α-isoproterenol administration, SMP30-WT mouse (19-week-old male). A large number of small granules and expansion of rER were observed in SMP30-WT mice after α-isoproterenol administration. Bar: 2 µm.

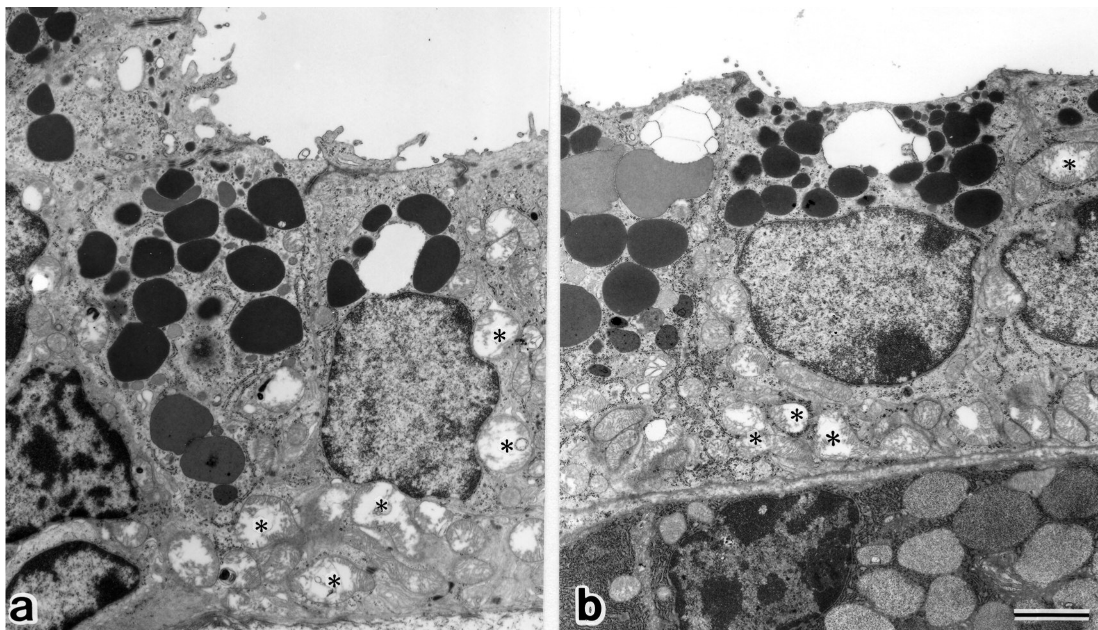

Fig. 9. Electron photomicrographs of the SMP30-KO submandibular gland with or without α-isoproterenol administration. **a**. SMP30-KO mouse (19-week-old male), no treatment. **b.** After α-isoproterenol administration, SMP30-KO mouse (19-week-old male). In the SMP30-KO mouse, little or no change was observed after α- isoproterenol administration. Swollen mitochondria (*). Bar: 2 µm.

consisting of both adrenergic and cholinergic nerve fibers (Schneyer et al., 1972; Takeda, 1978). Secretion of stored materials from granular ducts has been reported in response to stimulation by both cholinergic and adrenergic agents (Hanzen-Martin and Simon, 1986). They observed dramatic degranulation from granular ducts at 5 minutes after α-adrenergic agent (phnylephrine) stimulation. By 60 minutes following phenylephrine administration, the number of very small granules was increased. They considered the phenomena as a recovery. The response of granular ducts to ß-adrenergic agent (isoproterenol) was much less dramatic than that observed following α-adrenergic agent. However, at 60 minutes following isoproterenol stimulation, most granular duct cells contained abundant small granules. The increasing of small secretory granules observed in this study was considered as a phenomenon of recovery.

In contrast, in KO mice, the administration of α-isoproterenol may fail to transduce Ca-signaling, which presumably results in the lack of changes in organelles and secretory granules. Therefore, the response of granular cells to the adrenergic stimulant was affected by knocking out the SMP30 gene. We postulate that SMP30 is related to Ca^{2+} signaling mediated by the activation of beta-adrenoceptors.

In the present study, intercalated and striated duct cells also showed the immunoreactivity of SMP30. Northern blot and immunohistochemical analyses showed that SMP30 mRNA was strongly and specifically expressed in rat kidney. In the kidney, immunoreactivity was detected in proximal tubular epithelium, and less-intense immunoreactivity was observed in distal and collecting tubular epithelia (Fujita et al., 1992). It is hypothesized that granular duct cells develop from the cells near the intercalated duct (Jacoby and Leeson, 1959; Srinivason and Chang, 1975). Although the exact function of intercalated ducts is unknown, intercalated and striated ducts are believed to involve in the transport of electrolytes to saliva. Especially, striated ducts and distal tubules have many similarities in the structure and function. Striated ducts transport potassium into saliva and reabsorbing sodium ions (Thaysen et al., 1954). It is suggested that SMP30 relates to a signal transduction pathway consist of selective transport of electrolytes in the duct system of submandibular glands.

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