Tissue Injury-induced Reactive Oxygen Species Cause Dysfunction of Parotid Acinar Cells via Erk-novel PKC Activation

（組織傷害で発生する活性酸素による Erk-novel PKC 経路を介した耳下腺腺房細胞の機能低下）

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Abstract

Dysfunction of salivary glands and decrease in saliva are serious problems in clinical dentistry since saliva maintains oral health. Tissue injuries caused by γ-irradiation or autoimmune syndromes induce atrophy of salivary acinar cells. We have previously reported that cell isolation processes from rat parotid glands by digestion with enzymes mimic tissue injuries and result in decrease of expression of amylase, which indicates dysfunction of acinar cells. In this study, we found that a non-selective PKC inhibitor, Ro31-8220, and an inhibitor of MEK1/2, U0126, suppressed the decrease of amylase activity caused by cell isolation. Gö6983, an inhibitor for novel PKC, also suppressed its decrease although Gö6976, a specific inhibitor for conventional PKC, did not. Time-dependent increase in phosphorylation of novel PKCs during culture was detected. Their phosphorylation was inhibited by addition of Ro31-8220 or Gö6983, but not by Gö6976. Phosphorylation of novel PKC was inhibited by U0126 while Erk1/2 activation was not suppressed by Ro31-8220, which suggests that activation of Erk1/2 is upstream of phosphorylation of novel PKCs. Addition of diphenyleneiodonium suppressed the activation of Erk1/2. Therefore, tissue injury during cell isolation generates reactive oxygen species, which causes dysfunction of parotid acinar cells via activation of Erk1/2 and novel PKCs.

Introduction

Hyposecretion of saliva is a serious problem in clinical dentistry since saliva maintains the environment of the oral cavity. Decrease of saliva and consequent dry mouth lead to severe dental caries, periodontal disease, and mucosal infections (1, 2). Radiotherapy for head and neck cancers and autoimmune diseases such as Sjögren syndrome result in hyposecretion of saliva through atrophy of acinar cells because primary saliva is produced by acinar cells. Tissue injuries caused by irradiation and inflammation may lead to apoptosis or dysfunction of acinar cells, but the mechanism is unclear.

To clarify mechanisms of salivary gland dysfunction, we have established a system for primary culture of parotid acinar cells (3). Using the primary culture system, we have found that the process of cell isolation using digestive enzymes induced the stress signal mediated by Src and p38 MAP kinases (MAPK). p38 MAPK was phosphorylated during cell isolation, which is inhibited by Src kinase inhibitors (4). The signal pathway via Src-p38 MAPK induces the dedifferentiation of parotid acinar cells into duct-like cells (5). For example, the expression of acinar cell markers such as amylase and aquaporin-5 (AQP5) decreased and duct cell markers such as claudin-4 increased during the culture. These changes were retarded by addition of inhibitors against Src or p38 MAPK, but are not completely suppressed. Therefore, other pathways than Src-p38 MAPK are expected to be involved in the dysfunction process. It is useful to find pathways that induce dysfunction of salivary glands for development of remedy for xerostomia.

In the previous study, we have already found that tissue injury during cell isolation also induced activation of Erk1/2 (4). U0126, an inhibitor for MEK1/2, suppressed the decrease of amylase activity in parotid acinar cells by repression of the activation of Erk1/2.

Unlike activation of p38 MAPK, however, activation of Erks was not inhibited by addition of Src kinase inhibitors. Thus, the pathway mediated by Erk1/2 is different from that of Src-p38 MAPK. We investigated the signal transduction pathway mediated by Erk1/2 and focused on the role of members of protein kinase C (PKC). The PKC family consists of more than 10 isozymes and is divided into three subfamilies. PKC-α, -β, and -γ are called as conventional PKC that require both Ca^{2+} and diacylglycerol (DAG) for activation. Second group of PKC (PKC-δ, ε, η, θ) is called as novel PKC and requires DAG but not Ca^{2+}. Another group is atypical PKC (PKC-ι, λ, ζ), which requires neither Ca^{2+} nor DAG. We have already reported that a novel PKC, PKC-δ, is expressed in parotid acinar cells and mediates stimulus-dependent amylase secretion (6). In this study, we found that novel PKCs are also involved in the stress signal transduction that induces dysfunction of parotid acinar cells.

Materials and Methods

Preparation and culture of isolated acinar cells

Parotid glands were taken from male Sprague-Dawley rats (150 - 200 g each) anesthetized with sodium pentobarbital. The experiment conforms with institutional guidelines for the use of experimental animals and was approved by the Experimental Animal Ethical Committee of Nihon University School of Dentistry at Matsudo. Acinar cells were isolated by digestion with collagenase A and hyaluronidase in isolating buffer (Hanks' balanced salt solution containing 20 mM Hepes/NaOH, pH7.4) as described previously (7). The cells were over 90% viable, as determined by Trypan blue exclusion. Cells were diluted to 0.3 mg/ml with Waymouth's medium containing 10% rat serum, ITS-X supplement, 1 μM hydrocortisone, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 nM cystatin. Medium was changed at 1

day after the cell isolation. Cells were used for experiments immediately after isolation and after culture. Kinase inhibitors were purchased from Calbiochem (San Diego, CA).

Immunoblot analysis

Rabbit polyclonal anti-phospho-panPKC antibody, anti-phospho-PKC-α/β, anti-phospho-PKC-δ, anti-PKC-δ, anti-PKC-ε, and anti-phospho-Erk1/2 antibodies were purchased from Cell signaling Technology Japan (Tokyo, Japan). Rabbit polyclonal anti-Erk1/2 antibody was purchased from Biosource International Inc (Camarillo, CA). Mouse monoclonal anti-occludin antibody was purchased from Zymed laboratories Inc (South San Francisco, CA). Cells were harvested and lysed in homogenizing buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, 50 mM β–glycerophosphate, 20 mM Hepes/NaOH, pH 7.4) containing 1 × Complete Protease Inhibitor Cocktail. Proteins were separated by SDS-PAGE, and were transferred to Hybond-LFP membranes (GE Healthcare, Buckinghamshire, UK). The same amounts of proteins were applied to each lane of SDS-PAGE. The membranes were blocked at room temperature for 50 minutes in Blocking Agent (GE Healthcare) and blotted with antibodies. Immunoreactivity was determined by using an ECL plex Western blotting kit (GE Healthcare) and the images were acquired by using Typhoon Trio (GE Healthcare) and the fluorescence intensities were measured with ImageQuant (GE Healthcare).

Amylase assay

The activity of amylase was measured by the method reported previously (3), modified for incubation at 30°C for 5 minutes. In this method, one unit of amylase is defined as the

quantity of enzyme that liberates 1 mg of maltose per minute at 30°C. Relative amylase activity is defined as amylase activity included in 1 mg proteins.

Immunofluorescence microscopy

Cells were cultured in collagen I-coated glass base dishes in the absence or presence of 2.5 μM Ro31-8220. After fixation with 10% formalin in PBS, permeabilization with 0.2% Triton X-100, and blocking with goat IgG and bovine serum albumin, cells were labeled with anti-amylase (1:100) and anti-occludin (1:100) antibodies, followed with AlexaFluor 568-anti-rabbit (1:50) and AlexaFluor 488-anti-mouse antibodies. Images were acquired by using LSM-510 laser confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All values are reported as means ± SE. Studies with more than two groups were evaluated by an analysis of variance (ANOVA). The ANOVA included a Dunnett's multiple-comparison test. $P < 0.05$ was considered significant.

Results

Inhibition of Erk1/2 and PKC suppressed the decrease of amylase activity

Amylase is one of the main components secreted from parotid acinar cells and can be used as an acinar cell marker. During culture of primary parotid acinar cells, amylase activity is rapidly declined, which suggests dysfunction of acinar cells. To find a method to rescue the dysfunction of parotid glands, we studied the effect of kinase inhibitors on the decrease of amylase activity. We have already found that U0126, a MEK1/2 inhibitor, retarded the rapid

decrease of relative amylase activity (ref. 4 and Fig. 1A) and that the pathway is different from the Src-p38 MAPK signaling pathway. Because activation of PKC has been reported to induce an apoptotic program in salivary acinar cells (8), we examined the effect of PKC inhibitors. We found that Ro31-8220, which inhibits both conventional and novel PKCs (9), also suppressed the decline of amylase activity. Then, we examined the effect of other PKC inhibitors. Gö6976 hardly have an effect while Gö6983 significantly suppressed the decrease of amylase activity (Fig. 1B). Gö6976 is known to inhibit conventional PKC but not novel PKC (10). Gö6983 have an inhibitory effect on novel PKC in addition with conventional PKC (11). Therefore, novel PKC may be involved in the process of salivary gland dysfunction.

Maintenance of secretory granules by inhibition of PKC activity

The maintenance of secretory granules is a good index of differentiation degree of salivary acinar cells. We observed granules in the control and Ro31-8220-treated cells by labeling with anti-amylase antibody. In the control culture, most cells form monolayers while some form semispherical clusters (12). The cells in the clusters retain secretory granules better than those in the monolayers, which lose most granules after 3-days culture (ref. 12; Fig. 2A). In the Ro31-8220-treated culture, the cells even in the monolayers also retained granules better than in the control culture (Fig. 2B). Therefore, inhibition of PKC suppressed the dysfunction of salivary acinar cells.

Phosphorylated forms of PKC-δ and -ε increase during the culture

Because the activity of PKC is involved in the stress signal that induces dysfunction of parotid acinar cells, we examined the activation of PKC during the culture. The activity of

PKC is regulated by phosphorylation events. Phosphorylation of Ser at the carboxy terminus indicates activation of conventional and novel PKCs. We used anti-phospho-panPKC antibody, which recognizes phosphorylated form of PKC-α, β, δ and ε isoforms. This antibody detected two bands with molecular weights of 78 and 85 kDa in the cultured cells (Fig. 3). Because PKC-α/β was not detected by immunoblotting analysis using a specific antibody for PKC-α/β (data not shown), the two bands correspond with PKC-δ (78 kDa) and -ε (85 kDa), respectively. The lower band was also reacted with anti-phosphorylated PKC-δ antibody. Thus, the phosphorylated forms of two novel PKCs increased over time during the culture.

Signal transduction pathway involved in phosphorylation of novel PKC

To confirm that the two bands detected with anti-phospho-panPKC antibody are novel PKCs, the effect of PKC inhibitors was examined. In the presence of Ro31-8220 and Gö6983, which inhibits both conventional and novel PKCs, the band intensities were decreased. In contrast, Gö6976, a specific inhibitor for conventional PKC, did not affect their phosphorylation (Fig. 4A). Because the effects of U0126 and Ro31-8220 on maintenance of amylase activity are similar, the effect of U0126 on the phosphorylation of PKC was examined. U0126 diminished the phosphorylation of both PKC-δ and -ε. This result indicates that activity of MEK/2 is necessary for activation of novel PKC.

On the other hand, Src kinase inhibitor PP1 slightly suppressed only PKC-ε and increased phosphorylation of PKC-δ to a small extent (Fig. 4B). In keratinocytes, Src kinase phosphorylates tyrosine residues of PKC-δ, resulting its inactivation. The anti-phospho-panPKC antibody that we used recognizes phosphorylated Ser^{643} of PKC-δ and Thr^{710} of PKC-ε, but not phosphorylated tyrosine residues. Although we have not investigated

the tyrosine phosphorylation of PKC-δ, it is likely that inhibition of Src kinase activity reduced tyrosine phosphorylation of PKC-δ and consequently enhanced its activity.

Phosphorylated forms of Erk1/2 were detected after cell isolation, which indicates their activation. In the presence of U0126, which inhibits MEK1/2 (MAPK kinases), the activation of Erk1/2 was completely suppressed. In contrast, Ro31-8220 did not affect the phosphorylation of Erk1/2 (Fig. 5A). Therefore, phosphorylation of novel PKC is downstream of activation of Erk1/2.

Reactive oxygen species induce activation of Erk1/2

Reactive oxygen species (ROS) has been reported to be involved in the pathophysiology of Sjögren's syndrome and irradiation-induced dysfunction of salivary glands (13, 14). Cellular stresses caused by tissue injuries and irradiation may induce generation of ROS. Because diphenyleneiodonium (DPI), an NAD(P)H oxidase inhibitor, can be used to inhibit ROS production (15), we examined the effect of DPI on the activation of Erk1/2. In the presence of DPI, the phosphorylation level of Erk1/2 during cell isolation was decreased (Fig. 5B). This result suggests that enzyme digestion of salivary glands causes ROS generation, which induces dysfunction of acinar cells via activation of Erk1/2.

Discussion

We have previously shown that enzyme digestion of parotid glands causes dedifferentiation and dysfunction of acinar cells. The signal pathway mediated by Src and p38 MAP kinases plays an essential role in the dedifferentiation process. In this study, we found another

pathway of ROS-induced stress signal involved in activation of Erks and novel PKCs. In salivary glands, γ-irradiation induces apoptosis of acinar cells via ROS generation (16). In general, ROS induces apoptosis or epithelial-mesenchymal transition of epithelial cells and leads to fibrosis. Although ROS had been thought to nonspecifically attack biological molecules as a toxin, it is now considered to mediate physiological signaling pathway to protect from cell damage (17). However, the signaling pathway in salivary acinar cells is not well known.

Erks are involved in many biological process and have been reported to regulate ROS-induced cell death in hepatocytes (18). We found that Erks are activated during cell isolation of parotid acinar cells and inhibition of Erks retarded the decrease of amylase. In addition, cells retained amylase activity in the presence of inhibitors for novel PKC, which indicates that novel PKCs also participate in the dedifferentiation process. In a previous report, activation of novel PKC was necessary for Erk-mediated Na-K-ATPase phosphorylation in rat parotid acinar cells (19). On the contrary, PKC activation was reported to inactivate Erks in the process of apoptosis of salivary cells (8). In our study, inhibition of novel PKC did not suppress the phosphorylation of Erks (Fig. 5A). Because the signal pathways to activate Erk are various, Erks were probably activated by upstream components other than PKCs in this dedifferentiation signal pathway. On the other hand, activity of Erks was essential for phosphorylation of novel PKC (Fig. 4B). These results suggest that activation of novel PKC is downstream of Erk signaling pathway. Because Erks regulate transcription directly and indirectly, there was a possibility that inhibition of Erks reduced the amount of PKCs. In our study, however, the protein levels of PKC-δ and -ε were not changed by U0126 (Fig. 4B), suggesting that Erks regulate the phosphorylation, but not expression levels of novel PKCs.

Because the inhibitors of Erks and novel PKCs have a protective effect on the decrease of amylase in acinar cells, there is a possibility that regulation of the signal pathway can protect the salivary glands from irreversible dysfunction.

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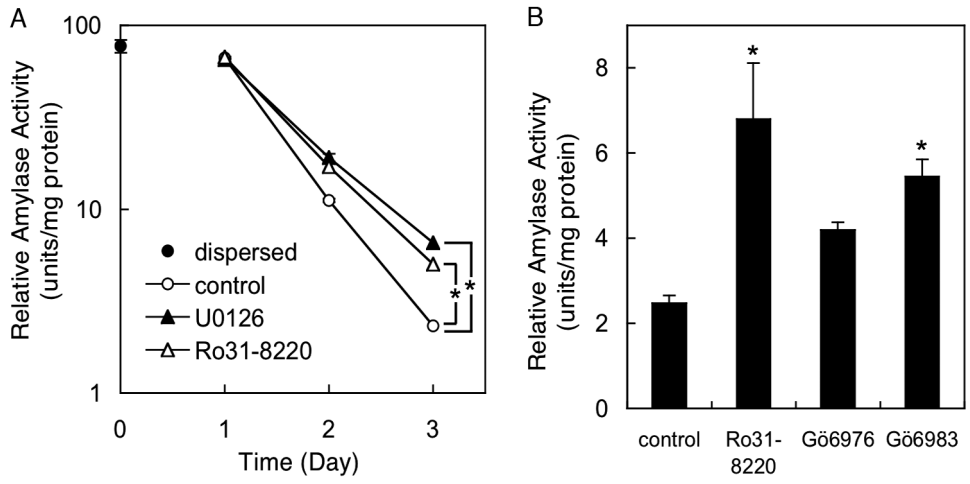

Fig. 1 Effects of kinase inhibitors on reduction of amylase activity during cell culture.

A: Cells were harvested just after cell isolation and at 1, 2 and 3 days for amylase assay. Reduction of relative amylase activity was suppressed in the presence of 25 μM U0126 or 2.5 μM Ro31-8220 compared with the control culture (*$p < 0.05$). B: Ro31-8220 (2.5 μM) and Gö6983 (8 μM) significantly suppressed the decrease of amylase activity compared with the control culture (*$p < 0.05$) while there is no significant difference between relative amylase activities in the lysates of the control and Gö6976 (8 μM)-treated cells.

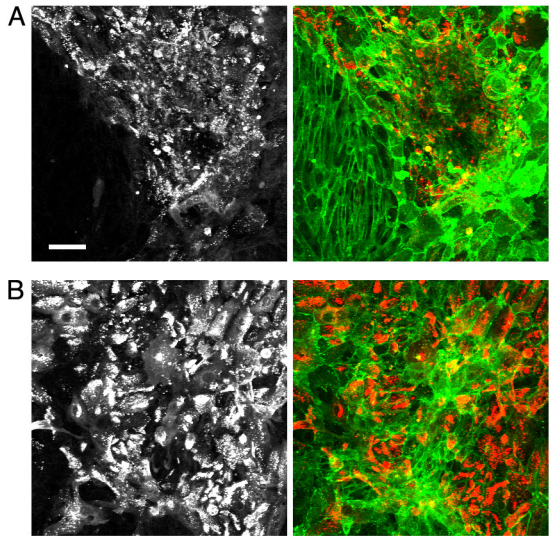

Fig. 2 Maintenance of secretory granules by inhibition of PKC activity.

Cells were cultured in the absence (A) and presence (B) of Ro31-8220 for 3 days. The cells were fixed and stained with anti-amylase antibody to visualize secretory granules (left panels). The right panels are merged images labeled with antibodies for amylase (red) and occludin (green). Bar, 50 μm.

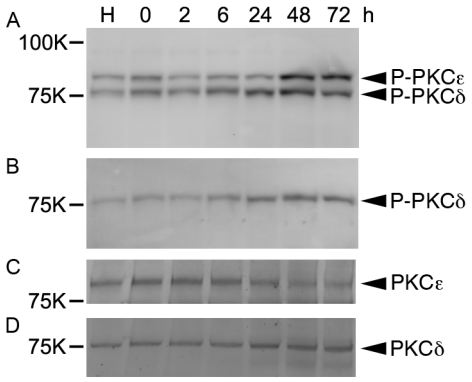

Fig. 3 Change of phosphorylation levels of PKC-δ and -ε during culture.

Cells were harvested just after cell isolation and at 2, 6, 24, 48, and 72 h after isolation. Homogenates (H) of parotid glands were prepared before cell isolation and were used as a control. Cell lysates (15 μg each) were loaded to 7.5% SDS-PAGE, transferred and visualized using a polyclonal anti-phospho-panPKC (A) or anti-phospho-PKC-δ (B), anti-PKC-ε (C), and anti-PKC-δ (D) antibodies.

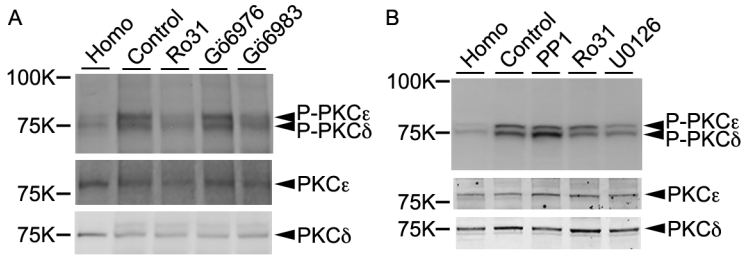

Fig. 4 Inhibition of phosphorylation of PKCs by kinase inhibitors.

Cells were harvested at 3 days after cell isolation. Homogenates (Homo) of parotid glands were prepared before cell isolation. The cells cultured in the absence of kinase inhibitors were used as control. Cell lysates (15 μg each) were used for immunoblotting analysis of phosphorylation of novel PKCs. A: Ro31-8220 (Ro31) and Gö6983 diminished the phosphorylation of PKCs while Gö6976 had no effect. B: Addition of U0126 to the culture medium also decreased the phosphorylation levels of novel PKCs while PP1 only slightly suppressed activation of PKC-ε.

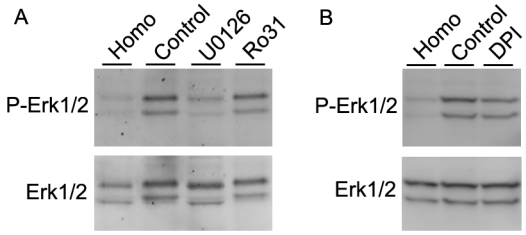

Fig. 5 Involvement of ROS generation in Erk activation

Cells were harvested just after cell isolation. Homogenates (Homo) of parotid glands were prepared before cell isolation. The cells isolated in the absence of kinase inhibitors were used as control. Cell lysates (15 μg each) were used for immunoblotting analysis of Erk activation. A: Ro31-8220 (Ro31) had no effect on activation of Erk1/2 while U0126 completely suppressed their phosphorylation. B: Addition of 10 μM DPI decreased phosphorylation levels of Erk1/2.