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Effect of Oxidative Stress on Secretory Function in Salivary Gland Cells

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1. Introduction

Reactive oxygen species (ROS) such as superoxide radical anion, singlet oxygen, hydrogen peroxide and hydroxyl radical are products of oxidative metabolism (Kourie, 1998). Low levels of ROS contribute to important signaling pathways to regulate key biological responses, including cell migration, mitosis and apoptosis (Goldschmidt-Clermont & Moldovan, 1999). For instance, endogenous oxidants protected the vasculature by inhibiting endothelial exocytosis that would otherwise lead to vascular inflammation and thrombosis, because endogenous hydrogen peroxide inhibited thrombin-induced exocytosis of granules from endothelial cells (Matsushita et al., 2005). In rat aortic smooth muscle cells, reduction in the intracellular concentration of hydrogen peroxide by the overexpression of catalase within cellular peroxisomes resulted in suppression of DNA synthesis and cell proliferation, and induction of apoptotic cell death (Brown et al., 1999). On the other hand, ROS are known to be pathogenic factors that induce cellular alterations in different cell types. For example, ROS are considered to be involved in the pathogenesis of postischemic endothelial dysfunction, because hydrogen peroxide induces Ca^{2+} oscillations in human aortic endothelial cells (Hu et al., 1998). In pancreatic β cells, hydrogen peroxide interferences glucose metabolism, which leads to the inhibition of insulin secretion (Krippert-Drews et al., 1999). In mesangial cells, hydrogen peroxide disturbs Ca^{2+} mobilization, which is considered to be involved in renal injury (Meyer et al., 1996). In neurons, hydrogen peroxide induces apoptotic cell death (Whittemore et al., 1995).

In salivary glands, ROS are involved in alteration of the functions. Oxidative stress demonstrated to induce alteration of secretory function of the rat submandibular gland, because reduction of submandibular saliva components such as protein and calcium was observed in the rat treated with lead acetate (Abdollahi et al., 1997, 2003), which induces oxidative stress (Pande & Flora, 2002). Irradiation, a major treatment modality administered for head and neck cancer, induces hypofunction of the salivary glands and consequent xerostomia (Nagler, 2002; de la Cal et al., 2006), in which ROS are believed to be involved in the hypofunction (Nagler et al., 1997, 2000; Takeda et al., 2003). Regarding Sjögren’s syndrome, an autoimmune disease which progressively destroys exocrine glands including the salivary glands, ROS has been suggested to be involved in the onset and pathology of...
Sjögren’s syndrome (Fox, 2005; Ryo et al., 2006). These findings suggest that oxidative stress from ROS causes salivary gland dysfunction (Vitolo et al., 2004).

Under conditions of oxidative stress, the thiols in cysteine residues within proteins are the most susceptible target among oxidant-sensitive molecules (Biswas et al., 2006; Jacob et al., 2006). There are some thiol-modulating reagents by different mechanisms. Ethacrynic acid, a once commonly used loop diuretic drug, is highly electrophilic and preferentially conjugates with glutathione enzymatically and non-enzymatically, and decreases reduced glutathione (GSH) in the mitochondrial pool (Habig et al., 1974; Meredith & Reed, 1982; Yamamoto et al., 2002). L-buthionine-S,R-sulfoximine (BSO) is an irreversible inhibitor of γ-glutamylcysteine synthetase, a rate-limiting enzyme in GSH biosynthesis (Griffith & Meister, 1985). Such thiol-modulating reagents are useful for the study with effects of thiol-oxidation on cell functions.

In salivary parotid acinar cells, stimulation of β-adrenergic receptors provokes release of amylase, a digestive enzyme. The receptor stimulation by β-adrenergic agonists such as isoproterenol (IPR) activates adenylate cyclase via heterotrimeric GTP-binding protein (G-protein), which leads to an increase in intracellular cAMP levels. The increased cAMP subsequently activates cAMP-dependent protein kinase, which has been well recognized to be essential for consequent exocytotic amylase release (Butcher & Putney, 1980; Quissell et al., 1982; Turner & Sugiyama, 2002). In this study, we investigated effects of the thiol-modulating reagents ethacrynic acid on amylase release induced by β-adrenergic receptor activation in rat parotid gland cells.

2. Materials and methods

2.1 Materials

Bovine serum albumin (Fraction V, BSA), collagenase A were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Trypsin (type-I), trypsin inhibitor (type-IS), IPR, N(6),2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP), forskolin, ethacrynic acid, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO). Mastiparan, cysteine, glutathione (reduced form, GSH), BSO, sodium sulfosalicylate (SSA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Vasoactive intestinal peptide (VIP) was obtained from Peptide Institute (Osaka, Japan). The GSSG/GSH Quantification Kit was obtained from Dojindo (Kumamoto, Japan).

2.2 Preparation of parotid acinar cells

All animal protocols were approved by the Laboratory Animal Committee of the Nihon University. Parotid acinar cells were prepared as previously described (Satoh et al., 2008). Sprague-Dawley rats (male, 200–250 g) were intraperitoneally anesthetized with pentobarbital (50 mg/kg), and the parotid glands were removed and placed in a small volume of Krebs-Ringer-bicarbonate (KRB) solution with the following composition (mM): 116 NaCl, 5.4 KCl, 0.8 MgSO4, 1.8 CaCl2, 0.96 NaH2PO4, 25 NaHCO3, 5 Hepes (pH 7.4) and 11.1 glucose. KRB solution was equilibrated with an atmosphere of 95% O2/5% CO2. After being minced with a razor, the parotid glands were treated with KRB solution containing 0.5% BSA in the presence or absence of enzyme. First, the glands were incubated with
trypsin (0.2 mg/ml) at 37°C for 5 min, after which the trypsin-treated glands were removed by centrifugation at 200 g for 1 min. The glands were subsequently incubated in Ca²⁺-Mg²⁺-free KRB solution containing 2 mM EGTA and trypsin inhibitor (0.2 mg/ml) at 37°C for 5 min. After the solution was removed by centrifugation (200 g for 1 min), the glands were incubated in Ca²⁺-Mg²⁺-free KRB solution without trypsin inhibitor at 37°C for 5 min. After the solution was removed by centrifugation (200 g for 1 min), the glands were incubated in Ca²⁺-Mg²⁺-free KRB solution with collagenase A (0.75 mg/ml) at 37°C for 20 min. The suspension was passed through eight layers of nylon mesh to separate the dispersed cells from undigested connective tissue and then was placed on KRB solution containing 4% BSA. After centrifugation (50 g for 5 min), the cells were suspended in appropriate amounts of KRB solution containing 0.5% BSA and 0.02% trypsin inhibitor.

2.3 Amylase release
Parotid acinar cells prepared as described above were stimulated by IPR (1 μM), forskolin (100 μM), mastoparan (50 μM), IBMX (1 mM), db-cAMP (100 μM), carbachol (10 μM), or VIP (10 μM) at 37°C for 20 min. When the effects of the thiol-modulating agents (EA and BSO) were examined, cells were preincubated with the agents for 10 min, and then stimulated. The cell suspensions were passed through a filter paper (Whatmann #1). Amylase activity in the filtrates was measured according to the method described previously (Bernfeld, 1955). Total amylase activity was measured in acinar cells homogenized in 0.01% Triton X-100, and amylase released was described as % of total.

2.4 Total glutathione measurement
Dispersed parotid acinar cells were collected by centrifugation at 10,000 g for 15 s and immediately mixed with 160 μl of 10 mM HCl. The mixture was frozen and thawed three times over, mixed with 40 μl of 5% SSA and then centrifuged at 8,000 g for 10 min. The supernatant was collected and diluted twice for further analysis. Total glutathione was measured by Dojindo GSSG/GSH Quantification Kit. Samples were incubated at 37°C for 10 min and then measured optical density at 405 nm by a micro plate reader (Bio-Rad). Total protein concentrations were determined by the Lowry method (1951).

3. Results
3.1 Effect of ethacrynic acid on IPR-Induced amylase release in parotid acinar cells
We first examined effect of the thiol-modulating reagent ethacrynic acid on amylase release in rat parotid acinar cells. After preincubation in the absence or presence of ethacrynic acid (250 μM) for 10 min, the cells were stimulated with the β-agonist IPR (1 μM) or vehicle (control) for 20 min. As Fig. 1 summarizes, IPR induced amylase release in a time dependent manner in the absence of ethacrynic acid, but the IPR-induced amylase release was partially inhibited in the presence of ethacrynic acid. Ethacrynic acid had no effect on amylase release from the cell non-stimulated. In the cells preincubated with 100, 250 or 500 μM ethacrynic acid and then stimulated with IPR for 20 min, ethacrynic acid inhibited the IPR-induced amylase release in a dose dependent manner, as Fig. 2 shows. These results suggest that the amylase release regulated by β-receptor activation is reduced by thiol-modulation.
Fig. 1. Inhibition of IPR-induced amylase release by ethacrynic acid in rat parotid acinar cells. After pretreatment of ethacrynic acid (250 μM, EA) or vehicle for 10 min, cells were incubated with (triangles) or without (circles) 1 μM IPR. Value are means ± SE from 5 independent experiments. *P < 0.05

Fig. 2. Dose-dependent effect of ethacrynic acid on IPR-induced amylase release. After preincubation with 0, 100, 250 or 500 μM ethacrynic acid (EA) for 10 min, rat parotid acinar cells were incubated with (closed columns) or without (open column) 1 μM IPR for 20 min. Values are means ± SE from 3 independent experiments. **P < 0.01

3.2 Relief of the inhibitory effect of ethacrynic acid on IPR-induced amylase release by GSH

To confirm the contribution of thiol-modulation to the inhibition of IPR-induced amylase release by ethacrynic acid, we examined effect of thiol-reducing reagents on the effect of
ethacrynic acid. When parotid acinar cells pretreated with ethacrynic acid (250 μM) in absence or presence of GSH (10 mM) or cysteine (10 mM) were stimulated with IPR (1 μM) for 20 min, GSH relieved the inhibitory effect of ethacrynic acid on IPR-induced amylase release, but less cysteine, as Fig. 3 summarizes. These results support that thiol-modulation causes the inhibitory effect of ethacrynic acid on IPR-induced amylase release, although the less effect of cysteine is obscure. GSH and cysteine had no effect on amylase release in the cells non-stimulated (data not shown).

**3.3 No effect of ethacrynic acid on VIP- and carbachol-induced amylase release**

Although β-receptor stimulation dominantly provokes amylase release, stimulation of VIP and muscarinic receptors also evokes amylase release via the increases in intracellular cAMP and Ca²⁺ concentrations, respectively, in rat parotid acinar cells (Scott & Baum, 1985; Yoshimura & Nezu, 1991). Then we next examined the effect of ethacrynic acid on amylase release induced by VIP and carbachol, a muscarinic agonist. When the cells were stimulated with VIP (10 μM) and carbachol (10 μM) for 20 min, amylase release was evoked, although the responses of both secretagogues were lower than that of IPR. However, ethacrynic acid (250 μM) had no effect on VIP- and carbachol-induced amylase release, as shown in Table 1.
Table 1. No effect of ethacrynic acid on VIP- and carbachol-induced amylase release in rat parotid acinar cells. After pretreatment of ethacrynic acid (250 μM, EA) or vehicle for 10 min, cells were stimulated with 1 μM IPR, 10 μM VIP or 10 μM carbachol (CCh) for 20 min. Value are means ± SE from 5 independent experiments. *P < 0.05

| Agonist | EA | Amylase release (%) of total |
|---------|----|-----------------------------|
| (-)     | -  | 3.5 ± 0.4                   |
|         | +  | 2.3 ± 0.4                   |
| IPR     | -  | 13.6 ± 1.7                  |
|         | +  | 9.6 ± 1.5*                  |
| VIP     | -  | 5.5 ± 0.4                   |
|         | +  | 7.7 ± 1.3                   |
| CCh     | -  | 5.0 ± 0.8                   |
|         | +  | 4.2 ± 0.4                   |

3.4 No effect of ethacrynic acid on amylase release induced by activators of cAMP signaling pathway

It is well known that β-receptor stimulation provokes amylase release via the increase in intracellular cAMP levels in rat parotid acinar cells (Turner & Sugiya, 2002). Then we examined the effect of ethacrynic acid on amylase release induced by activators of cAMP signaling pathway. When parotid acinar cells were incubated with forskolin (100 μM), mastoparan (50 μM), db-cAMP (1 mM) and IBMX (1 mM), a cell-permeable cAMP analogue, an adenylate cyclase activator, a G-protein activator and a cyclic nucleotide phosphodiesterase inhibitor, respectively, for 20 min, amylase release was induced. However, the effects of these drugs on amylase release were not changed even in the cells treated with ethacrynic acid (250 μM), as shown in Table 2. These observations imply that ethacrynic acid has no effect on the cAMP signaling pathway in rat parotid acinar cells.

Table 2. No effect of ethacrynic acid on amylase release induced by cAMP signaling activators. After pretreatment of ethacrynic acid (250 μM, EA) or vehicle for 10 min, rat parotid acinar cells were incubated with forskolin (100 μM), mastoparan (50 μM), db-cAMP (1 mM) or IBMX (1 mM) for 20 min. Value are means ± SE from 5 independent experiments.
3.5 No effect of ethacrynic acid on the intracellular glutathione level

Since EA has been reported to deplete the intracellular glutathione (GSH) (Meredith & Reed, 1982; Dhanboora & Babson, 1992), we determined total amount of glutathione in the rat parotid acinar cells treated with ethacrynic acid (250 μM). As Table 3 shows, however, ethacrynic acid had no effect on total amount of glutathione in the cells. Then we next examined effect of the glutathione biosynthesis inhibitor BSO on IPR-induced amylase release. However, BSO (1 mM) had no effect on IPR-induced amylase release, as shown in Fig. 4. These observations suggest that the reduction of glutathione levels is not caused for the inhibitory effect of ethacrynic acid on IPR-induced amylase release.

Table 3. No effect of ethacrynic acid on total glutathione contents. After treatment of ethacrynic acid (250 μM, EA) or vehicle for 30 min, total glutathione were measured. Values are means ± SE from 3 independent experiments.

| EA   | Total glutathione (nmol / mg protein) |
|------|--------------------------------------|
| (-)  | 12.67 ± 0.72                         |
| (+)  | 13.97 ± 0.12                         |

Fig. 4. No effect of BSO on IPR-induced amylase release. After preincubation with 1 mM BSO or vehicle for 10 min, rat parotid acinar cells were incubated with (triangles) or without (circles) 1 μM IPR. Values are means ± SE from 3 independent experiments.

4. Discussion

Amylase release in parotid acinar cells occurs via the two distinct processes, constitutive release and regulatory release (Turner & Sugiya, 2002). The regulatory release is induced by
the activation of receptors, whereas the constitutive release is continuously observed without receptor activation. In this study, we demonstrated that the thiol-modulating reagent ethacrynic acid inhibits regulatory amylase release provoked by $\beta$-adrenergic receptor stimulation.

Ethacrynic acid has been reported to induce a rapid depletion of glutathione (GSH), subsequent intracellular ROS elevation, and consequent cell injury (Miccadi et al., 1988; Dhanbhooora & Babson, 1992). In fact, depletion of glutathione by treatment with 2-cyclohexene-1-one has been demonstrated to result in inhibition of carbachol-induced amylase release in guinea pig exocrine pancreatic acini (Stenson et al., 1983). In rat pancreatic acinar cells, thiol modulating agents including ethacrynic acid have been reported to reduce the intracellular glutathione levels and inhibition of caerulein-stimulated amylase release (Yu et al., 2002). However, we demonstrated here that ethacrynic acid had no effect on the level of glutathione. Furthermore, the glutathione biosynthesis inhibitor BSO had no effect on IPR-induced amylase release. These observations strongly suggest that the inhibitory effect of ethacrynic acid is not due to depletion of glutathione. Ethacrynic acid had no effect on amylase release induced by cAMP signaling activators and control release and failed to inhibit the effect of IPR in the presence of GSH. Over 90% of cell viability in the cells treated with ethacrynic acid was confirmed by trypan blue extrusion. Therefore, it is also unlikely that cell injury induced by ethacrynic acid causes the inhibition of IPR-induced amylase release.

In the regulatory amylase release, cAMP-dependent signaling pathway is involved. Namely, stimulation of $\beta$-adrenergic receptors activates adenylate cyclase via heterotrimeric G-protein, which leads to an increase in intracellular cAMP level. Subsequently, cAMP-dependent protein kinase is activated, which causes exocytotic amylase release (Butcher & Putney, 1980; Quissell et al., 1982; Turner & Sugiya, 2002). However, ethacrynic acid failed to inhibit amylase release induced by the G-protein activator mastoparan, the adenylate cyclase activator forskolin, the cyclic nucleotide phosphodiesterase inhibitor IBMX and the cell-permeable cAMP analogue db-cAMP. These results suggest that the cause of the inhibition of IPR-induced amylase release by ethacrynic acid is distinct from the disturbance of cAMP signaling. VIP is another agonist, which induces amylase release via cAMP signaling in rat parotid acinar cells (Scott & Baum, 1985; Inoue et al., 1985). However, ethacrynic acid failed to inhibit VIP-induced amylase release, supporting that EA has no effect on cAMP signaling. Taken together, it is most likely that thiol-modulation of $\beta$-adrenergic receptors results in the inhibition of IPR-induced amylase release.

In rat parotid acinar cells, the thiol-oxidizing compound diamide has been demonstrated to reduce the binding affinity of $\beta$-adrenergic receptors for ligands and consequently inhibit IPR-induced amylase release (Guo et al., 2010). Diamide had also no effect on mastoparan- or forskolin-induced amylase release and failed to inhibit IPR-induced amylase release in the presence of thiol-reducing reagents, dithiothreitol and GSH, as well as ethacrynic acid described in this paper. Therefore, ethacrynic acid probably leads to thiol-oxidation of $\beta$-adrenergic receptors, which results in the reduction of IPR-induced amylase release. Conserved cysteine residues in an extracellular domain of the human $\beta$-adrenergic receptor have been suggested to be involved in ligand binding assessed by site-directed mutagenesis (Fraser, 1989). Therefore, it is conceivable that such cysteine residues of $\beta$-adrenergic receptor are oxidized by ethacrynic acid. It has been considered that ethacrynic acid is not
an oxidant but depletes glutathione by conjugation (Meredith & Reed, 1982). However, currently, independent effects on depletion of intracellular glutathione of ethacrynic acid have been demonstrated (Aizawa et al., 2003; Lu et al., 2009). Therefore, ethacrynic acid appears to have a direct effect as a thiol-oxidating reagent.

Protein thiols are typically maintained in the reduced state. GSH is the most abundant intracellular SH and represents one of the major intracellular defense systems against mediators of oxidative stress (Meister & Tate, 1976). The reducing conditions in cells are primarily maintained by exceedingly large ratio of GSH to GSSG. IPR-induced amylase release inhibited by ethacrynic acid was restored by GSH. Therefore, the antioxidant system by GSH probably plays an important role in maintaining cellular defenses under oxidative stress in rat parotid acinar cells. On the other hand, despite this reducing environment, the formation of mixed disulfides between protein thiols and glutathione has been observed, a process known as S-glutathionylation (Dalle-Donne et al., 2005). S-glutathionylation is considered to occur under physiological conditions and is a reversible cellular response to mild oxidative stress. Involvement of S-glutathionylation in regulating ß-adrenergic receptor function under mild oxidative stress in rat parotid acinar cells would be a further study.

5. Conclusion
In this study, we demonstrated that ethacrynic acid, a thiol-modulating reagent, inhibited amylase release induced by ß-adrenergic agonist in rat parotid acinar cells. The effect of ethacrynic acid was independent of depletion of glutathione in the cells. Ethacrynic acid failed to inhibit amylase release induced by activators of cAMP signaling pathway, suggesting that the inhibitory effect of ethacrynic acid on amylase release induced by ß-adrenergic agonist is caused by the thiol-modulation of ß-adrenergic receptors.

6. Acknowledgements
This study was supported in part by a Grant-in-Aid for Scientific Research from the JSPS (#21592375), a Nihon University Multidisciplinary Research Grant for 2011 and a Grant of "Strategic Research Base Development" Program for Private Universities from MEXT, 2010-2014 (S1001024).

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