Oxidative stress impairs the calcification ability of human dental pulp cells

Satomi Shirawachi¹, Katsuhiro Takeda¹*, Tomoya Naruse¹, Yohei Takahasi¹, Jun Nakanishi¹, Satoru Shindo² and Hideki Shiba¹

Abstract

Background: The relationship between internal root resorption and oxidative stress has not yet been reported. This study aimed to add molecular insight into internal root resorption. The present study was conducted to investigate the effect of hydrogen peroxide (H₂O₂) as an inducer of oxidative stress on the calcification ability of human dental pulp cells (hDPCs) and the involvement of inositol 1, 4, 5-trisphosphate (IP3).

Material and methods: hDPCs (Lonza, Basel, Switzerland) were exposed to H₂O₂. Cell viability and reactive oxygen species (ROS) production were then evaluated. To investigate the effect of H₂O₂ on the calcification ability of hDPCs, real-time PCR for alkaline phosphatase (ALP) mRNA expression, ALP staining, and Alizarin red staining were performed. Data were compared with those of hDPCs pretreated with 2-aminoethyldiphenylborate (2-APB), which is an IP3 receptor inhibitor.

Results: H₂O₂ at concentrations above 250 µM significantly reduced cell viability (P<0.01). More ROS production occurred in 100 µM H₂O₂-treated hDPCs than in control cells (P<0.01). 2-APB significantly decreased the production (P<0.05). H₂O₂-treated hDPCs showed significant reductions in ALP mRNA expression (P<0.01), ALP activity (P<0.01), and mineralized nodule deposition compared with negative control cells (P<0.01). 2-APB significantly inhibited these reductions (P<0.01, P<0.05 and P<0.01, respectively). Data are representative of three independent experiments with three replicates for each treatment and values are expressed as means±SD.

Conclusion: To the best of our knowledge, this is the first study documenting the involvement of IP3 signaling in the calcification ability of human dental pulp cells impaired by H₂O₂.

Keywords: Oxidative stress, Human dental pulp cells, 2-aminoethyldiphenylborate

Introduction

Internal root resorption in permanent teeth is characterized by destruction of intraradicular dentin and dental tubules along the root canal wall [1]. It occurs in pathological conditions, including trauma, infections, or unknown causes [1, 2]. Concern and curiosity about resorption of dental structures are not recent. Numerous theories have been proposed as a possible cause of internal root resorption. One study reported that dental pulp cells have an innate ability to attenuate dentin resorption by inhibiting osteoclastogenesis [3]. Therefore, it is assumed that the damaged dental pulp cells are probably related to the onset of internal root resorption. However, the underlying pathology is not fully understood.

Dental pulp cells can differentiate into odontoblasts and generate a mineralizing matrix, particularly during reparative dentin formation associated with injury or disease [4, 5]. Odontoblasts, which are organized in a layer...
at the dentin-pulp interface, secrete type I collagen, osteocalcin (OCN), alkaline phosphatase (ALP), and other noncollagenous proteins [6]. The impairment of reparative dentin formation in permanent teeth may be a key factor implicated in internal root resorption.

Oxidative stress is a damaging response and refers to excessive intracellular levels of reactive oxygen species (ROS). Hydrogen peroxide (H$_2$O$_2$) is one of the major ROS. At low physiological levels in the nanomolar range, H$_2$O$_2$ is the major agent signaling through specific protein targets that engage in metabolic regulation and stress responses to support cellular adaptation to a changing environment and stress. Many previous studies reported the effects of oxidative stress on pulp cells [7–9]. One study showed that the oxidative stress of human dental pulp cells mediated by H$_2$O$_2$ promotes reduction of odontoblastic capability [8]. On the other hand, Matsu et al. [9] found an increase in osteopontin (OPN) and osteocalcin (OCN) in H$_2$O$_2$-treated human dental pulp cells. Further investigations seem to be needed to clarify the mechanism. In addition, the relationship between internal root resorption and oxidative stress has not yet been reported. This study focused on H$_2$O$_2$ as an inducer of oxidative stress.

2-Aminoethyldiphenylborate (2-APB) was originally described as a membrane-permeant inhibitor of the inositol 1, 4, 5-trisphosphate (IP$_3$) receptor and it was also used as one of the store-operated Ca$^{2+}$ entry (SOCE) inhibitors [10]. Yamamura et al. [11] reported that oxidative stress reduced SOCE, and the decrease was recovered by 2-APB in brain capillary endothelial cells.

The present study was conducted to add molecular insight into root canal resorption. In the current study, it was demonstrated for the first time that IP$_3$ signaling is involved in the calcification ability of H$_2$O$_2$-treated human dental pulp cells. A possible mechanism to explain the influence of the oxidative stress caused by H$_2$O$_2$ on the calcification ability of human dental pulp cells is presented. The present study may provide a clue to help clarify a mechanism of onset of internal root resorption.

Materials and methods

Reagents

Hydrogen peroxide (H$_2$O$_2$) and 2-aminoethoxydiphenylborane (2-APB) were purchased from FUJIFILM Wako Pure Chemicals Corporation (Osaka, Japan) and Bio-Techne Corporation (Minneapolis, MN, USA), respectively.

Cell culture

Human dental pulp cells (hDPCs) were obtained from Lonza (Basel, Switzerland). Cells were cultured in growth medium (GM) (Eagle's Minimum Essential Medium, ALPHAC™ modification (MEM-α) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum; Sigma-Aldrich, MO, USA), 100 IU/mL penicillin (FUJIFILM Wako Pure Chemicals Corp.), and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemicals Corp.) (medium A). The cells were incubated in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C. Cell cultures between the sixth and ninth passages were used in this study.

To differentiate into odontoblast-like cells, the cells were cultured in osteo-odontogenic medium (OM), MEM-α supplemented with 2.5% heat-inactivated FBS (Sigma-Aldrich), 100 IU/mL penicillin (FUJIFILM Wako Pure Chemicals Corp.), 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemicals Corp.), 10 mM β-glycerophosphate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and 50 mg/mL ascorbic acid (FUJIFILM Wako Pure Chemicals Corp.).

WST assay

Cell viability was assessed by the WST assay using the Cell Counting Kit-8 according to the manufacturer’s protocol (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were plated at a density of 5.0 × 10$^4$ cells/well in 96-well plates and maintained in 0.1 mL of medium A. After 24-h incubation, culture medium was replaced to serum-free medium with H$_2$O$_2$ at concentrations of 0, 50, 100, 250, 500, and 1000 μM (final concentration) and incubated for 24 h in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C. Then, CCK-8 solution was added, and the cells were incubated for another hour. Absorbance at 450 nm was then measured with the reference wavelength at 620 nm using a microplate reader (Multiskan™ FC; Thermo Fisher Scientific, CA, USA). Data are representative of three independent experiments with three replicates for each treatment.

Intracellular reactive oxygen species measurements

Measurement of intracellular ROS was performed by dichlorodihydrofluorescein diacetate (DCFH-DA) (ROS Assay Kit-Highly Sensitive DCFH-DA; Dojindo Laboratories). Cells were seeded at a density of 1.0 × 10$^4$ cells in 6-well plates and incubated overnight in medium A. The medium was replaced to serum-free medium with 10 μM 2-APB or PBS for control cells. One hour later, the cells were stimulated with 100 μM H$_2$O$_2$ for 2 h in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C. DCFH-DA solution was added after 3-h incubation, and the cells were then incubated for another 30 min. Fluorescence signals were observed using an all-in-one fluorescence microscope (BZ-X700; Keyence, Tokyo, Japan). Data are representative of three independent experiments with three replicates for each treatment.
ALP staining
To evaluate alkaline phosphatase activity, the cells were stained by BCIP-NBT solution (SIGMA FAST™/BCIP®/NBT; Sigma-Aldrich). hDPCs were seeded into 48-well plates at a density of 1.0 × 10^4 cells/well in medium A. After the cells reached 70–80% confluency, the culture medium was replaced to fresh MEM-α supplemented with 2.5% FBS (Sigma-Aldrich), 100 IU/mL penicillin (FUJIFILM Wako Pure Chemicals Corp.), and 100 µg/mL streptomycin (FUJIFILM Wako Pure Chemicals Corp.) (medium B). The cells were treated with 10 µM 2-APB or PBS for control cells. One hour later, the cells were stimulated with 100 µM H2O2 for 6 h. Then, medium was replaced to PBS for control cells. One hour later, the cells were stimulated with 100 µM H2O2 for 6 h. The medium was changed on the third day. Total RNA from each culture was extracted using RNA iso plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s protocol. cDNA was synthesized from 250 ng of total RNA in a final volume of 10 µL with the Rever Tra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo Co., Ltd, Osaka, Japan) with a thermal cycler (Veriti™ 96-Well Thermal Cycler; Life Technologies, CA, USA).

ALizarin red staining
hDPCs were seeded into 6-well plates at a density of 1.0 × 10^5 cells/well and cultured in medium A. After the cells reached 70–80% confluency, the culture medium was changed on the third day. Total RNA from each culture was extracted using RNA iso plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s protocol. cDNA was synthesized from 250 ng of total RNA in a final volume of 10 µL with the Rever Tra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo Co., Ltd, Osaka, Japan) with a thermal cycler (Veriti™ 96-Well Thermal Cycler; Life Technologies, CA, USA).

Isolation of total RNA and reverse transcription
hDPCs were seeded into 6-well plates at a density of 1.0 × 10^5 cells/well and cultured in medium A until sub-confluent. The cells were treated with 10 µM 2-APB or PBS for control cells. One hour later, the cells were stimulated with 100 µM H2O2 for 6 h. The cells were stimulated with 100 µM H2O2 for 6 h. The cells were stimulated with 100 µM H2O2 for 6 h. The cells were stimulated with 100 µM H2O2 for 6 h.

Statistical analysis
Each result is presented as a mean ± standard error of the mean. All experiments were performed in at least triplicate. Comparisons between two groups were evaluated by an unpaired two-tailed Student’s t-test. For comparisons of more than two groups, one-way ANOVA was followed by Tukey’s test for multiple comparisons. Significance was set at p < 0.05.

Results
hDPCs underwent severe cell death after high-dose H2O2 (250, 500 and 1000 µM) treatment for 24 h as determined by the WST assay (Fig. 1A). H2O2 at 50 and 100 µM did not decrease cell viability. ROS were increased in 100 µM H2O2-treated hDPCs (Fig. 1B). Therefore, in the present study, H2O2 at 100 µM, which is enough to promote ROS production, was used.

To determine whether H2O2 has an effect on the calcification ability of hDPCs in the present experimental system, the effects of H2O2 on hDPCs cultured in osteo-inductive medium were tested via ALP activity. After osteogenic differentiation for 7 days, H2O2 at 100 µM significantly decreased ALP activity in hDPCs (P < 0.01) (Fig. 2A, B).
Real-time PCR was used to determine the effect of H$_2$O$_2$ addition on the mRNA expression of ALP. H$_2$O$_2$ significantly decreased the ALP mRNA levels compared with control cells ($P < 0.01$). 2-APB reversed the decrease significantly ($P < 0.05$) (Fig. 3A).

The percentages of the BCIP-NBT stained area of the control group, positive control group (cultured in osteo-inductive medium), H$_2$O$_2$ group and H$_2$O$_2$/2-APB treated group were 24.4 ± 1.3%, 76.9 ± 5.9%, 45.2 ± 0.6%, and 62.6 ± 4.1%, respectively. H$_2$O$_2$ down-regulated ALP activity compared with positive control cells (cultured in osteo-inductive medium). 2-APB significantly rescued the decrease of ALP activity in H$_2$O$_2$-treated cells ($P < 0.01$) (Fig. 3B, C).

The percentages of the Alizarin red-stained area of the control group, positive control group (cultured in osteo-inductive medium), H$_2$O$_2$ group, and H$_2$O$_2$/2-APB treated group were 19.1 ± 3.6%, 67.1 ± 3.2%, 26.9 ± 4.8%, and 57.1 ± 1.5%, respectively. Alizarin red staining showed that 2-APB significantly increased H$_2$O$_2$-decreased hDPC calcification ($P < 0.01$) (Fig. 4A, B).

ROS were increased in 100 μM H$_2$O$_2$-treated hDPCs (Fig. 5A(a, b). 10 μM 2-APB inhibited the ROS production induced by 100 μM H$_2$O$_2$ (Fig. 5A(c)). 10 μM 2-APB did not affect the ROS production(Fig. 5A(d)). FITC fluorescence intensity of the H$_2$O$_2$/2-APB treated group was significantly decreased compared to that of the H$_2$O$_2$-treated group ($P < 0.05$) (Fig. 5B).

Discussion

H$_2$O$_2$ promotes ROS production in various cells, such as human periodontal ligament cells, human retinal epithelial cells, and vascular endothelial cells [12–14]. H$_2$O$_2$ can induce oxidative destruction of organs and tissues,
associated with other inflammatory responses when overexpressed in cells [15]. The effect of H$_2$O$_2$ in a living system is dependent on the type of cell, its concentration, its physiological state, and duration of exposure [16, 17]. Deng et al. [18] reported that, like other ROS molecules, a high concentration of H$_2$O$_2$ is cytotoxic to cells. In the present study, high-dose H$_2$O$_2$ (250, 500 and 1000 μM) significantly reduced the viability of hDPCs. Loss of cell viability is one characteristic of apoptotic cells. Therefore, in the present study, to avoid the influence of apoptosis, H$_2$O$_2$ at 100 μM, which is enough to promote ROS production without inducing apoptosis, was used.
Fig. 3. 2-APB reverses H$_2$O$_2$-decreased ALP mRNA expression and ALP activity of hDPCs. **A** ALP mRNA was determined by real-time PCR. Graphs show the ratio of ALP mRNA to GAPDH mRNA. Values represent means±SD of three cultures. *p<0.05, **p<0.01: differs significantly from the control. **B**(a) A macroscopic view of wells showing hDPCs cultured in growth medium, hDPCs cultured in osteo-odontogenic medium, hDPCs treated with 100 μM H$_2$O$_2$ in osteo-odontogenic medium, and hDPCs treated with 10 μM 2-APB before the addition of 100 μM H$_2$O$_2$ in osteo-odontogenic medium. (b) **B**(a) panels have been converted to RGB stack, and “blue slice” images were picked up. (c) The thresholds of **B**(b) images were adjusted, and a limited threshold area is highlighted in red. **C** The stained area was quantified using the software NIH Image J® on digitized photomicrographs **B**(c) captured by a Windows-based computer. Data are shown as means±SD of three independent experiments. *p<0.05, **p<0.01, one-way ANOVA with Tukey’s test.
Alkaline phosphatase (ALP), involved with the initial phase of dentin matrix biomineralization, promotes dephosphorylation of extracellular matrix proteins, providing inorganic phosphate [19]. In the present study, H$_2$O$_2$ decreased ALP activity in hDPCs cultured in osteo-inductive medium. These data are in
Fig. 5. 2-APB inhibits the ROS production induced by H$_2$O$_2$ in hDPCs. A ROS production in hDPCs. Fluorescence signals were detected with a confocal microscope. (a) control, (b) H$_2$O$_2$ 100 μM, (c) H$_2$O$_2$ 100 μM + 2-APB 10 μM, (d) 2-APB 10 μM. Bars: 100 μm. B The graph shows quantitative results of ROS production from three independent experiments. Per well, three pictures were taken at random. FITC fluorescence intensity from an area of 2 mm square from each picture was analyzed using Image J software on digitized photomicrographs captured by a Windows-based computer. Data represent means ± SD. *P < 0.05, **P < 0.01, one-way ANOVA with Tukey’s test.
agreement with those from previous studies [8, 20]. Correlated with ALP activity, the present study also showed decreased ALP mRNA expression by \( \text{H}_2\text{O}_2 \) in hDPCs.

2-APB has been described as a membrane-permeant inhibitor of the IP\(_3\) receptor, and the inhibition of SOCE by 2-APB was taken as evidence for IP\(_3\) receptor activation of calcium release-activated Ca\(^{2+}\) channels [21]. It has been reported that the increase in intercellular Ca\(^{2+}\) concentration regulates cell functions, such as proliferation, differentiation, and migration [22]. A previous study reported that oxidative stress reduced SOCE, and the decrease was reversed by 2-APB in brain capillary endothelial cells [11]. In the present study, the \( \text{H}_2\text{O}_2 \)-decreased human dental pulp cell calcification ability was reversed by 2-APB. In addition, an important criterion for the characterization of odontoblastic cells is their ability to mineralize the collagenous matrix they secrete. In the present study, 2-APB rescued the \( \text{H}_2\text{O}_2 \)-decreased amounts of mineralization of the extracellular matrix, judged by Alizarin red staining. Contrary to the present findings, it has been reported that vascular smooth muscle cell calcification was reduced by 2-APB [23, 24]. 2-APB is also used as an inhibitor of transient receptor potential potential channels (TRPCs), in which Ca\(^{2+}\) entry was inhibited directly, rather than as a result of the inhibition of IP\(_3\) [25]. Further experiments are needed to clarify the detailed mechanism underlying the relationships between IP\(_3\), SOCE, TRPC, and the calcification ability of human dental pulp cells.

It has been reported that 2-APB reduced ROS production in neutrophils and prevented ROS-induced cardiomyocyte death [26, 27]. The present study showed for the first time that 2-APB reduces ROS production in hDPCs. Therefore, administration of 2-APB may represent a promising therapeutic strategy for the treatment of ROS-related endodontic disease. Further experiments beyond the scope of this paper are required to determine the relationship between ROS and endodontic disease.

In summary, \( \text{H}_2\text{O}_2 \) decreased the calcification ability of human dental pulp cells, and the reduction was reversed by 2-APB. To the best of our knowledge, this is the first study documenting the involvement of IP\(_3\) signaling in the calcification ability of human dental pulp cells. The results of the present study add molecular insight into internal root resorption and may provide a clue to the development of a new therapeutic agent for such resorption in endodontic therapy.

Abbreviations

- hDPCs: Human dental pulp cells; \( \text{H}_2\text{O}_2 \): Hydrogen peroxide; ROS: Reactive oxygen species; 2-APB: 2-Aminoethyldiphenylborate.
