

Title	Hydrogen peroxide induces expression and activation of AMP-activated protein kinase in a dental pulp cell line
Author(s) Alternative	Fukuyama, Y; Ohta, K; Okoshi, R; Kizaki, H; Nakagawa, K
Journal	International endodontic journal, 41(3): 197-203
URL	http://hdl.handle.net/10130/559
Right	The definitive version is available at www.blackwell-synergy.com

Abstract

Aim To investigate the effects of hydrogen peroxide on cell viability and expression and activation of AMP-activated protein kinase (AMPK) in rat dental pulp cell line RPC-C2A.

Methodology RPC-C2A cells derived from rat dental pulp were maintained in MEM supplemented with 10% FBS at 37°C, in a humidified atmosphere at 5% CO₂. Cells were cultured in the presence or absence of H₂O₂ for up to 60 min at concentrations of from 0.1 mM to 3.0 mM. Cell viability was analyzed by WST-1 reduction assay. Expression of AMPK subunit isoforms was analyzed by Western blotting using antibodies to the catalytic α 1 and regulatory β 1 and γ 1 subunit isoforms. The effect of silencing AMPK α 1 on cell viability was determined using siRNA.

Results Exposure to H₂O₂ decreased cell viability time- and dose-dependently. The catalytic AMPK α 1 subunit and its activated form, phospho-AMPK α , increased with exposure to H₂O₂ time- and dose-dependently, whereas the regulatory β 1 and γ 1 subunits showed no change. Downregulation of AMPK α 1 resulted in a reduction in cell viability in H₂O₂-treated cells at a concentration of 0.1 mM for 30 min incubation, indicating an increased sensitivity to H₂O₂.

Conclusions Reactive oxygen induced energy fuel gauge enzyme AMPK α expression and

its activation by phosphorylation in RPC-C2A cells, suggesting that AMPK is essential for protection against H₂O₂-induced non-apoptotic cell death. Therefore, AMPK may be a therapeutic modulation target for treatment of dentin-pulp complex injured by reactive oxygen.

Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), are involved in a variety of responses in inflammatory diseases, as well as in cancer (Salvemini *et al.* 2006, Fruehauf & Meyskens 2007). In dental pulp tissue, ROS and antioxidants play an important role in modulation of inflammation through metabolic reaction and morphological alteration of pulp cells (Baumgardner & Sulfaro 2001, Shimauchi *et al.* 2001, Esposito *et al.* 2003).

AMP-activated protein kinase (AMPK), a serine/threonine protein kinase, functions as an energy sensor in metabolic adaptation to stress, including ROS (Choi *et al.* 2001, Leverve *et al.* 2003). It is a heterotrimeric enzyme composed of a catalytic α subunit ($\alpha 1$ and $\alpha 2$) and regulatory β ($\beta 1$ and $\beta 2$) and γ subunits ($\gamma 1$, $\gamma 2$ and $\gamma 3$) (Stapleton *et al.* 1996, Woods *et al.* 1996). The main AMPK subunit isoforms in RPC-C2A cells were catalytic $\alpha 1$ and regulatory $\beta 1$ and $\gamma 1$ (Fukuyama *et al.* 2007). AMPK is activated by an increase in cellular AMP concentration resulting from ATP depletion and by phosphorylation on Thr172 of the catalytic α subunit by upstream kinases (Carling 2004, Hardie 2004).

The aim of this study was to investigate the effects of H₂O₂ on cell viability, as assessed by water-soluble tetrazolium salt (WST-1) reduction assay and the expression of

the major AMPK subunit isoforms in rat dental pulp RPC-C2A cells by Western blotting.

Materials and methods

Cell culture

RPC-C2A cell line, established from the dental pulp of the rat incisor, has high alkaline phosphatase (ALP) activity and is capable of differentiating into odontoblast-like cells (Kasugai *et al.* 1988). Cells were provided by S. Kasugai (Department of Pharmacology, Faculty of Dentistry, Tokyo Medical and Dental University, Japan) and maintained in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 10 mg/ml kanamycin at 37°C in a humidified atmosphere at 5% CO₂.

Cell viability assay

Cells were seeded in 96-well plates at a density of 5×10^4 cells/well and cultured for 24 h. After addition of H₂O₂ (Wako Pure Chemical, Osaka, Japan) to a final concentration of 0.1, 0.25, 0.5, 1.0 or 3.0 mM, the cells were then further cultured for 5, 10, 30 or 60 min. After treatment with H₂O₂, catalase (Sigma-Aldrich, St. Louis, MO, USA) was added at 200 U/ml to inactivate H₂O₂, followed by incubation for an additional 10 min. Thereafter, cell viability was measured by WST-1 reduction assay using the Cell Counting Kit

(Dojindo, Kumamoto, Japan) and the Micro-plate Reader Model 3550 (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Cell viability was also determined by trypan blue exclusion test. After inactivating H₂O₂, an equal volume of 0.4 % Trypan Blue Stain (Invitrogen) was added, followed by incubation for 3 min at room temperature, after which, samples were obtained for cell counts.

Western blotting

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well and cultured for 24 h. H₂O₂ was then added to a final concentration of 0.25, 0.5, 1.0 or 3.0 mM, followed by culture for an additional 10, 30 or 60 min. After incubation, the cells were collected, washed once in phosphate-buffered saline, and then sonicated in ice-cold lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich) at 20 W for 2 min. Supernatant obtained by centrifugation at $10\,000 \times g$ for 15 min at 4°C was used for analysis.

Proteins (5 µg) were separated on 10% SDS-polyacrylamide gel and blotted onto an Immobilon-P Transfer membrane (Millipore, Bedford, MA, USA), which was then

incubated in blocking buffer containing 5% skim milk in TBS-T (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20) for 1 h. It was then incubated with anti-phospho-AMPK α (1:2000, Cell Signaling Technology, Beverly, MA, USA), anti- α 1 (1:1000, Upstate, Charlottesville, VA, USA), anti- β 1 (1:1000), anti- γ 1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-actin (1:10,000, Sigma-Aldrich) overnight at 4°C. Blots were detected using the ECL plus Detection Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Images were scanned and the densitometric value of each band was analyzed using the NIH Image (Scion Corporation, Frederick, MD, USA).

RNA interference

The sequence of small interference (si) RNA for AMPK α 1 was 5'-UUAAAUGGUGAUCAUUCGAGGAAAGA-3'. Both the experimental and control siRNAs were obtained from Invitrogen. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well for 24 h. siRNA was delivered to cells in Opti-MEM (Invitrogen) with Lipofectamine RNAiMAX (Invitrogen) at 37°C. After 5 h incubation, the medium was replaced with MEM containing 10% FBS, and the cells were cultured for an additional 19 h. Expression of AMPK α 1 was analyzed by Western blotting. H₂O₂ was then added to a

final concentration of 0.1 mM, followed by culture for an additional 30 min. Cell viability was analyzed by WST-1 reduction assay.

Statistical analysis

The Mann-Whitney U-test was used for statistical analysis of the cell viability data.

Densitometric data from Western blotting were statistically analyzed by one-way ANOVA.

Differences were considered statistically significant at $p < 0.05$.

Results

Effects of H₂O₂ on cell viability

Cell viability as assessed by WST-1 reduction assay was almost identical to that determined by the trypan blue exclusion test (Fig. 1A) at each concentration of H₂O₂ and incubation period. Therefore, WST-1 reduction assay was used for assessment of cell viability. H₂O₂ at 0.1 mM decreased cell viability to about 92%, 87%, and 85% of that of the controls at 10 min, 30 min, and 60 min incubation, respectively. However, at higher concentrations of above 0.25 mM, cell viability showed a reduction at 5 min, which decreased time- and dose-dependently. Cell viability at 30 min was approximately 85%, 64%, 55%, and 30% of that of the controls at 0.25, 0.5, 1, and 3 mM H₂O₂, respectively (Fig. 1B).

During incubation with H₂O₂, dead cells floated. Dead cells were observed in the presence of 50 μM pan-caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) (R&D Systems) at the same level as in the absence of Z-VAD, and neither chromatin condensation in floating cells, nor DNA ladder formation was observed by Hoechst 33342 staining and electrophoresis on an agarose gel, respectively (data not shown).

Effects of H₂O₂ on expression of major AMPK subunit isoforms and activation of AMPK

To determine whether expression and activation of AMPK in pulp cells were regulated by oxidative stress, expression of the main AMPK subunit isoforms, catalytic $\alpha 1$ and regulatory $\beta 1$ and $\gamma 1$, and activation by phosphorylation of the α subunit were investigated after exposure to H₂O₂ by Western blotting using the corresponding antibodies and phospho-AMPK α , respectively. AMPK $\alpha 1$ protein showed an approximately 1.6 fold-increase at 30 min with 0.5 mM H₂O₂ (Figs 2A-C). However, the levels of regulatory subunits $\beta 1$ and $\gamma 1$ showed no change (Fig. 2A), and no induction of $\alpha 2$, $\beta 2$, $\gamma 2$ or $\gamma 3$ were observed (data not shown). Phospho-AMPK α increased dose-dependently, reaching a maximum value with incubation at 3 mM H₂O₂ (Figs 2A and 2B). At 0.5 mM H₂O₂, phospho-AMPK α increased by approximately 3.4fold at 30 min, reaching almost the maximum level (Fig. 2C).

Effects of downregulation of AMPK $\alpha 1$ on cell viability

To clarify the role of AMPK under H₂O₂ treatment, the effect of silencing AMPK $\alpha 1$ on cell viability was investigated by using siRNA, as AMPK $\alpha 1$ is the main isoform of the

catalytic subunit in RPC-C2A cells. At 24 h incubation with siRNA, expression of AMPKa 1 protein decreased to approximately 70% of that in the cells transfected with control siRNA (Fig. 3A). Downregulation of AMPKa 1 induced a reduction in cell viability of approximately 30% of that in cells transfected with control siRNA in the absence of H₂O₂, whereas in the presence of H₂O₂ at 0.1 mM for 30 min incubation, cell viability showed a further reduction to about 20% of that of the control (Fig. 3B).

Discussion

The present study showed that H₂O₂ at concentrations of above 0.1 mM reduced RPC-C2A cell viability, with no DNA ladder formation or nuclear condensation.

Exposure to H₂O₂ induced expression of AMPK α 1, a prominent catalytic subunit isoform in such cells, activating it through phosphorylation. Downregulation of AMPK α 1 reduced cell viability and sensitized cells to H₂O₂.

Many cell mediators and enzymes are involved in lesions resulting from inflammation of dental pulp (Forman *et al.* 2001). Lee *et al.* (2006) and Schweikl *et al.* (2006) showed that dental materials induced oxidative stress, resulting in mutagenicity and apoptosis in cultured pulp cells. It has been reported that H₂O₂ induced apoptosis and necrosis in a variety of cells, depending on concentration and exposure time (Garg & Chang 2003, Bae *et al.* 2007). Apoptotic cell death and necrosis were induced in human T-lymphoma Jurkat cells treated with 0.05 mM and 0.5 mM H₂O₂, respectively (Saito *et al.* 2006). The present study showed that dead cells floated, even in the presence of Z-VAD-FMK, a pan-caspase inhibitor, and neither chromatin condensation nor DNA ladder formation was observed, suggesting that H₂O₂ at concentrations of above 0.1 mM induces necrosis rather than apoptosis in RPC-C2A cells.

It has been reported that AMPK is highly sensitive to oxidative stress in a variety of cells such as NIH-3T3 cells (Choi *et al.* 2001), mouse Neuro 2a neuroblastoma cells (Jung *et al.* 2003) and rat skeletal muscle (Toyoda *et al.* 2004), and that it is activated by H₂O₂ in a time- and dose-dependent manner. The results of the present study showed that AMPKα1 expression increased in a time- and dose-dependent manner by incubation with H₂O₂ at concentrations of between 0.1 and 3 mM, they also showed that AMPK was activated by exposure to H₂O₂, as evidenced by increased phospho-AMPKα. It has been reported that H₂O₂ evokes intercellular ATP depletion (Tavazzi *et al.* 2000), and that the profile of AMPK activation by H₂O₂ is associated with an increase in the AMP: ATP ratio (Choi *et al.* 2001). Taken together with the results of this study, these suggest that exposure to H₂O₂ increases the AMP:ATP ratio, resulting in activation of AMPKα. As AMPK acts as an intercellular energy sensor maintaining energy balance within cells during oxidative stress (Choi *et al.* 2001), increased expression and activation of AMPKα1 under treatment with H₂O₂ suggests an important role for AMPK in pulp cells in the maintenance of energy homeostasis under oxidative stress.

To further explore the role of AMPK in H₂O₂ response, AMPKα1 was downregulated using siRNA, and cell viability and sensitivity to H₂O₂ were analyzed. When the cells

were transfected with siRNA, AMPK α 1 was downregulated to approximately 70% of that in the control cells, and cell viability decreased to approximately 29% of that of the controls after 24 h incubation. This downregulation was cytotoxic to a similar level to that seen in the cells treated with H₂O₂ at 1 mM for 60 min or at 3 mM for 30 min. Even in the presence of a 1.5 to 3-fold increase in expression of AMPK α and a 3 to 7-fold increase in activation with H₂O₂, cell death was induced by approximately 10% at 0.1 mM, however, the level of cytotoxicity was less than that observed in AMPK α -downregulated cells, with cell viability of 20% at 0.1 mM. It has been reported that activation of AMPK has protective effects against ischemia and ischemia-reperfusion injury in rat hepatocytes (Peralta *et al.* 2001), heart (Russell *et al.* 2004, Dyck & Lopaschuk 2006), kidney (Mount *et al.* 2005) and brain (McCullough *et al.* 2005). Taken together with the results of this study, these suggest that dental pulp cells are more sensitive to H₂O₂ than other tissues, and that AMPK α 1 regulates sensitivity to H₂O₂ and is essential for cell survival via energy adaptation in dental pulp cells. Therefore, AMPK may be a therapeutic modulation target for treatment of dentin-pulp complex injured by reactive oxygen.

Conclusions

RPC-C2A cells were susceptible to oxidative stress, which induced AMPK expression and activation. In addition, AMPK was essential in the protection of RPC-C2A cells.

Acknowledgements

The present study was supported by a Grant -in-Aid for Scientific Research (No. 15591978 for H. K.) from the Ministry of Education, Culture, Sports, Science and Technology. We would like to thank Jeremy Williams, Laboratory of International Dental Information, Tokyo Dental College, for his assistance with the English of this manuscript

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Figure legends

Figure 1. Effects of H₂O₂ on cell viability. RPC-C2A cells were incubated in presence of H₂O₂ for times and concentrations indicated. (A) After treatment with 0 or 0.25 mM H₂O₂ and 30 min incubation, trypan blue staining assays were performed. (B) Cell viability was analyzed by WST-1 reduction assay, and is expressed as percentage of control. Data represent means \pm SD of three independent experiments.

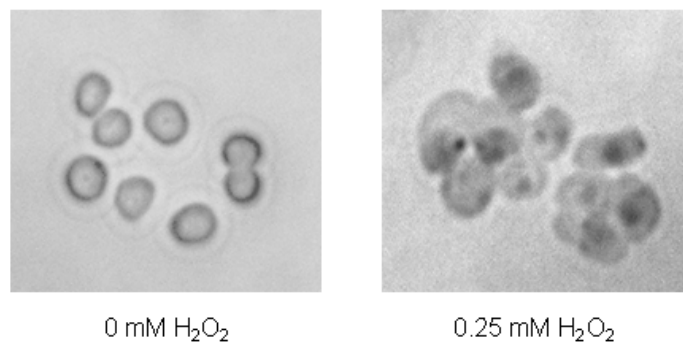
Figure 2. Effects of H₂O₂ on expression of main AMPK subunit isoforms. RPC-C2A cells were incubated in presence of H₂O₂ for times and concentrations indicated. Expression of AMPK subunit isoforms was analyzed by Western blotting (A). Each protein level was normalized to expression of β -actin, and relative protein levels to that at 0 mM are shown in Figure (B, C). Blot values represent means \pm SD of three independent experiments. * p<0.05 vs 0 min.

Figure 3. Effects of AMPKa 1 silencing on cell proliferation. RPC-C2A cells were transfected with control siRNA or siRNA against AMPK a1, and incubated for 24 hrs. (A) Expression of AMPKa 1 was analyzed by Western blotting. Each protein level was

normalized to expression of β -actin, and relative protein level to that of control is shown in Figure. Blot values represent means \pm SD of three independent experiments. * $p < 0.05$ vs control. (B) Cells were then treated with 0.1 mM H_2O_2 for 30 min. Cell proliferation was analyzed by WST-1 reduction assay, and is expressed as percentage of control. Data represent means \pm SD of three independent experiments. * $p < 0.05$.

Figure 1

(A)



(B)

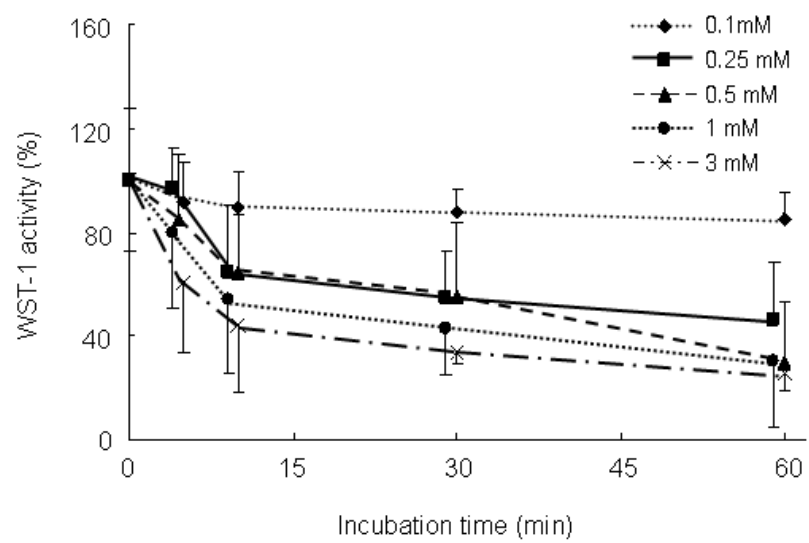
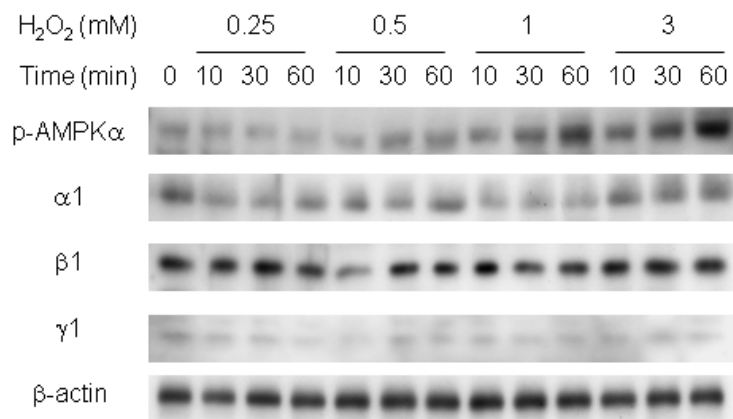
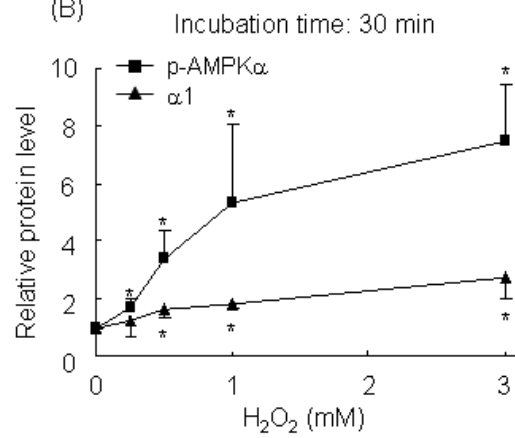


Figure 2

(A)



(B)



(C)

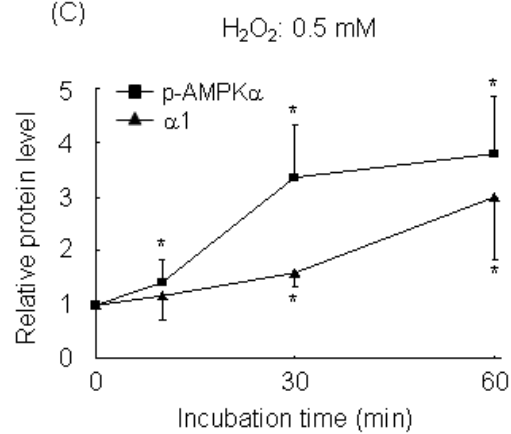


Figure 3

