doi:10.1111/j.1365-2591.2007.01337.x

Hydrogen peroxide induces expression and activation of AMP-activated protein kinase in a dental pulp cell line

Y. Fukuyama[1], K. Ohta[2,*], R. Okoshi[2], H. Kizaki[2] & K. Nakagawa[1]

Departments of [1]Endodontics, Pulp and Periapical Biology and [2]Biochemistry, Tokyo Dental College, Chiba City, Japan

Abstract

Fukuyama Y, Ohta K, Okoshi R, Kizaki H, Nakagawa K. Hydrogen peroxide induces expression and activation of AMP-activated protein kinase in a dental pulp cell line. *International Endodontic Journal*, **41**, 197–203, 2008.

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% CO_2. Cells were cultured in the presence or absence of H_2O_2 for up to 60 min at concentrations of from 0.1 to 3.0 mmol L^{-1}. Cell viability was analysed by WST-1 reduction assay. Expression of AMPK subunit isoforms was analysed by Western blotting using antibodies to the catalytic α1 and regulatory β1 and γ1 subunit isoforms. The effect of silencing AMPKα1 on cell viability was determined using siRNA.

Results Exposure to H_2O_2 decreased cell viability in a time- and dose-dependent manner. The catalytic AMPKα1 subunit and its activated form, phospho-AMPKα, increased with exposure to H_2O_2 in a time- and dose-dependent manner, whereas the regulatory β1 and γ1 subunits showed no change. Downregulation of AMPKα1 resulted in a reduction in cell viability in H_2O_2-treated cells at a concentration of 0.1 mmol L^{-1} for 30 min incubation, indicating an increased sensitivity to H_2O_2.

Conclusions Reactive oxygen induced energy fuel gauge enzyme AMPKα expression and its activation by phosphorylation in RPC-C2A cells, suggesting that AMPK is essential for protection against H_2O_2-induced nonapoptotic cell death. Therefore, AMPK may be a therapeutic modulation target for treatment of the dentine–pulp complex injured by reactive oxygen.

Keywords: adenosine monophosphate-activated protein kinase (AMPK), dental pulp cells, H_2O_2.

Received 28 May 2007; accepted 6 August 2007

Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), 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 the modulation of inflammation through metabolic reaction and morphological alteration of pulp cells (Baumgardner & Sulfaro 2001, Shimauchi *et al.* 2001, Esposito *et al.* 2003).

Adenosine monophosphate-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 (α1 and α2) and regulatory β (β1 and β2) and γ subunits (γ1, γ2 and γ3) (Stapleton *et al.* 1996, Woods *et al.* 1996). The main AMPK subunit isoforms in RPC-C2A cells were catalytic α1 and regulatory β1 and γ1 (Fukuyama *et al.* 2007). AMPK is activated by an increase in cellular AMP concentration resulting

Correspondence: Dr Yoshiko Fukuyama, Department of Endodontics, Pulp and Periapical Biology, Tokyo Dental College, 1-2-2, Masago, Mihama-ku, Chiba City 261-8502, Japan (Tel.: +81 43 270 3950; fax: +81 43 270 3951; e-mail: gakofukuyama@tdc.ac.jp).

*Present address: Department of Biology, Tokyo Medical University, 6-1-1 Shinjyuku, Shinjyuku-ku, Tokyo 160-8402, Japan.

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 H_2O_2 on cell viability, as assessed by a 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^{-1} kanamycin at 37 °C in a humidified atmosphere at 5% CO_2.

Cell viability assay

Cells were seeded in 96-well plates at a density of 5×10^4 cells per well and cultured for 24 h. After the addition of H_2O_2 (Wako Pure Chemical, Osaka, Japan) to a final concentration of 0.1, 0.25, 0.5, 1.0 or 3.0 mmol L^{-1}, the cells were then further cultured for 5, 10, 30 or 60 min. After treatment with H_2O_2, catalase (Sigma-Aldrich, St Louis, MO, USA) was added at 200 U mL^{-1} to inactivate H_2O_2, 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 H_2O_2, 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 six-well plates at a density of 5×10^5 cells per well and cultured for 24 h. H_2O_2 was then added to a final concentration of 0.25, 0.5, 1.0 or 3.0 mmol L^{-1}, 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 mmol L^{-1} Tris–HCl (pH 7.4), 10 mmol L^{-1} NaCl, 3 mmol L^{-1} $MgCl_2$, 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 ***g*** for 15 min at 4 °C was used for the 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 mmol L^{-1} Tris–HCl (pH 7.4), 150 mmol L^{-1} NaCl, 0.1% Tween 20] for 1 h. It was then incubated with anti-phospho-AMPKα (1 : 2000; Cell Signaling Technology, Beverly, MA, USA), anti-α1 (1 : 1000; Upstate, Charlottesville, VA, USA), anti-β1 (1 : 1000), anti-γ1 (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 analysed using the NIH Image (Scion Corporation, Frederick, MD, USA).

RNA interference

The sequence of small interference (si) RNA for AMPKα1 was 5′-UUAAAUGGUGAUCAUCGAGGAA AGA-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 per well for 24 h. siRNA was delivered to cells in Opti-MEM (Invitrogen) with Lipofectamine RNAiMAX (Invitrogen) at 37 °C. After 5 h of incubation, the medium was replaced with MEM containing 10% FBS, and the cells were cultured for an additional 19 h. Expression of AMPKα1 was analysed by Western blotting. H_2O_2 was then added to a final concentration of 0.1 mmol L^{-1}, followed by culture for an additional 30 min. Cell viability was analysed by WST-1 reduction assay.

Statistical analysis

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

from Western blotting were statistically analysed by a one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

Effects of H_2O_2 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 H_2O_2 and incubation period. Therefore, WST-1 reduction assay was used for the assessment of cell viability. H_2O_2 at 0.1 mmol L^{-1} decreased cell viability to about 92%, 87% and 85% of that of the controls at 10, 30 and 60 min of incubation respectively. However, at higher concentrations of above 0.25 mmol L^{-1}, cell viability showed a reduction at 5 min, which decreased in a time- and dose-dependent manner. 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 mmol L^{-1} H_2O_2 respectively (Fig. 1b).

During incubation with H_2O_2, dead cells floated. Dead cells were observed in the presence of 50 μmol L^{-1} pan-caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK) (R&D Systems, Minneapolis, MA, USA) 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 Höechst 33342 staining and electrophoresis on an agarose gel respectively (data not shown).

Effects of H_2O_2 on the expression of major AMPK subunit isoforms and activation of AMPK

To determine whether the expression and activation of AMPK in pulp cells were regulated by oxidative stress, expression of the main AMPK subunit isoforms, cata-

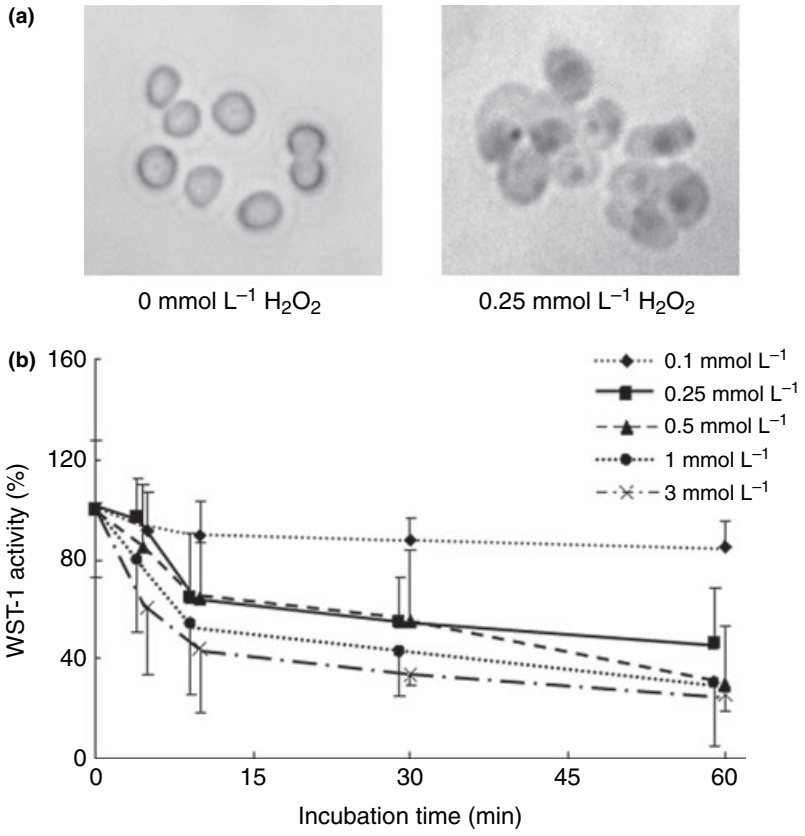

Figure 1 Effects of H_2O_2 on cell viability. RPC-C2A cells were incubated in the presence of H_2O_2 for times and concentrations indicated. (a) After treatment with 0 or 0.25 mmol L^{-1} H_2O_2 and 30 min of incubation, trypan blue staining assays were performed. (b) Cell viability was analysed by WST-1 reduction assay, and is expressed as percentage of control. Data represent means ± SD of three independent experiments.

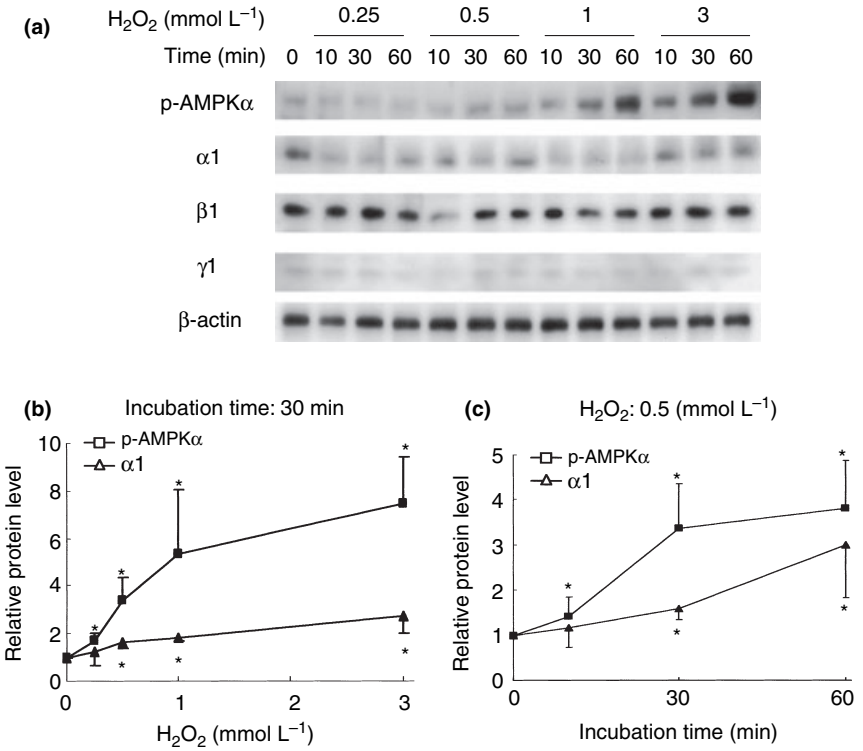

Figure 2 Effects of H_2O_2 on the expression of main AMPK subunit isoforms. RPC-C2A cells were incubated in the presence of H_2O_2 for times and concentrations indicated. Expression of AMPK subunit isoforms was analysed by Western blotting (a). Each protein level was normalized to expression of β-actin, and relative protein levels to that at 0 mmol L^{-1} are shown in (b,c). Blot values represent means ± SD of three independent experiments. *$P < 0.05$ vs 0 min.

lytic α1 and regulatory β1 and γ1, and activation by phosphorylation of the α subunit were investigated after exposure to H_2O_2 by Western blotting using the corresponding antibodies and phospho-AMPKα respectively. AMPKα1 protein showed an approximately 1.6-fold increase at 30 min with 0.5 mmol L^{-1} H_2O_2 (Fig. 2a–c). However, the levels of regulatory subunits β1 and γ1 showed no change (Fig. 2a), and no induction of α2, β2, γ2 or γ3 were observed (data not shown). Phospho-AMPKα increased in a dose-dependent manner, reaching a maximum value with incubation at 3 mmol L^{-1} H_2O_2 (Fig. 2a,b). At 0.5 mmol L^{-1} H_2O_2, phospho-AMPKα increased by approximately 3.4-fold at 30 min, reaching almost the maximum level (Fig. 2c).

Effects of downregulation of AMPKα1 on cell viability

To clarify the role of AMPK under H_2O_2 treatment, the effect of silencing AMPKα1 on cell viability was investigated by using siRNA, as AMPKα1 is the main isoform of the catalytic subunit in RPC-C2A cells. At 24 h of incubation with siRNA, expression of AMPKα1 protein decreased to approximately 70% of that in the cells transfected with control siRNA (Fig. 3a). Downregulation of AMPKα1 induced a reduction in cell viability of approximately 30% of that in cells transfected with control siRNA in the absence of H_2O_2, whereas in the presence of H_2O_2 at 0.1 mmol L^{-1} 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 H_2O_2 at concentrations of above 0.1 mmol L^{-1} reduced RPC-C2A cell viability, with no DNA ladder formation or nuclear condensation. Exposure to H_2O_2 induced the expression of AMPKα1, a prominent catalytic subunit isoform in such cells, activating it through phosphorylation. Downregulation of AMPKα1 reduced cell viability and sensitized cells to H_2O_2.

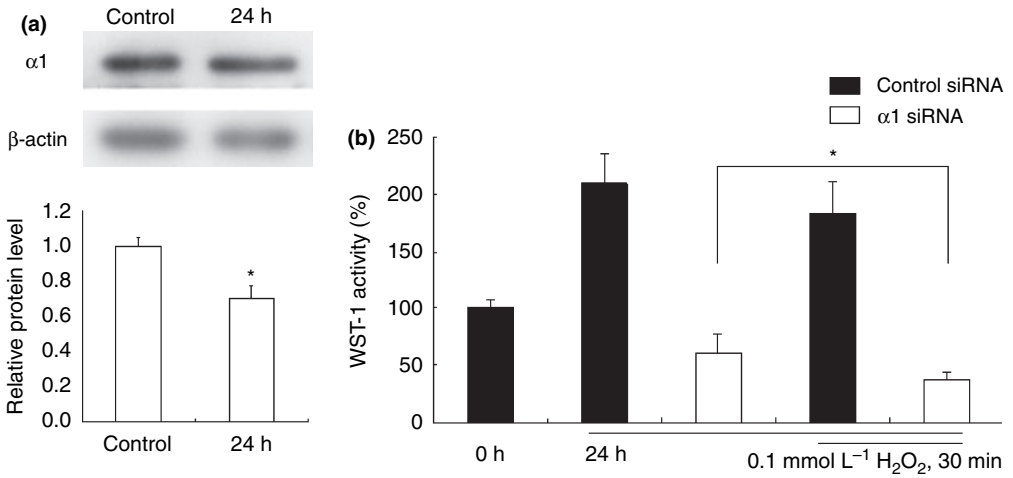

Figure 3 Effects of AMPKα1 silencing on cell proliferation. RPC-C2A cells were transfected with control siRNA or siRNA against AMPKα1, and incubated for 24 h. (a) Expression of AMPKα1 was analysed by Western blotting. Each protein level was normalized to expression of β-actin, and relative protein level to that of control is shown. Blot values represent means ± SD of three independent experiments. $^*P < 0.05$ vs control. (b) Cells were then treated with 0.1 mmol L^{-1} H_2O_2 for 30 min. Cell proliferation was analysed by WST-1 reduction assay, and is expressed as percentage of control. Data represent means ± SD of three independent experiments. $^*P < 0.05$.

Many cell mediators and enzymes are involved in lesions resulting from inflammation of dental pulp (Forman & Torres 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 H_2O_2 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 and 0.5 mmol L^{-1} H_2O_2 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 H_2O_2 at concentrations of above 0.1 mmol L^{-1} 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.* 2004) and rat skeletal muscle (Toyoda *et al.* 2004), and that it is activated by H_2O_2 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 H_2O_2 at concentrations of between 0.1 and 3 mmol L^{-1}, they also showed that AMPK was activated by exposure to H_2O_2, as evidenced by increased phospho-AMPKα. It has been reported that H_2O_2 evokes intercellular ATP depletion (Tavazzi *et al.* 2000), and that the profile of AMPK activation by H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 response, AMPKα1 was downregulated using siRNA, and cell viability and sensitivity to H_2O_2 were analysed. When the cells were transfected with siRNA, AMPKα1 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 H_2O_2 at 1 mmol L^{-1} for 60 min or at 3 mmol L^{-1} for 30 min. Even in the presence of a 1.5- to threefold increase in the expression of AMPKα and a three- to sevenfold increase in activation with H_2O_2, cell death was induced by approximately 10% at 0.1 mmol L^{-1}; however, the level of cytotoxity was less than that

observed in AMPKα-downregulated cells, with cell viability of 20% at 0.1 mmol L^{-1}. It has been reported that the activation of AMPK has protective effects against ischaemia and ischaemia–reperfusion injury in rat hepatocytes (Peralta *et al.* 2001), heart (Russell *et al.* 2003, 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 H_2O_2 than other tissues, and that AMPKα1 regulates sensitivity to H_2O_2 and is essential for cell survival via energy adaptation in dental pulp cells. Therefore, AMPK may be a therapeutic modulation target for treatment of the dentine–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.

References

Bae JY, Ahn SJ, Han W, Noh DY (2007) Peroxiredoxin I and II inhibit H_2O_2-induced cell death in MCF-7 cell lines. *Journal of Cellular Biochemistry* **101**, 1038–45.

Baumgardner KR, Sulfaro MA (2001) The anti-inflammatory effects of human recombinant copper-zinc superoxide dismutase on pulp inflammation. *Journal of Endodontics* **27**, 190–5.

Carling D (2004) The AMP-activated protein kinase cascade – a unifying system for energy control. *Trends in Biochemical Sciences* **29**, 18–24.

Choi SL, Kim SJ, Lee KT *et al.* (2001) The regulation of AMP-activated protein kinase by H_2O_2. *Biochemical and Biophysical Research Communications* **287**, 92–7.

Dyck JR, Lopaschuk GD (2006) AMPK alterations in cardiac physiology and pathology: enemy or ally? *Journal of Physiology* **574**, 95–112.

Esposito P, Varvara G, Murmura G, Terlizzi A, Caputi S (2003) Ability of healthy and inflamed human dental pulp to reduce hydrogen peroxide. *European Journal of Oral Sciences* **111**, 454–6.

Forman HJ, Torres M (2001) Redox signaling in macrophages. *Molecular Aspects of Medicine* **22**, 189–216.

Fruehauf JP, Meyskens FL Jr (2007) Reactive oxygen species: a breath of life or death? *Clinical Cancer Research* **13**, 789–94.

Fukuyama Y, Ohta K, Okoshi R, Suehara M, Kizaki H, Nakagawa K (2007) Hypoxia induces expression and activation of AMPK in rat dental pulp cells. *Journal of Dental Research* **86**, 903–7.

Garg TK, Chang JY (2003) Oxidative stress causes ERK phosphorylation and cell death in cultured retinal pigment epithelium: prevention of cell death by AG126 and 15-deoxy-delta 12, 14-PGJ_2. *BMC Ophthalmology* **3**, 5.

Hardie DG (2004) The AMP-activated protein kinase pathway – new players upstream and downstream. *Journal of Cell Science* **117**, 5479–87.

Jung JE, Lee J, Ha J *et al.* (2004) 5-Aminoimidazole-4-carboxamide-ribonucleoside enhances oxidative stress-induced apoptosis through activation of nuclear factor-κB in mouse Neuro 2a neuroblastoma cells. *Neuroscience Letters* **354**, 197–200.

Kasugai S, Adachi M, Ogura H (1988) Establishment and characterization of a clonal cell line (RPC-C2A) from dental pulp of the rat incisor. *Archives of Oral Biology* **33**, 887–91.

Lee DH, Lim BS, Lee YK, Ahn SJ, Yang HC (2006) Involvement of oxidative stress in mutagenicity and apoptosis caused by dental resin monomers in cell cultures. *Dental Materials* **22**, 1086–92.

Leverve XM, Guigas B, Detaille D *et al.* (2003) Mitochondrial metabolism and type-2 diabetes: a specific target of metformin. *Diabetes & Metabolism* **29**, 6S88–94.

McCullough LD, Zeng Z, Li H, Landree LE, McFadden J, Ronnett GV (2005) Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. *Journal of Biological Chemistry* **280**, 20493–502.

Mount PF, Hill RE, Fraster SA *et al.* (2005) Acute renal ischemia rapidly activates the energy sensor AMPK but does not increase phosphorylation of eNOS-Ser1177. *American Journal of Physiology. Renal Physiology* **289**, F1103–15.

Peralta C, Bartrons R, Serafin A *et al.* (2001) Adenosine monophosphate-activated protein kinase mediates the protective effects of ischemic preconditioning on hepatic ischemia-reperfusion injury in the rat. *Hepatology* **34**, 1164–73.

Russell RR 3rd, Li J, Coven DL *et al.* (2003) AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *Journal of Clinical Investigation* **114**, 495–503.

Saito Y, Nishio K, Ogawa Y *et al.* (2006) Turning point in apoptosis/necrosis induced by hydrogen peroxide. *Free Radical Research* **40**, 619–30.

Salvemini D, Doyle TM, Cuzzocrea S (2006) Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. *Biochemical Society Transactions* **34**, 965–70.

Schweikl H, Spagnuolo G, Schmalz G (2006) Genetic and cellular toxicology of dental resin monomers. *Journal of Dental Research* **85**, 870–7.

Shimauchi H, Takayama S, Narikawa-Kiji M, Shimabukuro Y, Okada H (2001) Production of interleukin-8 and nitric oxide in human periapical lesions. *Journal of Endodontics* **27**, 749–52.

Stapleton D, Mitchelhill KI, Gao G *et al.* (1996) Mammalian AMP-activated protein kinase subfamily. *Journal of Biological Chemistry* **271**, 611–4.

Tavazzi B, Pierro DD, Amorini AM *et al.* (2000) Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress. *European Journal of Biochemistry* **267**, 684–9.

Toyoda T, Hayashi T, Miyamoto L *et al.* (2004) Possible involvement of the α1 isoform of 5′AMP-activated protein kinase in oxidative stress-stimulated glucose transport in skeletal muscle. *American Journal of Physiology Endocrinology Metabolism* **287**, E166–73.

Woods A, Cheung PC, Smith FC *et al.* (1996) Characterization of AMP-activated protein kinase β and γ subunits. Assembly of the heterotrimeric complex *in vitro*. *Journal of Biological Chemistry* **271**, 10282–90.