Original

Suppression of parotid acinar cell dysfunction by the free radical scavenger 3-methyl-1-phenyl-2-pyrazolin-5-one

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Abstract: Salivary gland atrophy and consequent hyposalivation are serious problems in clinical dentistry, as saliva regulates the environment of the oral cavity. To clarify the mechanisms underlying salivary gland dysfunction, a system for primary culture of parotid acinar cells has been established. It has been reported previously that the process of cell isolation from parotid glands triggers stress signaling mediated by Src and p38 mitogen-activated protein (MAP) kinase (p38), leading to dedifferentiation of acinar cells, and that an nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor suppresses this activation of Src and p38, suggesting that reactive oxygen species initiate the dedifferentiation signal. The present study examined the effect of a free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (also termed MCI-186 or edaravone), on activation of the stress signal and the secretory function of parotid acinar cells. Activation of p38 during cell isolation was suppressed by addition of MCI-186. The retention of the activity of amylase, a major salivary protein, and the number of amylase-containing secretory granules were improved by isolation and culture in the presence of MCI-186. In addition, calcium elevation upon stimulation with a muscarinic agonist was

higher in MCI-186-treated cells than in untreated cells. These results suggest that MCI-186 (edaravone) is a promising agent for prevention of salivary gland dysfunction.

Keywords: salivary glands; dry mouth; hyposalivation; oxidative stress; calcium signaling; exocrine.

Introduction

Saliva is essential for maintenance of the oral environment. Hyposecretion of saliva causes serious caries, periodontitis, and oral mucosal infections due to poor oral hygiene (1). In addition, the associated decrease in salivary proteins, which have lubrication properties, also impairs mastication, swallowing and articulation. Therapeutic radiation for head and neck cancers causes apoptosis and atrophy of acinar cells and an increase of duct cells and connective tissues in salivary glands. Thus, many such patients suffer an irreversible decrease in saliva after radiotherapy. In addition, chronic inflammatory conditions, such as Sjögren's syndrome, can induce tissue damage and decrease parenchymal cells. Therefore, approaches for protecting salivary gland cells from such damage are urgently needed.

It is difficult to maintain the functions of exocrine cells, such as salivary acinar cells, in primary cultures or as cell lines. This is one of the major problems hindering studies of salivary acinar cell function. To study the mechanisms allowing maintenance of salivary acinar cell secretory function, a system for primary culture of parotid acinar cells that maintain their capacity to generate secretory granules (SGs) and stimulus-dependent secretion of sali-

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vary proteins has been established (2). However, it was found that this capacity gradually decreased, and that expression of differentiation markers changed during culture (3); the expression levels of acinar markers, such as amylase and aquaporin-5, decreased, while ductal markers and mesenchymal markers increased. Inhibitors of Src and p38 mitogen-activated protein (MAP) kinase (p38) suppressed these changes and increased the expression of acinar marker proteins. These results suggest that the Src-p38 signaling pathway plays a role in triggering cell dedifferentiation to ensure survival in response to cellular stress (4). Diphenyleneiodonium (DPI), nicotinamide adenine dinucleotide phosphate (NADP) oxidase inhibitor, suppresses activation of Src and p38 (5). Therefore, reactive oxygen species (ROS) may be generated during cell isolation, and these may activate Src. It has been suggested that oxidative stress is involved in the development of Sjögren's syndrome (6) and hyposalivation after γ -irradiation (7).

However, the use of DPI to suppress stress signals carries a risk of side effects, because DPI also has inhibitory activities against other enzymes, such as nitric oxide synthetases. The present study was performed to examine the effect of 3-methyl-1-phenyl-2-pyrazolin-5-one (also known as MCI-186 or edaravone), which has been approved for the treatment of acute-phase brain infarction and amyotrophic lateral sclerosis (8,9), on the dedifferentiation signaling and secretory function of parotid acinar cells.

Materials and Methods

Materials

Rabbit polyclonal anti-amylase and mouse monoclonal anti-occludin antibodies were purchased from Merck (Darmstadt, Germany). Mouse monoclonal anti-p38 and rabbit polyclonal anti-phosphorylated p38 antibodies were purchased from BD Biosciences (Sparks, MD, USA) and R&D Systems (Minneapolis, MN, USA), respectively. MCI-186 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Culture medium and supplements were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation and culture of isolated acinar cells

All animal experiments were carried out in accordance with institutional and national guidelines for the care and use of experimental animals and were approved by the Experimental Animal Committee of the Nihon University School of Dentistry at Matsudo (AP15MD018). Parotid glands were removed from male Sprague-Dawley rats (weighing 150–200 g) under inhalation anesthesia

with 3% sevoflurane. Acinar cells were isolated from the glands as described previously (10). Briefly, finely minced glands were digested with collagenase and hyaluronidase in isolation buffer (Hanks' balanced salt solution containing 20 mM HEPES-NaOH, pH7.4) in the absence or presence of MCI-186. The isolated cells were diluted to 0.3 mg/mL protein in Waymouth's medium containing 10% rat serum, ITS-X supplement, 1 μ M hydrocortisone, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 10 nM cystatin, and cultured at 37°C in 5% CO₂. The medium was changed 1 day after cell isolation. MCI-186 was dissolved in dimethylsulfoxide (DMSO) to a concentration of 50 mM as a stock solution, and then diluted 1:1,000 to a final concentration of 50 μ M for use. The same volume of DMSO was added to the control medium (final, 0.1%).

SDS-PAGE and immunoblotting analysis

Cells were lysed with 20 mM HEPES-NaOH (pH7.4) containing 0.1% Triton X-100 and 1 $\times$ Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The proteins were separated by SDS-PAGE and transferred to Hybond-LFP membranes (GE Healthcare, Chicago, IL, USA). The membranes were blocked at room temperature for 1 h in blocking reagent (GE Healthcare) and probed with the primary antibodies. Immunoreactivity was determined using ECL-Plex (GE Healthcare), and images were acquired using Typhoon Trio (GE Healthcare). The intensities of the immunoreactivities were quantified using ImageQuantTL software (GE Healthcare). The intensity of immunoreactivity in each MCI-treated sample was normalized to that in an untreated sample on the same blotting membrane. After the normalized values of total and phosphorylated p38 had been acquired, the ratio of the phosphorylated form to total p38 was calculated in each experiment.

Immunofluorescence microscopy of cultured acinar cells

Acinar cells were plated on collagen I-coated dishes (Iwaki, Tokyo, Japan) and cultured for 3 days. The cells were then treated with 10% formalin in phosphate-buffered saline (PBS) for 5 min and subsequently treated with 0.2% Triton X-100 in PBS for 10 min. After blocking with bovine serum albumin and preimmune goat IgG, the cells were labeled with the primary antibodies, followed by the respective Alexa Fluor-labeled secondary antibodies. Images were acquired using a 100 $\times$ 1.46 α Plan-Apochromat oil objective lens on an LSM-510 laser confocal microscope (Carl Zeiss, Oberkochen, Germany). Optical sections were taken along the z-axis

at 0.5- μ m intervals. The number of granules was counted by spot analysis with Imaris software (Bitplane, Zurich, Switzerland). Amylase-positive spots were selected with a threshold diameter of 0.5 μ m. When the software did not count the spots because of overcrowding, they were excluded because it was not possible to judge whether they were granules.

Amylase release assay

Cells cultured in dishes were incubated with release buffer (20 mM HEPES-NaOH, pH 7.4, 128 mM NaCl, 4 mM KCl, 1.2 mM MgCl₂, 1 mM CaCl₂, 1 mM NaH₂PO₄, 5 mM hydroxybutyrate) in the absence or presence of 10⁻⁶ M isoproterenol (Iso) at 37°C for 15 min. After incubation, the solution was removed, and the remaining cells were harvested. Amylase activity was measured using a method reported previously (2). One unit of amylase was defined as the quantity of enzyme that liberates 1 mg of maltose during 1 min at 30°C.

Measurement of intracellular Ca²⁺ mobilization

Isolated parotid acinar cells were loaded with 2 μ M fura-2 AM (Thermo Fisher) in loading buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 10 mM glucose, 1.25 mM probenecid, and 0.2% bovine serum albumin) at 37°C for 30 min. The cells were rinsed three times and suspended in Ca²⁺-free buffer. The fluorescence of the fura-2-loaded cells was measured by using CAF-110 spectrofluorometer (Jasco, Tokyo, Japan) with excitation at 340 and 380 nm, and emission at 510 nm. Elevation of the intracellular Ca²⁺ concentration was evaluated as the increase in the ratio of fluorescence intensities at 340 nm and 380 nm (F340/F380).

Statistical analyses

All values are reported as means $\pm$ SEM. Statistical analyses of the differences in the means of experimental pairs were evaluated by the paired *t*-test. The *P* values obtained are indicated in the figure legends when statistically significant.

Results

Activation of p38 during cell isolation is suppressed by addition of MCI-186

It had been shown previously that p38 was activated during cell isolation from parotid glands, detected as an increase in its phosphorylation (4). Here, acinar cells were isolated from rat parotid glands in the absence and presence of MCI-186. Phosphorylation of p38 was detected by immunoblotting in the cells that had been

Fig. 1 Suppression of p38 activation by addition of MCI-186.

(A) Immunoblotting analysis of lysates from control (–) and MCI-186-treated (+) cells with antibodies against the phosphorylated form (anti-P-p38) and total p38 (anti-p38). (B) Quantitative analysis of the immunoblots. After the intensity of immunoreactivity in the MCI-treated sample had been normalized to that in an untreated sample (control) on the same blotting membrane, the ratio of the phosphorylated form to total p38 (P-p38/p38) was calculated in each experiment. Phosphorylation of p38 was significantly suppressed by the addition of MCI-186 (***P* < 0.01, paired *t*-test, *n* = 4).

isolated without MCI-186 (Fig. 1). In cells that had been isolated in the presence of MCI-186, however, the phosphorylated p38 was significantly decreased, although the amount of total p38 was unchanged.

Maintenance of amylase activity and secretory granules in MCI-186-treated cells

The activity of amylase, the most abundant salivary protein in parotid acinar cells, was examined to evaluate the degree of cell differentiation. After 3 days of culture in the absence and presence of MCI-186, the cells were harvested and their amylase activity was measured as the relative activity per mg of protein in the cell lysate. The relative amylase activity in the MCI-186-treated cells was significantly higher (1.5-fold) than that in the control cells (Fig. 2).

Salivary proteins like amylase are stored in SGs until stimulation. SGs are released through secretagogue-dependent exocytosis, which is an important function of exocrine cells. In primary culture, the acinar cells spread over the dish surface and formed a monolayer, and the number of SGs decreased gradually during culture (3). When SGs were labeled with an anti-amylase antibody at 3 days after isolation, the distribution of granules in the cells was not uniform (Fig. 3A); some cells lost most of their granules, whereas others retained many. To compare the number of remaining granules in the control

Fig. 2 Effect of MCI-186 on the decrease in relative amylase activity during culture.

The relative amylase activity (units/mg protein) in the lysates from control and MCI-186-treated cells after 3 days of culture is shown. Although the relative amylase activity was lower than that in the cells just after isolation, the addition of MCI-186 suppressed the decrease in amylase activity (* $P < 0.05$, paired t -test, $n = 4$).

and MCI-186-treated cells (Fig. 3A, B), all the granules were counted in images ($142.86 \mu\text{m} \times 142.86 \mu\text{m}$) of the confluent monolayer, and the number of granules per unit area was calculated. MCI-186-treated cells retained more granules (1.7-fold) than the control cells (Fig. 3C). The difference in the number of granules between the two cultures was consistent with the remaining amylase activity.

Maintenance of stimulus-dependent amylase secretion in both control and MCI-186-treated cells

The cells were cultured in the absence and presence of isoproterenol (Iso) and the medium was collected for measurement of the released amylase activity, determined as the percentage of the total activity in whole cells. A small amount of amylase was released without stimulation, and Iso enhanced the release from both control and MCI-186-treated cells (Fig. 4). Addition of Iso increased amylase secretion in control and MCI-168-treated cells 2.8-fold and 3.0-fold, respectively. This enhancement was comparable to that observed in the cells just after isolation (2). Stimulus-dependent exocytosis capacity was not greatly impaired, even after 3 days of culture, although the synthesis of the amylase protein declined. MCI-168 did not promote the response to secretagogues, although it increased the number of remaining granules.

Enhancement of the increase in intracellular Ca^{2+} concentration in MCI-186-treated cells

Muscarinic stimulation increases the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which is essential for fluid secretion of saliva. Control cells isolated without MCI-186 responded to $10 \mu\text{M}$ carbachol (CCh). The rapid increase

Fig. 3 Effect of MCI-186 on the number of secretory granules retained in cultured acinar cells.

Immunofluorescence microscopy of control (A) and MCI-186-treated (B) cells labeled with anti-amylase (red) and anti-occludin (green) antibodies. Optical sections were taken along the z -axis at $0.5\text{-}\mu\text{m}$ intervals, and the images shown are single z -planes. Spots labeled with the anti-amylase antibody correspond to secretory granules (SGs). Bars, $20 \mu\text{m}$. (C) Quantitative analysis of the density of SGs in the cells. Values are the number of granules per unit area. MCI-186-treated cells retained significantly more granules than the control cells (* $P < 0.05$, t -test, $n = 8$).

in $[\text{Ca}^{2+}]_i$ observed in the absence of extracellular Ca^{2+} indicates the release of Ca^{2+} from the intracellular Ca^{2+} pool (Fig. 5A). Addition of extracellular Ca^{2+} (final concentration 2.5 mM) induced an additional and sustained increase in $[\text{Ca}^{2+}]_i$, corresponding to Ca^{2+} influx from the extracellular buffer. The CCh-dependent increase in $[\text{Ca}^{2+}]_i$ was higher in MCI-186-treated cells than in control cells (Fig. 5A). The increase from the baseline to the peak F340/F380 was significantly higher in MCI-treated cells than in control cells (Fig. 5B).

Discussion

MCI-186 is a free radical scavenger widely used to treat ischemic brain damage and neurodegenerative diseases (8,9). It is also thought to protect other organ systems such as the cardiovascular system, and to suppress autoimmune disease (11–13). The present study demonstrated that MCI-186 slightly improved the function of isolated salivary acinar cells.

MCI-186 treatment suppressed the phosphorylation

Fig. 4 Response of cultured acinar cells to β -adrenergic stimulation.

At 3 days after cell isolation, cultured acinar cells were incubated with or without (w/o) 10^{-6} M isoproterenol (Iso) at 37°C for 15 min. Amylase activity in the medium and cell lysate was measured, and the amount of released amylase is shown as a percentage of the total amylase. There was no significant difference between the control and MCI-186-treated cells ($n = 4$, paired t -test).

of p38, which is mediated by Src activation, as shown previously (4). Generation of ROS may be involved in Src activation, as the NADPH oxidase inhibitor DPI suppresses the activation of both Src and p38 (5). However, the viability of cultured cells at 24 h after isolation was decreased in the presence of DPI (data not shown). DPI may also inhibit enzymes that are essential for survival. In the presence of MCI-186, the cells survived and retained amylase-containing SGs. In general, primary cultures of exocrine cells rapidly lose their SGs (14,15). In the present primary culture system, the cells maintained the ability to generate SGs, but the amount of amylase decreased to less than 10% of that in the cells just after isolation (about 60 units/mg protein). In this study, the addition of MCI-186 increased amylase activity as well as the number of SGs. In contrast, amylase secretion was detected in both control and MCI-186-treated cells, and the enhancement of secretion induced by β -adrenergic stimulation was comparable to that observed in the cells just after isolation. Thus, during their generation, SGs may acquire the machinery required for stimulus-dependent exocytosis.

Muscarinic stimulation induces the secretion of water and ions from salivary acinar cells. Elevation of intracellular Ca^{2+} , mediated by the inositol triphosphate receptors IP_3R_2 and IP_3R_3 , is essential for this secretion (16). The increase in the F340/F380 ratio induced by addition of CCh in MCI-186-treated cells was significantly higher than the increase induced in control cells. This result suggested that fluid secretion capacity would be more

Fig. 5 Effect of MCI-186 on calcium mobilization in isolated parotid acinar cells.

(A) Cells were isolated from parotid glands in the absence and presence of MCI-186, and the freshly isolated cells were loaded with fura-2. Cells suspended in Ca^{2+} -free buffer (0 mM Ca^{2+}) were stimulated with $10 \mu\text{M}$ carbachol (CCh), and then 2.5 mM Ca^{2+} was added to the extracellular buffer. The values are shown as the ratio of the fluorescence intensities at 510 nm following excitation at 340 nm and 380 nm (F340/F380). (B) The increases in the F340/F380 ratio from the baseline to the peak response to CCh ($\Delta\text{F340/F380}$) were compared. The response to CCh in the MCI-186-treated cells was significantly higher than the response in the control cells ($*P < 0.05$, paired t -test, $n = 4$).

efficiently maintained in the presence of MCI-186. ROS production induced by irradiation and inflammatory signals has been reported to reduce Ca^{2+} signaling in salivary gland cells. Irradiation induces loss of STIM1, which is essential for Ca^{2+} influx from extracellular buffer and sustained elevation of intracellular Ca^{2+} (17). A phosphodiesterase inhibitor, rolipram, which suppresses ROS production, enhanced CCh-induced Ca^{2+} signaling in submandibular gland cells (18). During isolation from the glands, the cells were exposed to ROS, which may depress Ca^{2+} signaling. The presence of MCI-186 during cell isolation may have protected the cells from ROS and increased the CCh-induced Ca^{2+} response.

Although ROS are reportedly involved in the impairment of salivary gland function in Sjögren's syndrome (6) and after irradiation (7), the intracellular signaling pathways responsible remain to be determined. In primary culture, ROS activated the Erk1/2-novel PKC pathway in addition to the Src-p38 pathway (19). It is expected that multiple pathways would participate in the salivary gland dysfunction caused by irradiation and inflammation. It has been shown previously that γ -irradiation induces changes in the expression patterns of intercellular adhesion molecules *in vivo*, and similar changes were also observed in cultured acinar cells (20). Thus, the responses triggered by cell isolation and by γ -irradiation may have common properties.

In conclusion, MCI-186 improves both the Ca^{2+} response and protein exocytosis in salivary acinar cells through inhibition of Src-p38 signaling. Although the

effect of MCI-186 was small, salivary acinar cells have the potential to recover from dysfunction *in vivo* if the damage is not too severe. For example, the secretory function of salivary glands is temporarily impaired by γ -irradiation, but restored after several months when the radiation doses are lower (21). MCI-186 may increase the threshold for irreversible dysfunction. Further *in vivo* studies are needed to examine the possible clinical use of MCI-186 to prevent salivary gland dysfunction.

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Conflict of interest

None.

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