

Title	Hypoxia induces expression and activation of AMPK in rat dental pulp cells
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ABSTRACT

AMP-activated protein kinase (AMPK) is a stress-responsive enzyme involved in cell adaptation to an energy crisis. We hypothesized that hypoxia suppresses oxidative phosphorylation and ATP production, resulting in AMPK activation to protect cells. We investigated the effects of hypoxia on cell proliferation, the expression of AMPK and hypoxia-inducible factor 1 α (HIF-1 α), the activation of AMPK, and the relationship between AMPK and HIF-1 α expression in rat dental pulp RPC-C2A cells. AMPK in the cells was composed of catalytic α 1, and regulatory β 1 and γ 1 subunit isoforms. Cell proliferation was initially suppressed under hypoxia, but it increased thereafter, together with an increase in the expression of AMPK and HIF-1 α , and the activation of AMPK. Down-regulation of AMPK α 1 by siRNA inhibited cell proliferation under both normoxia and hypoxia, revealing that AMPK induction and activation were required for cell proliferation, although HIF-1 α expression under hypoxia was not affected.

KEY WORDS: AMPK, HIF-1 α , hypoxia, dental pulp cells.

Hypoxia Induces Expression and Activation of AMPK in Rat Dental Pulp Cells

INTRODUCTION

Blood circulation in dental pulp plays several crucial roles in maintaining pulp tissue homeostasis. A reduction in pulpal blood flow frequently occurs following the use of local anesthetics containing vasoconstrictors (Kim, 1986), pulpal inflammation, and other stresses, resulting in hypoxia and subsequent pulp damage. Dental pulp cell responses to hypoxia play a critical role in pulp homeostasis, both physiologically and pathologically. Recently, it has been reported that various stresses, including hypoxia and heat stress, induced pulp cell responses such as cell cycle arrest and apoptosis (Kitamura *et al.*, 2005, 2006; Ueno *et al.*, 2006). Hypoxia changes cellular energy status (Rajpurohit *et al.*, 1996). However, little is known about the relationship between hypoxia-induced pulp cell responses and cellular energy status.

AMP-activated protein kinase (AMPK), a serine/threonine protein kinase, is an enzyme that monitors cellular energy status and responds to a variety of stresses. It is a heterotrimeric enzyme composed of a catalytic α subunit (α 1 and α 2) and regulatory β (β 1 and β 2) and γ subunits (γ 1, γ 2, and γ 3) (Stapleton *et al.*, 1996; Woods *et al.*, 1996). AMPK is activated by an increase in cellular levels of AMP resulting from ATP depletion, and by phosphorylation on Thr172 of the catalytic subunit by upstream kinase (Carling, 2004; Hardie, 2004). Moreover, AMPK is activated and adapts cells to hypoxia by adjusting the energy level required for cell survival (Beauloye *et al.*, 2001). Hypoxia-inducible factor 1 α (HIF-1 α) is a key cellular mediator of hypoxic response as a global regulator of oxygen homeostasis (Semenza, 2000).

In this study, we investigated the effects of hypoxia on cell proliferation and expression of AMPK subunit isoforms and HIF-1 α at the initial stage of incubation, and the relationship between the expression of these molecules in rat dental pulp RPC-C2A cells.

MATERIALS & METHODS

Cell Culture

Rat dental pulp RPC-C2A cells, established and kindly provided by Kasugai *et al.* (1988), were maintained in minimum essential medium (MEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 mg/mL kanamycin at 37°C in a humidified atmosphere at 5% CO₂ with 20% O₂. As hypoxic conditions, cells were cultured in a humidified atmosphere at 5% CO₂ with 2% O₂.

Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 5 x 10⁴ cells/well and cultured for 24 hrs, and then further cultured for an additional 6, 12, or 24 hrs under normoxic or hypoxic conditions. Cell viability was measured by water-soluble tetrazolium salt (WST-1) reduction activity, with a cell counting kit (Dojindo, Kumamoto, Japan). WST-1 was added at a final concentration of 0.5 mM and

incubated at 37°C for 2 hrs, after which we analyzed the reduced product of WST-1, formazan, by measuring absorbance at a wavelength of 405 nm and a reference wavelength of 620 nm with a Micro-plate Reader, Model 3550 (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR (qRT-PCR)

Cells were cultured in six-well plates at a density of 5×10^5 cells/well for 24 hrs. Total RNA was isolated by means of a BioRobot EZ1 (Qiagen, Hilden, Germany) and EZ1 RNA Tissue Mini Kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 5 μ g total RNA with the use of 1x Reaction Buffer (50 mM Tris-HCl, pH 8.3, containing 40 mM KCl and 7 mM MgCl₂), 10 mM dithiothreitol, 0.25 mM dNTPs, 100 units ReverScript II (Nippon Gene, Tokyo, Japan), 100 units ribonuclease inhibitor, and oligo dT primer. PCR primers specific to each AMPK subunit isoform were designed according to NCBI gene sequences. Primers for AMPK α 1 were: (forward) 5'-TGTGACAAGCACATTTTCCAA-3' and (reverse) 5'-CCGATCTCTGTGGAGTAGCAG-3'. Primers for AMPK α 2 were: (forward) 5'-TGAAGCAGCTGGACTTTGAAT-3' and (reverse) 5'-CTCCGATTGTCAACCAGGTAA-3'. Primers for AMPK β 1 were: (forward) 5'-TGAACAAGGACACGGGCAT-3' and (reverse) 5'-GTTGGGCTCCGGAAGCA-3'. Primers for AMPK β 2 were: (forward) 5'-CCCTCACCTCCTCCAAGTTAT-3' and (reverse) 5'-GATGGGTTGCGCTAAGGA-3'. Primers for AMPK γ 1 were: (forward) 5'-GCTCCAAGCTGGTGGTATTTG-3' and (reverse) 5'-GGCAGCACGAACACCGTTA-3'. Primers for AMPK γ 2 were: (forward) 5'-ATACTTACCCACAAAA GAATCCTCAAG-3' and (reverse) 5'-AGCTCATCCAGGTT CTGCTTC-3'. Primers for AMPK γ 3 were: (forward) 5'-AGAGCCTAGGTGAAGTCATTGACA-3' and (reverse) 5'-GCTGGGTCTCATCCACCAA-3'. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: (forward) 5'-TGTTGGACCTCATGGCCTAC-3' and (reverse) 5'-CAGCAACTGAGGGCCTCTCT-3'. AMPK mRNA expression was quantified by real-time RT-PCR with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and qPCR Master Mix Plus for SYBR Green I (Eurogentec, Seraing, Belgium). PCR conditions included initial incubation at 95°C for 10 min, followed by 40 cycles consisting of 15 sec at 95°C and 60 sec at 60°C. Data were normalized against GAPDH expression as an internal control, and calculated by the comparative CT method. PCR products underwent electrophoresis on a 2% agarose gel in Tris-borate-EDTA (TBE) buffer.

Western Blotting

Cells were seeded in six-well plates at a density of 5×10^5 cells/well, cultured for 24 hrs, and then further cultured for an additional 1, 2, 4, or 6 hrs under hypoxic conditions. After incubation, the cells were collected and 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, St. Louis, MO, USA) 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 μ g) were separated on 10% SDS-polyacrylamide gels and blotted onto Immobilon-P Transfer membranes (Millipore, Bedford, MA, USA), which were then incubated in blocking buffer containing 5% skimmed milk in TBS-T [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] for 1 hr, after

which they were incubated with anti-phospho-AMPK α (1:2000, Cell Signaling Technology, Beverly, MA, USA), anti- α 1 (1:1000, Upstate, Charlottesville, VA, USA), anti- α 2 (1:1000, R&D systems, Minneapolis, MN, USA), anti- β 1 (1:1000), anti- β 2 (1:1000), anti- γ 1 (1:200), anti- γ 2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- γ 3 (1:1000, Abgent, San Diego, CA, USA), anti-HIF-1 α (1:1000, R&D systems), or anti- β -actin (1:10,000, Sigma-Aldrich) overnight at 4°C. Blots were detected with 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 determined by NIH Image (Scion Corporation, Frederick, MD, USA).

RNA Interference

The sequence of small interference (si) RNA for α 1 was 5'-UCCUUCGCACACGCAAAUAAUAGG-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 hrs. siRNA was delivered to cells in Opti-MEM (Invitrogen) with Lipofectamine RNAiMAX (Invitrogen) at 37°C. After 5 hrs of incubation, the medium was replaced with MEM containing 10% FBS, and the cells were cultured for an additional 43 hrs. Cells were further incubated under normoxic or hypoxic conditions for 24 hrs, and cell proliferation was analyzed by WST-1 reduction assay. Expression of HIF-1 α under normoxic or hypoxic conditions for 4 hrs was analyzed by Western blotting.

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 Hypoxia on Cell Proliferation

Under normoxic conditions, cells showed an approximately 2-fold and 2.7-fold proliferation at 12 and 24 hrs, respectively. Under hypoxic conditions, no proliferation was observed until 6 hrs; thereafter, cells began to proliferate at a similar rate to that of the controls (Fig. 1).

Expression of AMPK Subunit Isoforms

To explore mRNA expression of AMPK catalytic and regulatory subunit isoforms, the relative expression of each subunit isoform to that of α 1 was determined by qRT-PCR (Fig. 2). No expression of catalytic α 2 mRNA was detected by qRT-PCR, and neither was its PCR product detected by agarose gel electrophoresis when the number of reaction cycles was increased to 50 or the reaction was performed using another primer described by Liu *et al.* (Liu Y *et al.*, 2006). The main expressed regulatory subunit isoforms were β 1 and γ 1, and their relative levels to that of α 1 were approximately 1.2- and 1.9-fold, respectively. Expression of β 2 and γ 3 mRNAs was less than 6% of that of α 1, while expression of γ 2 was approximately 17% and 9% of that of α 1 and γ 1, respectively.

At the protein level, catalytic subunit isoform α 1 and regulatory β 1 and γ 1 were detected by Western blotting reflecting their expression of mRNAs (Fig. 2), while α 2, β 2, γ 2 and γ 3 were not detected (data not shown); therefore, the main

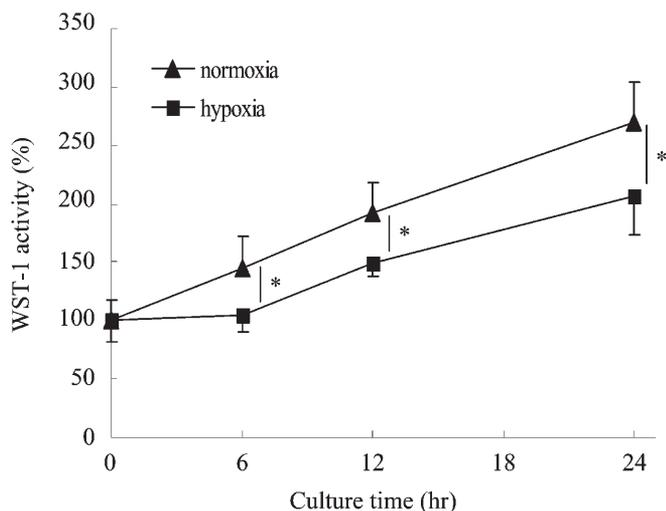


Figure 1. Effects of hypoxia on cell proliferation. RPC-C2A cells were incubated under normoxic (20% O₂) or hypoxic (2% O₂) conditions for times indicated. Cell proliferation was analyzed by WST-1 reduction assay, and is expressed as percentage of control. Data represent means \pm SD of three independent experiments. * $p < 0.05$.

AMPK subunit isoforms in the RPC-C2A cells were catalytic $\alpha 1$, and regulatory $\beta 1$ and $\gamma 1$, at both the mRNA and protein levels.

Expression of AMPK and HIF-1 α Proteins under Hypoxic Conditions

Inhibition of cell proliferation was observed early during incubation. Therefore, we investigated expression of AMPK and HIF-1 α under hypoxic conditions from 0 to 6 hrs incubation. Alpha1, $\beta 1$ and $\gamma 1$ began to increase with hypoxia at 1 hr, their expression at 6 hrs reaching a level approximately 2 to 4-fold higher than that at 0 hr. However, no induction of $\alpha 2$, $\beta 2$, $\gamma 2$ and $\gamma 3$ were observed under hypoxic conditions (data not shown). The activated form of the AMPK α subunit, phospho-AMPK α , showed an approximately 2.6-fold increase at 6 hrs (Fig. 3A). HIF-1 α protein showed an approximately 7-fold increase at 4 hrs, and an approximately 5-fold increase at 6 hrs (Fig. 3B).

Effects of Inhibition of AMPK $\alpha 1$ Expression by RNA Interference on Cell Proliferation and HIF-1 α Expression

To explore the role of AMPK under hypoxic conditions, we examined the effect of silencing AMPK $\alpha 1$ on cell proliferation and HIF-1 α expression by using siRNA, as AMPK $\alpha 1$ was the main isoform of the catalytic subunit in the RPC-C2A cells. AMPK $\alpha 1$ siRNA inhibited protein expression to about 30% of that of the control under the conditions used at 48 hrs (Fig. 4A). Cells with down-regulated AMPK $\alpha 1$ showed a reduction in cell proliferation of approximately 35% and 39% of the respective controls at 72 hrs incubation under both normoxic and hypoxic conditions, respectively (Fig. 4B). HIF-1 α expression in siRNA-transfected cells was analyzed after 4 hrs under normoxic and hypoxic conditions by Western blotting, as HIF-1 α expression exhibited the greatest increase at 4 hrs under hypoxic conditions. Increased HIF-1 α expression under hypoxic conditions in siRNA-transfected cells was observed at the same level as that in the control cells (Fig. 4C).

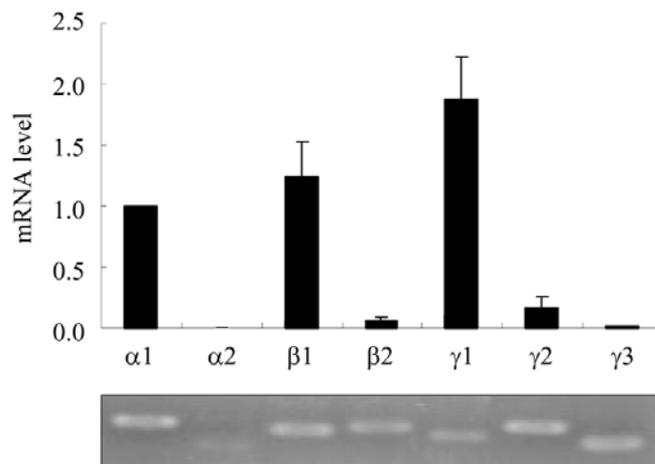


Figure 2. Expression of AMPK subunit isoforms mRNA. mRNA expression of AMPK subunit isoforms in RPC-C2A was analyzed by qRT-PCR. mRNA level was normalized to internal control, GAPDH, and relative mRNA level to that of $\alpha 1$, which was set at 1.0. Data represent means \pm SD of three independent experiments.

DISCUSSION

The present study showed that RPC-C2A cell proliferation under hypoxic incubation was initially suppressed, together with an increase in expression of AMPK and HIF-1 α with activation of AMPK. Furthermore, downregulation of AMPK $\alpha 1$ using siRNA revealed that AMPK played an important role in pulp cell proliferation under both normoxia and hypoxia, while HIF-1 α expression under hypoxia was not affected.

Early inhibition of cell proliferation may be explained by hypoxia-induced cell-cycle arrest inhibiting the molecules required for DNA synthesis (Ueno *et al.*, 2006), since cell proliferation in mammalian cells is energy-dependent under hypoxia (Liu L *et al.*, 2006). However, AMPK activates glucose transport through GLUT4 (Hardie *et al.*, 2004) and glycolysis by activation of an inducible activator enzyme, 6-phosphofructo-2-kinase (Marsin *et al.*, 2002), resulting in restoration of energy deprivation. In this study, the rate of cell proliferation after 6 hrs was found to be close to that of the control, presumably due to up-regulation and activation of AMPK. Amemiya *et al.* (2003) reported that pulp cell proliferation in primary culture was not reduced after long (48 hrs) incubation under hypoxia (Amemiya *et al.*, 2003). Taken together with our results, this suggests AMPK-mediated adaptation of cells to hypoxia.

The main AMPK subunit isoforms in the RPC-C2A cells were $\alpha 1$, $\beta 1$ and $\gamma 1$. In rat tissues, such as lung, kidney and brain, $\alpha 1$, $\beta 1$ and $\gamma 1$ are the predominant subunit isoforms (Cheung *et al.*, 2000), although in rat liver, the main catalytic subunit isoform is $\alpha 2$ (Stapleton *et al.*, 1997). The composition of each AMPK subunit isoform will vary according to the specific function of each tissue and species (Stapleton *et al.*, 1997). Cellular response to hypoxia is different between AMPK $\alpha 1$ and $\alpha 2$ (Neurath *et al.*, 2006). AMPK subunit composition in pulp cells observed in this study may reflect its specific role under stress.

Alpha1, $\beta 1$, $\gamma 1$ and phospho- α , an active form of the

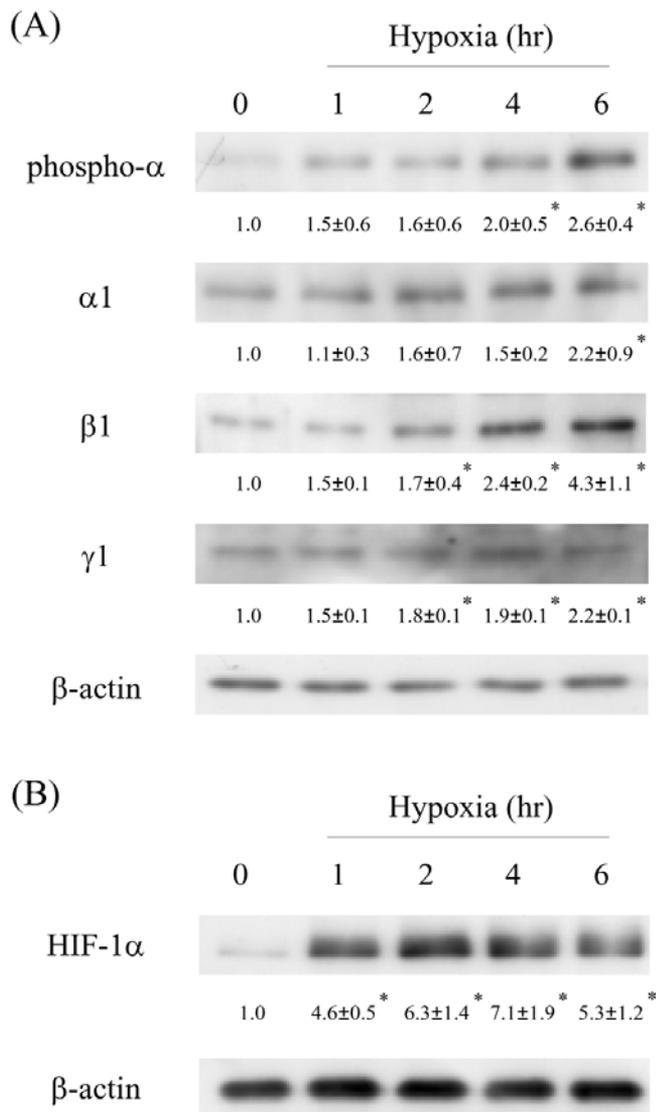


Figure 3. Effects of hypoxia on expression of major AMPK subunit isoforms and HIF-1 α . RPC-C2A cells were incubated under hypoxic conditions for the times indicated. Protein expressions of AMPK α 1, β 1 and γ 1 (A) and HIF-1 α (B) were analyzed by Western blotting. Each protein level was normalized to expression of β -actin, and relative protein levels to that at 0 hr are shown. Blot values represent means \pm SD of three independent experiments. * p <0.05 vs. 0 hr.

catalytic subunit, increased time-dependently under hypoxia. This suggests that AMPK plays an important role in pulp cell reactions to hypoxia, presumably in maintaining energy homeostasis. Indeed, it has been reported that hypoxia leads to activation of AMPK *via* an increase in the AMP: ATP ratio, and that AMPK acts as an intercellular energy sensor, maintaining energy balance during hypoxia in a variety of cells (Hardie *et al.*, 2001). To explore the role of AMPK in hypoxic cells, AMPK α 1 was silenced using siRNA at about 30% of the protein level. Cell proliferation was reduced under both normoxia and hypoxia, suggesting that AMPK activity is a prerequisite for cell proliferation.

HIF-1 α is an inducer of angiogenesis, and AMPK is

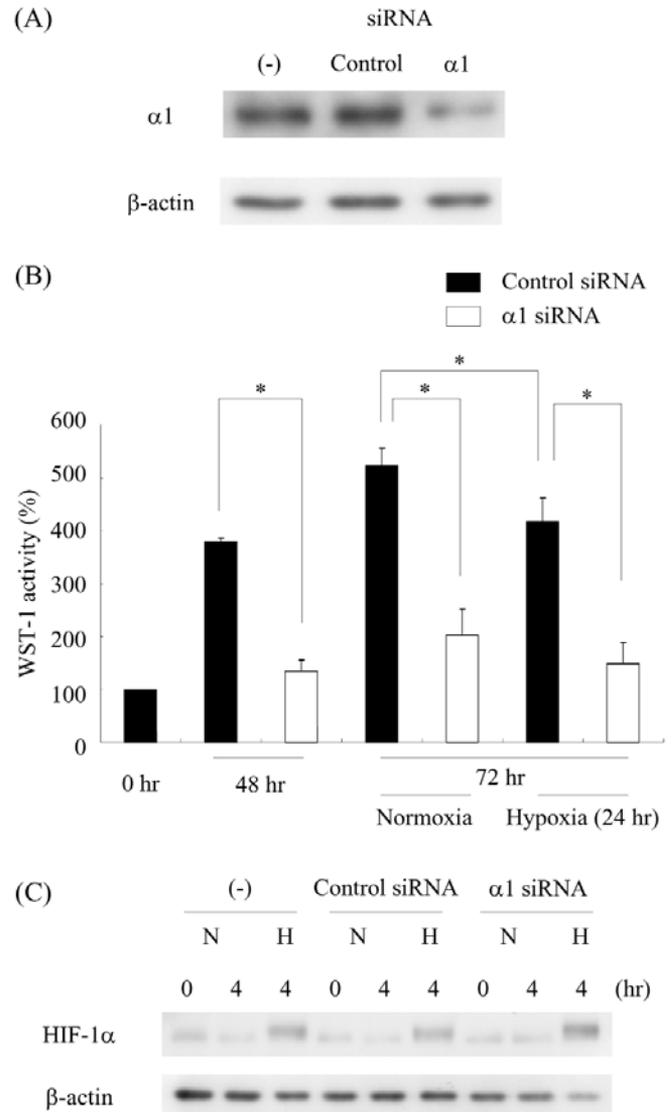


Figure 4. Effects of AMPK α 1 silencing on cell proliferation and AMPK α 1 and HIF-1 α expression. RPC-C2A cells were transfected with control siRNA or siRNA against AMPK α 1, and incubated for 48 hrs. (A) Expression of AMPK α 1 protein was analyzed by Western blotting. (B) Cells were then incubated under normoxic or hypoxic conditions for an additional 24 hrs. 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. (C) After transfection, cells were incubated under normoxic or hypoxic conditions for an additional 4 hrs. Expression of HIF-1 α protein was analyzed by Western blotting. N, normoxic conditions; H, hypoxic conditions.

involved in angiogenic responses in endothelial cells under hypoxia (Nagata *et al.*, 2003). Our results are also consistent with those of a study showing that HIF-1 α expression was induced in primary cultured rat pulp cells under hypoxia (Amemiya *et al.*, 2003). The present study showed that HIF-1 α was up-regulated under hypoxia, even in AMPK α 1 silenced cells, and that HIF-1 α expression increased earlier than AMPK phosphorylation and expression, suggesting that HIF-1 α is expressed independently of AMPK. HIF-1 α expression was also reported to be independent

of AMPK α expression under hypoxic conditions in mouse embryonic fibroblasts (Laderoute *et al.*, 2006).

In conclusion, we found that cell proliferation was initially suppressed under hypoxia, and that hypoxia induced expression of AMPK and HIF-1 α and activated AMPK, with subsequent initiation of cell proliferation. In addition, we showed that AMPK is required for pulp cells to adapt to hypoxia and to proliferate, although its expression is unrelated to that of HIF-1 α . These results of this study will help identify novel target for development of vital pulp therapy.

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