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Original Article

Hydrogen peroxide inhibits Ca^{2+} efflux through plasma membrane Ca^{2+}-ATPase in mouse parotid acinar cells

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ABSTRACT Intracellular Ca^{2+} mobilization is closely linked with the initiation of salivary secretion in parotid acinar cells. Reactive oxygen species (ROS) are known to be related to a variety of oxidative stress-induced cellular disorders and believed to be involved in salivary impairments. In this study, we investigated the underlying mechanism of hydrogen peroxide (H_2O_2) on cytosolic Ca^{2+} accumulation in mouse parotid acinar cells. Intracellular Ca^{2+} levels were slowly elevated when 1 mM H_2O_2 was perfused in the presence of normal extracellular Ca^{2+}. In a Ca^{2+}-free medium, 1 mM H_2O_2 still enhanced the intracellular Ca^{2+} level. Ca^{2+} entry tested using manganese quenching technique was not affected by perfusion of 1 mM H_2O_2. On the other hand, 10 mM H_2O_2 induced more rapid Ca^{2+} accumulation and facilitated Ca^{2+} entry from extracellular fluid. Ca^{2+} refill into intracellular Ca^{2+} store and inositol 1,4,5-trisphosphate (1 μM)-induced Ca^{2+} release from Ca^{2+} store was not affected by 1 mM H_2O_2 in permeabilized cells. Ca^{2+} efflux through plasma membrane Ca^{2+}-ATPase (PMCA) was markedly blocked by 1 mM H_2O_2 in thapsigargin-treated intact acinar cells. Antioxidants, either catalase or dithiothreitol, completely protected H_2O_2-induced Ca^{2+} accumulation through PMCA inactivation. From the above results, we suggest that excessive production of H_2O_2 under pathological conditions may lead to cytosolic Ca^{2+} accumulation and that the primary mechanism of H_2O_2-induced Ca^{2+} accumulation is likely to inhibit Ca^{2+} efflux through PMCA rather than mobilize Ca^{2+} ions from extracellular medium or intracellular stores in mouse parotid acinar cells.

INTRODUCTION

In the salivary gland, agonist-induced Ca^{2+} mobilization is the initial cellular event for fluid and amylase secretions [1]. Cytosolic Ca^{2+} can be mobilized from both external fluid and internal stores, and then rapidly eliminated to internal store and external space in parotid acinar cells [2]. It has been reported that oxidative stress is involved in salivary dysfunction caused by drugs and irradiation [3]. Reduction of submandibular saliva secretion has been observed in the rat treated with lead acetate, which induces oxidative stress [4]. Irradiation-induced hypofunction of the salivary glands has been believed to be involved with oxidative stress [5]. Furthermore, antioxidants have a protective effect on oxidative stress-induced salivary dysfunctions [6,7]. The impairment of salivary secretion in Sjogren's syndrome, an autoimmune disease which progressively destroys salivary glands, has been known to be generated by oxidative stress and be related to intracellular Ca^{2+} accumulation in parotid acinar cells [8,9].

Although reactive oxygen species (ROS) are normally generated from partial reduction of oxygen during the aerobic respiration, they cause oxidative damage to various biological molecules, thereby disrupting normal cellular function and integrity [10,11]. The superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are considered as primary ROS which interact with

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ion transporters in surface and internal membrane [12,13]. ROS are controlled by intracellular antioxidant enzymes and free radical scavengers which protect cells from oxidative stress under physiological conditions [14]. However, the imbalanced states by excessive production of ROS or reduction of antioxidants leading to morphological and functional damage of cells [15]. Although hydrogen peroxide-induced oxidative stress are correlated with overloaded intracellular Ca^{2+} levels, the mechanism of sustained Ca^{2+} overload has still been unclear due to cell-to-cell difference in Ca^{2+} transport molecules as shown by the following evidence: 1) The enhanced Ca^{2+} release from intracellular store [16-18], 2) The stimulated Ca^{2+} entry from extracellular medium [19-22] and 3) The attenuated Ca^{2+} efflux by inactivation of plasma membrane Ca^{2+}-ATPase (PMCA) or sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [23-25] in various cell types. In the present study, we have therefore characterized the effect of hydrogen peroxide on intracellular Ca^{2+} signals and the underlying mechanism of Ca^{2+} accumulation in mouse parotid acinar cells. Here we report that hydrogen peroxide could accumulate intracellular Ca^{2+} by reducing Ca^{2+} efflux through PMCA, rather than by enhancing Ca^{2+} mobilization from extracellular fluid or intracellular store in pathological conditions.

METHODS

Materials

Collagenase P was purchased from Roche Diagnostics GmbH (Mannheim, Germany), fura-2/AM and magfura-2/AM were from Thermo Fisher Scientific (Waltham, MA, USA), inositol 1,4,5-trisphosphate ($InsP_3$) was from Enzo Life Sciences (Farmingdale, NY, USA) and thapsigargin (TG) was from Tocris (Avonmouth, BS, UK). All other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of parotid acinar cells

Small cluster of parotid acinar cells (8-15 cells per experiment) were freshly isolated by collagenase digestion as described previously [26]. Briefly, the parotid gland was removed from male Balb/c mice (8-10 weeks) after CO_2 asphyxiation and cervical dislocation. The tissues were enzymatically digested with collagenase P in HEPES-buffered physiological saline for 30 min following gentle agitation. After isolation, parotid acinar cells were suspended in HEPES-buffered physiological saline containing 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 1 mM Na_2HPO_4, 1.28 mM $CaCl_2$, 10 mM HEPES and 5.5 mM glucose (PH 7.4) until ready for use. To ensure examination in a Ca^{2+}-free condition, HEPES-buffered physiological saline containing no added Ca^{2+} was replaced with 5 mM ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA). All experimental procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animal provided by NIH. All experiments adhered to Konyang University policies regarding the care and use of animals.

Intracellular Ca^{2+} measurements

The isolated parotid acinar cells were loaded with 5 μM Fura-2/AM for 1 h at room temperature for measurements of intracellular Ca^{2+}. Fura-2/AM loaded cells were mounted on cover glass in the perfusion chambers. Acinar cells were continuously perfused with HEPES-buffered physiological saline. The perfusion rate (1 ml/min) was controlled using an electronic perfusion system (Warner Instrument, CT, USA). Intracellular Ca^{2+} imaging was conducted using a TILL Photonics imaging system. Fura-2/AM loaded cells were excited alternately with light at 340 nm and 380 nm using a Polychrome V monochrometer (TILL Photonics, CA, USA). Fluorescence images emitted at 510 nm were captured using a Cool-SNAP HQ_2 camera (Photometrics, AZ, USA) attached to inverted microscope (Olympus Corp., Tokyo, JP).

Mn^{2+} quenching study to measurement of Ca^{2+} entry

Fura-2 is known to have a high affinity with Mn^{2+}, and thereby fura-2 fluorescence is easily quenched by binding to Mn^{2+}. Therefore, we used this property of Mn^{2+} to quench fura-2 fluorescence as an indicator of Ca^{2+} influx through plasma membrane. Fura-2/AM loaded cells were perfused with HEPES-buffered physiological saline. The perfusion solution was then switched to 1 mM Mn^{2+}-containing solution without EGTA or Ca^{2+} for 10 min to observe the extent of Mn^{2+} entry. Fura-2/AM loaded cells were excited with light at 360 nm, a wavelength insensitive to intracellular Ca^{2+} changes. Fluorescence images emitted at 510 nm were captured. Mn^{2+} quenching the fluorescence signal was normalized using values determined by treatment of 20 μM β-escin to permeabilize cell membrane at the end of the experiments.

Luminal Ca^{2+} measurements in endoplasmic reticulum

The isolated acinar cells were loaded with 3 μM magfura-2/AM for 1 h at room temperature and then attached on cover glass in the perfusion chambers. Cells were permeabilized by perfusion with 20 μM β-escin for 2 min in intracellular medium (ICM) containing 19 mM NaCl, 125 mM KCl, 10 mM HEPES and 1 mM EGTA (pH 7.3) as described previously [27]. To remove intracellular dye, the permeabilized cells were washed with ICM no containing β-escin for 15 min. Intracellular Ca^{2+} stores were subsequently refilled with Ca^{2+} by activation of SERCA. To activate SERCA, cells were perfused with ICM containing 0.650 μM $CaCl_2$ (free Ca^{2+}=200 nM), 3 mM Na_2ATP and 1.4 mM $MgCl_2$. After the stores were refilled with Ca^{2+}, SERCA activity was effectively

inactivated by removal of Mg^{2+} from ICM. The free Ca^{2+} was constantly maintained at 200 nM throughout all experiments. Fluorescence images emitted 505 nm were captured following alternate excitation at 340 nm and 380 nm using a TILL Photonics imaging system.

Statistical analysis

All results were presented as mean±S.E. Data were analyzed using the Student's *t* test. Differences were considered significant when the p value was less than 0.05. Ca^{2+} refill rates and Ca^{2+} release rates were estimated by fitting the changing fluorescence to a single exponential function. Relative Ca^{2+} entry and efflux were normalized to maximum value in each experiments using Origin program.

RESULTS

Hydrogen peroxide (H_2O_2)-induced intracellular Ca^{2+} accumulation

Initial experiments were performed to investigate the effect of H_2O_2 on the intracellular Ca^{2+} level in parotid acinar cells. The change of intracellular Ca^{2+} concentration was monitored in various concentrations of H_2O_2 (0.1-10 mM) in the presence of 1.28 mM extracellular Ca^{2+} in intact cells. As shown in Fig. 1A, the perfusion of H_2O_2 for 10 min resulted in slow increases of intracellular Ca^{2+} concentrations. The significant Ca^{2+} elevations were observed from 1 mM of H_2O_2 and more rapid Ca^{2+} accumulation was observed in 10 mM H_2O_2. After the cessation of H_2O_2 perfusion, the augmented Ca^{2+} was nearly returned to baseline at 1 mM of H_2O_2. Contrastively, the sustained Ca^{2+} increase was observed at 3 mM and 10 mM of H_2O_2, even if H_2O_2 was removed from the perfusate (Fig. 1B). These results suggest that H_2O_2 could remarkably accumulate in intracellular Ca^{2+} and that an excess dose of H_2O_2 could irreversibly alter Ca^{2+} homeostasis in parotid acinar cells.

Effects of H_2O_2 on Ca^{2+} entry from extracellular medium

Next, we compared the intracellular Ca^{2+} accumulation in the presence and the absence of extracellular Ca^{2+} to confirm whether H_2O_2 could facilitate Ca^{2+} entry from the extracellular medium. Intracellular Ca^{2+} accumulation steadily increased during treatment of H_2O_2 in both normal Ca^{2+} and Ca^{2+}-free mediums (Fig. 2A). Although, a slight difference was detected in initial values of Ca^{2+} accumulation, the final values of Ca^{2+} accumulation showed no difference as shown in Fig. 2B (values at 300 s; 0.064±0.012 Δ ratio *vs* 0.034±0.007 Δ ratio, values at 600 s; 0.123±0.023 Δ ratio *vs* 0.122±0.015 Δ ratio, presence and absence of extracellular Ca^{2+}, respectively). Thus, the entire Ca^{2+} accumulation was observed regardless of external Ca^{2+} existence.

In another experiment, the Mn^{2+} quenching test was performed to confirm Ca^{2+} entry. As shown in Fig. 3A, the perfusion of 1 mM and 3 mM H_2O_2 for 10 min failed to facilitate quenching of fura-2 fluorescence, whereas 10 mM H_2O_2 markedly accelerated quenching of fura-2 fluorescence. The relative Ca^{2+} entries at the end of the experiments were 57.14±8.73%, 62.99±6.03%, 63.56±6.79% and 86.21±1.77% in control, 1 mM, 3 mM and 10 mM H_2O_2-treated groups, respectively (Fig. 3B). These data indicate that the primary origin of accumulated Ca^{2+} induced by 1 mM H_2O_2 may not come from extracellular fluid because Ca^{2+}

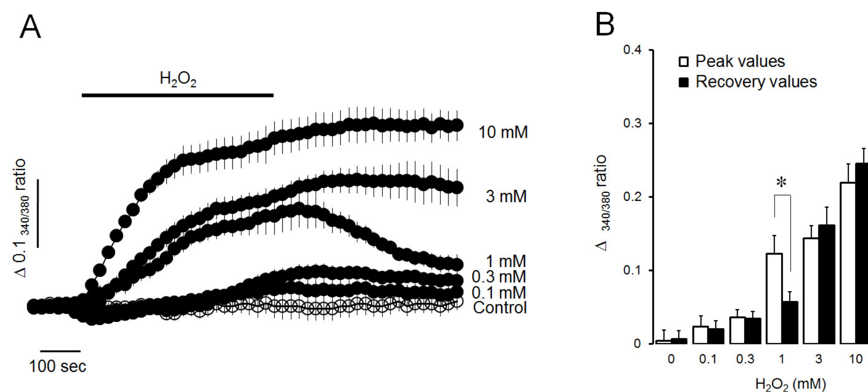

Fig. 1. Hydrogen peroxide (H_2O_2) produced intracellular Ca^{2+} accumulation in intact parotid acinar cells. (A) Effects of various concentrations (0.1-10 mM) of H_2O_2 (filled circles) for 10 min on Ca^{2+} accumulation in the presence of normal extracellular Ca^{2+}. (B) The peak (open bars) and recovery (filled bars) values of intracellular Ca^{2+} accumulation after 10 min of the perfusion and the removal of H_2O_2. The changes of the 340/380 ratio were expressed as means±SE obtained from at least seven separate experiments. The perfusion of H_2O_2 resulted in slow increases of intracellular Ca^{2+} concentrations. H_2O_2 at concentration of 1 mM effectively accumulate intracellular Ca^{2+} and nearly recovered to baseline after withdraw of H_2O_2. The sustained Ca^{2+} increase was still observed at higher concentration over 3 mM of H_2O_2 even if H_2O_2 was removed from the perfusate. The relatively rapid Ca^{2+} accumulation was observed in 10 mM H_2O_2. Asterisk indicates the recovery value obtained at 10 min after termination of H_2O_2 treatment is significantly different from the peak value obtained during H_2O_2 treatment ($p<0.05$).

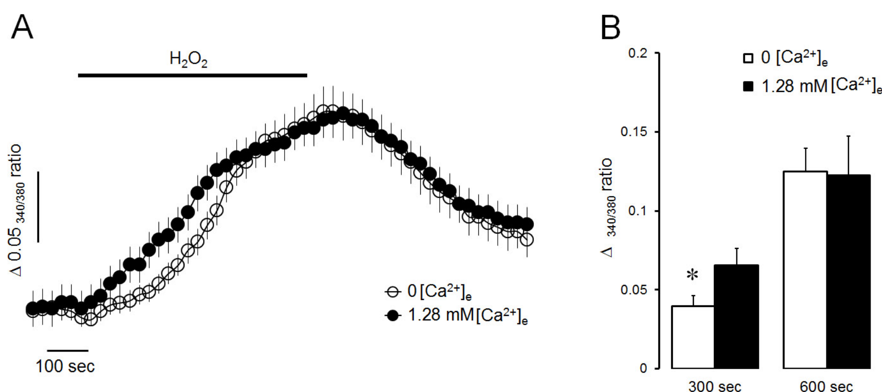

Fig. 2. H_2O_2 still enhanced Ca^{2+} accumulation in the absence of extracellular Ca^{2+} in intact parotid acinar cells. (A) Effects of 1 mM H_2O_2 on Ca^{2+} accumulation in the absence (open circles) or the presence (filled circles) of normal extracellular Ca^{2+}. (B) Intracellular Ca^{2+} accumulation at 300 s and 600 s after perfusion of 1 mM H_2O_2 in the absence (open bars) or the presence (filled bars) of normal extracellular Ca^{2+}. The values were expressed as means±SE obtained from seven (control) and five (H_2O_2) experiments. Although a slight difference was detected in initial values of Ca^{2+} accumulation at 300 s, the entire Ca^{2+} accumulation at 600 s was no difference. Asterisk indicates the value obtained in Ca^{2+}-free buffer is significantly different from the corresponding value obtained in normal Ca^{2+} buffer ($p<0.05$).

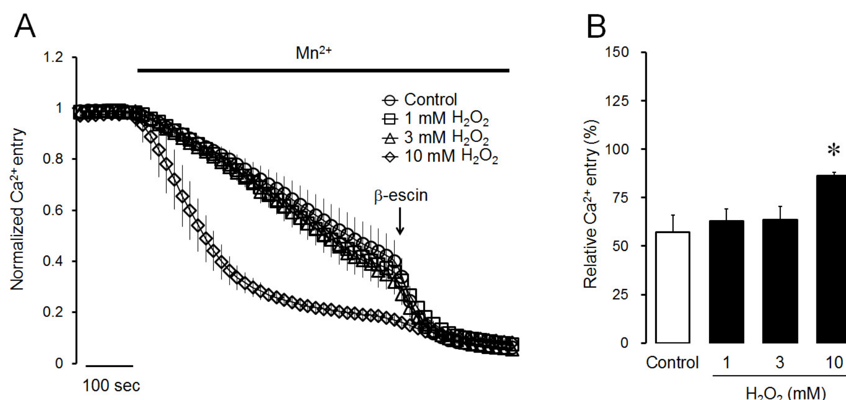

Fig. 3. Effects of H_2O_2 on Ca^{2+} entry using Mn^{2+} quenching test in intact parotid acinar cells. (A) Effects of various concentrations (1-10 mM) of H_2O_2 for 10 min on normalized Ca^{2+} entry. (B) Effects of H_2O_2 on relative Ca^{2+} entry at the end of experiments. The values were expressed as means±SE obtained from at least six separate experiments. H_2O_2 at the concentration of 1 mM (open squares) and 3 mM (open triangles) failed to facilitate Ca^{2+} entry, whereas 10 mM H_2O_2 (open diamonds) remarkably accelerated Ca^{2+} entry through plasma membrane. Asterisk indicates the value obtained in H_2O_2-treated experiments (filled bars) is significantly different from the corresponding value obtained in control experiments (open bar) ($p<0.05$).

accumulation was still observed in Ca^{2+}-free solution and Mn^{2+} quenching of fura-2 fluorescence was not facilitated in the presence of 1 mM H_2O_2. Therefore, we used a concentration of 1 mM H_2O_2 in the following experiments to identify the primary mechanism of Ca^{2+} accumulation. The next experiment was planned to evaluate whether H_2O_2 can influence Ca^{2+} transport through intracellular Ca^{2+} stores membrane.

Effects of H_2O_2 on Ca^{2+} transport through intracellular Ca^{2+} stores membrane

To determine the influence of H_2O_2 on Ca^{2+} transport through endoplasmic reticulum (ER) membrane, we employed unidirectional fluorescent ER Ca^{2+} measurements in permeabilized cells. Parotid acinar cells were loaded with a low-affinity Ca^{2+} dye magfura-2/AM and then permeabilized with β-escin to release the cytosolic dye. As shown in Fig. 4A, the perfusion with intracellular medium containing $CaCl_2$, $MgCl_2$ and ATP resulted in an increase of the fluorescence ratio that indicates an effective Ca^{2+} refill into the ER stores. In these conditions, the perfusion of 1 mM H_2O_2 failed to change Ca^{2+} refill rate into the ER stores (Fig. 4B). After the stores were refilled with Ca^{2+}, $MgCl_2$ was eliminated from the buffer to provide SERCA inactivation. Application of 1 μM $InsP_3$ caused significant Ca^{2+} release from the stores, but H_2O_2 had no effect on $InsP_3$-induced Ca^{2+} release rates (Fig. 4C). These findings indicate that 1 mM H_2O_2 led to neither Ca^{2+} refill nor $InsP_3$-induced Ca^{2+} release through the ER membrane.

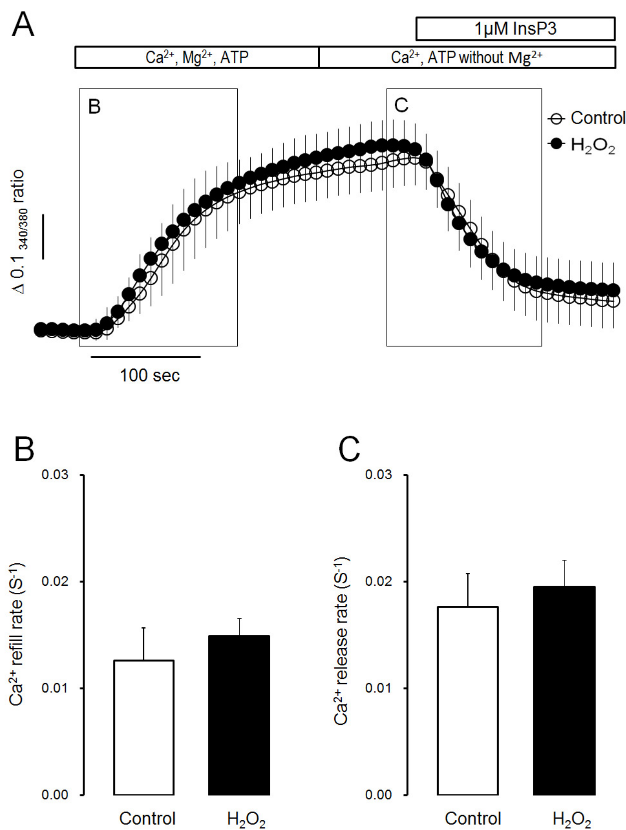

Fig. 4. H_2O_2 failed to modulate Ca^{2+} transport through endoplasmic reticulum (ER) membrane in permeabilized parotid acinar cells. (A) The effect of H_2O_2 on Ca^{2+} refill and $InsP_3$-induced Ca^{2+} release through ER membrane in permeabilized cells. (B) The effect of H_2O_2 on Ca^{2+} refill rates into intracellular Ca^{2+} stores. (C) The effect of H_2O_2 on $InsP_3$-induced Ca^{2+} release from Ca^{2+} stores. The values were expressed as means±SE obtained from five (control) and six (H_2O_2) experiments. ER Ca^{2+} stores were loaded with the buffer containing $MgCl_2$, Na2ATP and CaCl2. After Ca^{2+} loading, $MgCl_2$ was eliminated to SERCA inactivation at 60 s prior to 1 μM $InsP_3$ application (open circles and bars). The same procedure was repeated in H_2O_2-treated permeabilized cells (filled circles and bars). The perfusion of H_2O_2 resulted in change neither Ca^{2+} refill rate nor $InsP_3$-induced Ca^{2+} release rate through ER membrane in permeabilized cells.

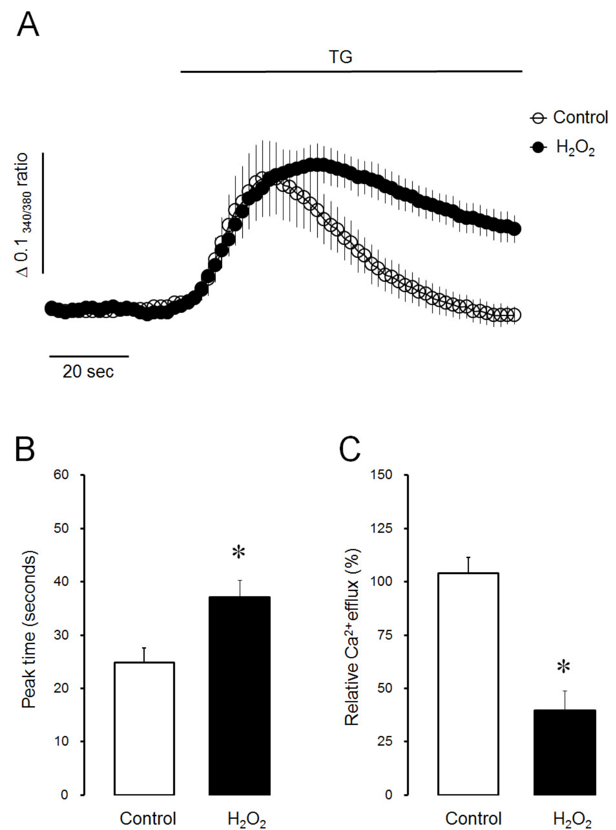

Fig. 5. H_2O_2 attenuated Ca^{2+} efflux in thapsigargin (TG)-treated intact acinar cells. (A) The effect of H_2O_2 on Ca^{2+} efflux during the depletion of ER Ca^{2+} store by TG treatment in Ca^{2+}-free buffer. (B) The effect of H_2O_2 on the peak time of intracellular Ca^{2+} elevation. (C) The effect of H_2O_2 on the relative Ca^{2+} efflux at the end of experiments. The values were expressed as means±SE obtained from seven (control) and five (H_2O_2) experiments. When H_2O_2 was treated with TG (filled circles and bars), the Ca^{2+} elevation was delayed and Ca^{2+} efflux was not fully recovered to baseline compared with control values (open circles and bars). Asterisks indicate the value obtained in H_2O_2-treated experiments is significantly different from the corresponding value obtained in control experiments ($p<0.05$).

H_2O_2 inhibits Ca^{2+} efflux through PMCA inactivation

To confirm the effect of H_2O_2 on PMCA activity, we evaluated the effect of H_2O_2 in Ca^{2+}-free conditions during the depletion of Ca^{2+} from the ER store by the treatment of 1 μM TG, a SERCA blocker. As shown in Fig. 5A, TG induced a transient Ca^{2+} elevation that indicates spontaneous Ca^{2+} release from the ER stores, and these responses were completely returned to baseline, which means there was Ca^{2+} efflux by plasma membrane Ca^{2+}-ATPase (PMCA) in control experiments. When H_2O_2 was treated with TG, the peak time of Ca^{2+} elevation was delayed (Fig. 5B, control; 24.83±2.76 s *vs.* H_2O_2; 37.17±3.17 s) and Ca^{2+} efflux was not fully returned to the control value in 1 mM H_2O_2-treated group (Fig. 5C, control; 103.95±7.62% *vs.* H_2O_2; 39.58±9.33%).

To further test the effect of H_2O_2 on PMCA activity in another set of experiments, Ca^{2+} store were initially depleted with 1 μM TG, and then Ca^{2+} entry and Ca^{2+} efflux was fully stimulated by adding and removing extracellular 1.28 mM Ca^{2+} in intact cells, respectively. In the control experiment, as shown in Fig. 6A, the addition of extracellular Ca^{2+} remarkably stimulated Ca^{2+} entry, and the elimination of extracellular Ca^{2+} resulted in a clear extrusion of intracellular Ca^{2+} to external space. In 1 mM H_2O_2-treated cells, the elevated intracellular Ca^{2+} level was not returned to baseline by withdrawal of extracellular Ca^{2+}. That means the extrusion of intracellular Ca^{2+} was markedly disrupted by treatment of 1 mM H_2O_2. Additionally, the perfusion of antioxidants with H_2O_2, either 30 μg/ml catalase or 2 mM dithiothreitol, completely protected the diminished Ca^{2+} efflux in TG-treated intact acinar cells (Fig. 6B). These evidences suggest that H_2O_2 may induce Ca^{2+} accumulation by inhibition of PMCA activity in mouse parotid

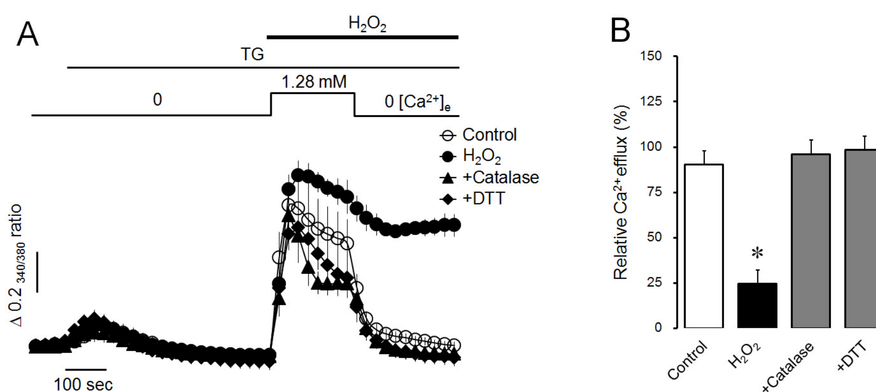

Fig. 6. H_2O_2 suppressed Ca^{2+} efflux through PMCA in intact parotid acinar cells. (A) The effect of H_2O_2 and antioxidants on Ca^{2+} efflux through PMCA. (B) The effect of H_2O_2 and antioxidants on relative Ca^{2+} efflux at the end of experiments. The values were expressed as means±SE obtained from at least five separate experiments. Ca^{2+} store were initially depleted with 1 μM TG, and then Ca^{2+} entry and Ca^{2+} efflux was fully stimulated by adding and removing extracellular 1.28 mM Ca^{2+}. Application of H_2O_2 (filled circles and bar) resulted in remarkable inhibition of Ca^{2+} efflux through PMCA compared with control values (open circles and bar) in TG-treated acinar cells. The antioxidants, 30 μg/ml catalase (filled triangles and gray bar) or 2 mM dithiothreitol (DTT, filled diamonds and gray bar), completely protected these diminished Ca^{2+} efflux by H_2O_2 in TG-treated intact acinar cells. Asterisk indicates the value obtained in H_2O_2-treated experiments is significantly different from the corresponding value obtained in control experiments ($p<0.05$).

acinar cells.

DISCUSSION

The present study provides evidence that the reactive oxygen species H_2O_2 accumulates cytosolic Ca^{2+} by attenuating Ca^{2+} efflux through PMCA rather than by mobilizing the Ca^{2+} from the extracellular space or intracellular Ca^{2+} store in mouse parotid acinar cells. Cytosolic free Ca^{2+} plays a crucial role in the salivary secretion of parotid acinar cells, and Ca^{2+} can be mobilized from both the external fluid and the internal Ca^{2+} stores such as endoplasmic reticulum, acidic store and mitochondria to elicit physiological responses [28-30]. Acetylcholine, a major agonist in the parotid gland, is known to initially mobilize Ca^{2+} from internal stores through activation of $InsP_3$ receptors, and to subsequently activate the store-operated Ca^{2+} entry from the external medium to refill the depleted stores [31,32]. After Ca^{2+} mobilization, cytosolic Ca^{2+} was rapidly eliminated to internal store and external space by SERCA and PMCA, respectively. Since the accumulation of intracellular Ca^{2+} causes cellular toxicity, including apoptosis and necrosis, basal intracellular Ca^{2+} concentrations are finely regulated to under about 10,000 folds compare to the extracellular space [33]. The balance between Ca^{2+} mobilization and Ca^{2+} elimination are regulated by various Ca^{2+} transporters expressed both in plasma membrane and ER membrane [34]. Oxidative stress is well known risk factor which induces cellular dysfunction of several tissues and organs [10,11], and oxidants-induced cellular dysfunction are closely linked with intracellular Ca^{2+} accumulation [12,13]. In this study, when parotid acinar cells were exposed to 1 mM H_2O_2 in normal Ca^{2+} buffer, there was a significant intracellular Ca^{2+} accumulation, and the elevated Ca^{2+} was nearly returned to baseline after the cessation of H_2O_2 perfusion. Generally, H_2O_2 is known to accumulate cytosolic Ca^{2+} without acute cell death at concentrations from 10 μM to 5 mM in various cell types [16-25]. Thus, mouse parotid acinar cells are thought to be relative resistance to H_2O_2.

To confirm the underlying mechanism of H_2O_2-induced Ca^{2+} accumulation, we measured Ca^{2+} transport through plasma membrane and ER membrane. Actually, H_2O_2-induced Ca^{2+} entry through plasma membrane is important for intracellular Ca^{2+} accumulation, and transient-receptor potential channels and store-operated Ca^{2+} channels are known to involved in Ca^{2+} overload in various cell types [19-22]. However, in our study, the entire Ca^{2+} accumulation induced by 1 mM H_2O_2 was not suppressed by the elimination of extracellular Ca^{2+} in intact acinar cells and Mn^{2+} quenching property was not facilitated by the perfusion of 1 mM H_2O_2. Since 10 mM H_2O_2 produced more rapid cytosolic Ca^{2+} elevation and accelerated Mn^{2+} quenching fura-2 fluorescence, higher concentrations of H_2O_2 may be required to promote Ca^{2+} influx in parotid acinar cells. Moreover, H_2O_2 failed to stimulate $InsP_3$-induced Ca^{2+} release for measurement Ca^{2+} transport through ER membrane in permeabilized acinar cells. These results suggest that H_2O_2 could accumulate cytosolic Ca^{2+} irrelevant of Ca^{2+} entry from external fluid and Ca^{2+} release from ER stores. Since H_2O_2 could mobilize Ca^{2+} from other TG-insensitive intracellular stores such as mitochondria [35,36], we evaluated the effect of H_2O_2 in Ca^{2+}-free conditions after the depletion of Ca^{2+} from the ER store by the pretreatment of 1 μM TG (Supplementary Fig. 1). TG induced a transient Ca^{2+} elevation, and these responses were completely returned to baseline. After return to baseline, the perfusion of 1 mM H_2O_2 failed to increase the Ca^{2+} release. It has been proposed that mitochondrial Ca^{2+} release by H_2O_2 could conceivably contribute to Ca^{2+} accumula-

tion when cytosolic Ca^{2+} is high, since H_2O_2 evoked cytosolic Ca^{2+} elevation when cells were pre-stimulated by cholecystokinin (CCK) but failed to evoke cytosolic Ca^{2+} elevation without pre-stimulation of CCK in ER Ca^{2+} stores were depleted pancreatic acinar cells [24]. Thus, 1 mM H_2O_2-induced Ca^{2+} accumulation is not due to mobilize Ca^{2+} from other intracellular Ca^{2+} stores or mitochondria in our study. On the other hand, when TG were treated with 1 mM H_2O_2 in the absence of extracellular Ca^{2+}, cytosolic Ca^{2+} elevation was delayed and enhanced Ca^{2+} was not fully recovered to baseline. Actually, slow intracellular Ca^{2+} elevation by SERCA inhibition was likely due to spontaneous release or leak of Ca^{2+} from the ER stores, and slow Ca^{2+} decline was due to extrusion of Ca^{2+} to the external space by PMCA or Na^+/Ca^{2+} exchanger (NCX) activation [37]. Although, NCX are also known to sensitive to oxidants [12,13], there is no evidence of the expression and the participation of NCX for Ca^{2+} extrusion in parotid acinar cells at the present time. Furthermore, Ca^{2+} extrusion was not affected when Na^+ was replaced with $NMDG^+$ to NCX inactivation in our preliminary study (data not shown). Since the removal of elevated Ca^{2+} from cytosol to the extracellular site was carried out mainly by PMCA under inhibition of SERCA by TG treatment in parotid acinar cells, this finding strongly suggests that H_2O_2 effectively suppressed PMCA activity. In another set of experiment, we further investigated the effect of H_2O_2 on Ca^{2+} efflux through PMCA. PMCA activity was assessed by a Ca^{2+} decrease to baseline by removing extracellular Ca^{2+} after store-operated Ca^{2+} entry was fully stimulated by adding extracellular Ca^{2+} in TG-treated cells. Here we show that the extrusion of intracellular Ca^{2+} was markedly disrupted by pretreatment of 1 mM H_2O_2 in TG-treated intact acinar cells. Actually, the initial Ca^{2+} levels were slightly higher in H_2O_2-treated cells than in control cells. These results were considered caused by blocking of Ca^{2+} efflux through PMCA, rather than by enhancing of the Ca^{2+} entry through activation of store-operated Ca^{2+} channels. Furthermore, the inhibitory effects of H_2O_2 on Ca^{2+} efflux were strongly protected by the adding of catalase, an enzyme degrading hydrogen peroxide, and DTT, a sulfhydryl reducing agent.

In fact, it has been reported that functionally important sulfhydryl group are present within PMCA and SERCA molecules that is localized within the catalytic and controlling centers of Ca^{2+}-ATPase [38-40]. We previously reported that H_2O_2 accumulates intracellular Ca^{2+} by attenuating SERCA activity in pancreatic acinar cells [25]. Interestingly, in this study, H_2O_2 failed to change Ca^{2+} refilling into the ER store through SERCA by the application of Ca^{2+} and Mg-ATP in permeabilized parotid acinar cells. As shown in supplement Fig. 2, three folds higher concentration of H_2O_2 are need to inhibit Ca^{2+} efflux through PMCA in pancreatic acinar cells compared to parotid acinar cells. Ca^{2+}-ATPase has distinct isoforms with different expression and regulation properties that caused diversity of Ca^{2+} signaling in various cell types [41]. It has been known that distinct Ca^{2+}-ATPase isoforms have different sensitivity to ROS due to locational difference of sulfhydryl group [42-44]. Thus, the different sensitivities of Ca^{2+}-ATPase to H_2O_2 between parotid and pancreatic acinar cells are thought to be due to differences in expression and regulation of Ca^{2+}-ATPase. In immunofluorescence study, PMCA1 is distributed throughout the plasma membrane, PMCA2 is localized to the basolateral membrane and PMCA4 is localized to the apical membrane in parotid acinar cell [45]. Even though PMCAs are dominantly expressed in apical membrane in pancreatic acinar cells [46,47], isoform specific distributions are not clear at the present time. Although the further studies are need to elucidate this issues, we predict from the present results that H_2O_2 could lead to accumulate cytosolic Ca^{2+} by attenuation of Ca^{2+} efflux through the oxidation of functional sulfhydryl groups of PMCA in parotid acinar cells.

From the above results, we concluded that H_2O_2 can accumulate intracellular Ca^{2+} by attenuating Ca^{2+} efflux through PMCA, rather than by mobilizing Ca^{2+} from extracellular medium or intracellular stores in parotid acinar cells. Thus the primary target for H_2O_2 excessively generated in pathological conditions is considered PMCA in mouse parotid acinar cells.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at http://pdf.medrang.co.kr/paper/pdf/Kjpp/Kjpp022-02-11-s001.pdf.

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