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Hypoxia induces apoptosis and autophagic cell death in human periodontal ligament cells through HIF-1α pathway

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Abstract

Objectives: Oxygen deficiency caused by occlusal trauma and smoking can be present in patients with periodontitis. However, biochemical events important in periodontal tissues during hypoxia remain unclear. The aim of this study was to investigate effects of hypoxia on apoptosis and autophagy of human periodontal ligament cells (PDLCs) *in vitro*.

Materials and methods: Human PDLCs were obtained and cultured *in vitro*. Cell viability, apoptosis, autophagy and gene and protein expression were measured in presence and absence of cobalt chloride ($CoCl_2$).

Results: $CoCl_2$ induced cytotoxicity of human PDLCs in a concentration-dependent manner dependent on macromolecular synthesis, and resulted in apoptosis and mitochondrial dysfunction. $CoCl_2$ also induced redistribution of autophagy marker LC3, increased ratio of LC3-IIto LC3-Iand function of lysosomes. Furthermore, $CoCl_2$ promoted expression of HIF-1α following upregulation of expressions of Bnip3. Significant increases in expression of IL-1β and MMP-8 were also observed. All these results were reversed by pre-treatment with antioxidant *N*-acetylcysteine.

Conclusions: Our data showed that $CoCl_2$ could induce cytotoxicity through mitochondria- apoptotic and autophagic pathways involved in HIF-1α. $CoCl_2$-treated PDLCs may serve as an *in vitro* model for studies of molecular mechanisms in periodontitis.

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Introduction

Periodontitis is one of the most common oral infectious diseases in humans and is characterized by inflammation and destruction of attachment apparatus supporting local and teeth. It is the most prevalent cause of tooth loss and is difficult to treat (1,2). A number of systemic and local risk factors have been associated with initiation and progression of periodontitis (3) and occlusal trauma has been considered to be a factor that accelerates inflammatory alveolar bone resorption, where periodontitis is present (4,5). It is well known that physiological blood flow in the periodontal ligament is slightly lower than that of either pulpal or cerebral blood flow (6,7), and blood flow is easily reduced by occlusal forces. Furthermore, histopathological study in an experimental occlusal trauma model in the dog (beagles) has demonstrated temporal obstruction of periodontal ligament (PDL) capillaries by mechanical compression, thrombus and subsequent reconstruction of blood vessels, resulting in marked resorption of alveolar bone (8). Thus, it is highly possible that hypoxia occurs in such traumatized PDL tissue.

Moreover, in cases of tooth transplantation or replantation, periodontal tissues containing osteogenic fibroblasts, which may differentiate into either cementoblasts or osteoblasts, decrease during severe ischaemia. If the periodontal ligament is exposed to a dry environment for more than 30 min after tooth extraction, its cells' ability to differentiate and to proliferate is lost (9,10). In

addition, one epidemiological study has reported that oxygen deficiency caused by smoking can be associated with periodontal tissue breakdown. Furthermore, growth of anaerobic bacterial biofilm may further lower oxygen tension in the vicinity of periodontal tissues (11). However, little is known of the behaviour of human periodontal ligament cells (PDLCs) under conditions of hypoxia *in vitro*.

Hypoxia-inducible factor (HIF)-1, a heterodimeric transcriptional factor, plays a central role in regulation of gene expression to maintain oxygen homeostasis (12). Under normoxic conditions, the HIF-1α subunit is rapidly degraded through ubiquitinylation by pVHL and proteosomes. This is triggered through post-translational hydroxylation at specific proline residues (13). However, under hypoxic conditions, HIF-1α evades hydroxylation and is translocated to the nucleus where it heterodimerizes with HIF-1α (14). Some metals are known to be hypoxic mimicking agents and these include cobalt chloride, nickel chloride and desferrioxamine (15). Recently, it has been reported that $CoCl_2$ treatment induces HIF-1α expression by binding to the PAS domain, resulting in blockage of HIF-1α-pVHL binding and thereby HIF-1α stability (16).

There are at least two types of programmed cell death, apoptosis and autophagic cell death (17) being the most well recognised. Apoptosis is characterized by a series of biochemical events that lead to a variety of morphological changes, such as loss of cell membrane asymmetry accompanied by phosphatidylserine translocation from the inner cell membrane to the cell surface, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (18,19). Autophagy on the other hand is a self-degradative process that is important for balancing sources of energy at critical times. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins and clearing damaged organelles (such as mitochondria, endoplasmic reticulum and peroxisomes) as well as eliminating intracellular pathogens. Thus, autophagy is generally regarded as an overall survival mechanism (20).

Hypoxia and $CoCl_2$ treatment have been reported to correlate with apoptotic and pro-apoptotic factors (21,22) and hypoxia also activates autophagy through effects that are dependent on target genes induced by HIF (23). The relationship between apoptosis and autophagy is now the subject of considerable research but effects of hypoxia on apoptosis and autophagy of periodontal ligament cells still largely remain unknown.

As an important component of the periodontal attachment apparatus, human PDLCs are significant in progress of periodontitis. In our previous study, we found that human PDL cell viability decreased in a dose-dependent manner after treatment with $CoCl_2$ for 48 h (24). Bearing this in mind, expression of HIF-1α and its correlation with apoptosis and autophagy in human PDLCs might be of great importance in development of periodontitis. In the present study, we have examined effects of hypoxia induced by $CoCl_2$ on human PDLCs and present evidence that hypoxia induces apoptosis and autophagic cell death.

Materials and methods

Human PDLC culture and $CoCl_2$-mimic hypoxic treatment

Human PDLCs were prepared from explants of periodontal ligaments of teeth extracted for orthodontic treatment, from healthy humans with their informed consent. PDLCs were isolated and cultured according to the method of Sun *et al.* (2) with minor modification. Periodontal ligament fragments were removed from the mid-third of the root and cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 20% foetal bovine serum (Gibco Biocult, Paisley, UK). Culture medium was changed every 7 days, for 10–20 days until confluent cell monolayers were formed. After four or five subcultures, homogeneous, slim, spindle-shaped cells were obtained.

For hypoxia treatment, PDLCs were seeded on plates by incubation with various concentrations of $CoCl_2$. In a proportion of experiments, PDLCs were pre-treated with *N*-acetylcysteine for 2 h prior to $CoCl_2$ exposure for 48 h.

Cell viability assay

Cell viability assays were performed using Cell Counting Kit-8 (CCK-8) (WST-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. PDLCs were plated into 96-well plates. After treatment, CCK-8 was added to culture media at 37 °C for 3 h. Absorbance was measured at 450 nm using an auto-microplate reader (Bio-Rad, Hercules, CA , USA). Cell viability was expressed as percentage of viable cells relative to untreated cells, using absorbance at 450 nm. All experiments were performed in five wells in three separate experiments.

Measurement of apoptosis and mitochondrial membrane potential

Percentage of cells actively undergoing apoptosis was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to manufacturer's instructions.

Briefly, after incubation with $CoCl_2$ for 48 h, PDLCs were harvested and resuspended in binding buffer. Cells were mixed with annexin VFITC and PI. After incubating for 15 min in the dark, analysis was performed by flow cytometry.

High-content screening was used to examine mitochondrial membrane potential (MMP) of the PDLCs. MitoTracker/Hoechst solution (Cellomics Inc, Pittsburgh, PA, USA) was added to wells of the 96-well plates, then, fixation solution was added to each well. Wells were aspirated, and each plate was washed once in wash buffer-M. Then plates were sealed and run on the ArrayScan HCS System. Intensity of red fluorescence represented MMP of the cells.

Detection of autophagy

PDLCs were plated in 12-well plates and treated with $CoCl_2$ for 48 h. After exposure, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% (v/v)Triton X-100, for 15 min. After blocking with 2% (w/v) bovine serum albumin, fixed cells were incubated overnight at 4 °C with anti-LC3 antibody (dilution 1:100, CST). Cultures were then washed and incubated for 2 h at room temperature in fluorescence-conjugated secondary antibody (Alexa Fluor 555-donkey anti-rabbit IgG, 1:200; Invitrogen Life Technologies, Carlsbad, CA, USA). Prevalence of autophagic cells was determined by LC3 antibody location, viewed by confocal microscopy (LSM-510; Carl Zeiss AG, Oberkochen, Germany) at excitation wavelength of 488 nm, and were photographed.

Induction of autophagy was assessed by detecting expression of Beclin1 or by increased ratio between LC-II and LC3-I, an indicator of autophagy induction, by immunoblotting.

Quantitative reverse transcription polymerase chain reaction

PDLCs were detached and homogenized using TRIZOL (Invitrogen, Carlsbad, CA, USA) and total RNA extracted. Real-time PCR was performed with ABI7500 instrumentation (Applied Biosystems, Foster City, CA, USA) in a total volume of 50 μl per glass capillary. Gene-specific primers used were: forward GTGCCA CATCATCACCATA and reverse CAAAGCGACAG ATAACACG for HIF-1α; forward TCCTTCCATCT CTGCTGCTCTC and reverse AAGGTGCTGGTGGA GGTTGTC for Bnip3; forward TTGAAGCTGATGGC CCTAAA and reverse ACAGGTGCATCGTGCACATA for interleukin-1β (IL-1β); forward GCATTCAGGCCA-TCTATGGAC and reverse TCCACGGAGTGTGGTGA TAGC for matrix metalloproteinase 8 (MMP-8); forward CCTGTACGCCAACACAGTGC and reverse ATACT CCTGCTTGCTGATCC for β-actin. Cycling protocols for all were identical. mRNA levels relative to that of housekeeping gene β-actin, were calculated by ΔΔCt method. Data were averaged from three dishes in each group.

Western blot analysis

PDLCs were washed in PBS and lysed in ice-cold SDS buffer; lysates were sonicated and protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL, USA). Proteins were separated using SDS–PAGE and transferred into PVDF membranes (Millipore, Billerica, MA, USA) which were incubated overnight at 4 °C, with the following primary antibodies, anti-: HIF-1α (Novus Biologicals, Inc, Littleton, CO, USA), Bnip3 (Abcam, Cambridge, MA, USA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation in appropriate horseradish peroxidase conjugated secondary antibody (Zhongshan Biotechnology, Beijing, China), membranes were incubated with ECL reagents for chemiluminescence then exposed to X-ray autoradiography film (Fuji, Tokyo, Japan).

Measurement of cytokines

Concentrations of cytokines IL-1β and MMP-8 in the cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) from commercial kits for determination of human IL-1β or MMP-8. Cell culture supernatants, samples or standard solutions were added to 96-well plates coated with murine monoclonal antibody for 2 h. After subsequent treatment for 1–2 h with alkaline phosphatase-conjugated polyclonal antibody, treation with *p*-nitrophenyl phosophate was carried out for 20 min. Trisodium phosphate solution was added to terminate the reaction. Absorbance of each well was measured at 450 nm, concentration was calculated from a standard curve and analysed statistically using Student's *t*-testing.

Method and antiserum specificity controls were included in each assay. Plate- to-plate variation controls were also employed when appropriate. IL-1β and MMP-8 concentrations were expressed as ng/ml.

Statistical analysis

Statistical analyses of the data were performed using analysis of variance (ANOVA) and Student–Newman–Keuls tests. Level of significance was set at $P < 0.05$.

Results

Cell viability assay

Our previous studies have shown that PDL cell viability decreases after treatment with $CoCl_2$ from 100 to 800 μM for 48 h (24). EC50 of $CoCl_2$ was found to be 169 μM (Fig. 1a). $CoCl_2$ at concentrations of 200 and 400 μM, for 48 h, which markedly reduced cell viability compared to controls, were used to induce PDLC injury in the subsequent experiments.

To determine transcription dependence of $CoCl_2$-induced cell death, inhibitors of macromolecular synthesis were applied. After pre-treatment with actinomycin D or cycloheximide for 2 h, cultures were exposed to 400 μM $CoCl_2$ for 48 h. Both RNA synthesis inhibitor actinomycin D and protein synthesis inhibitor cycloheximide, inhibited $CoCl_2$-induced cytotoxicity. Thus, it would seem that mRNA and protein syntheses are indeed required for induction of cytotoxicity by $CoCl_2$ (Fig. 1b,c).

Apoptosis and mitochondrial membrane potential assay

Flow cytometric analysis was used to present levels of apoptosis induced by $CoCl_2$. Levels of apoptosis were 3.805 ± 0.325%, 6.74 ± 0.12%, 11.88 ± 0.95%, of control cells and 200, 400 μM $CoCl_2$-treated cells for 48 h, respectively. When pre-treated with NAC, apoptosis was 8.47 ± 0.27% (Fig. 2a,b). These results demonstrate that $CoCl_2$ increased levels of apoptosis in concentration and time-dependent manner, which was abolished by treatment with an antioxidant.

To confirm the presence of apoptosis, we then measured loss of the mitochondrial membrane potential ($\Delta\psi m$), which is a common early event in apoptotic processes induced by a variety of stimuli (25). Integrity of $\Delta\psi m$ during apoptosis caused by $CoCl_2$ was monitored using MitoTracker red staining through the ArrayScan HCS System. Red fluorescence selectively accumulated in mitochondria and subsequently aggregated as a function of mitochondrial potential. Compared to control cells, $CoCl_2$ treatment substantially attenuated fluorescence of mitochondrial membranes and reduced membrane potential. After NAC pre-treatment, mitochondrial membrane potential recovered (Fig. 2c,d). These results indicate that $CoCl_2$ had induced disruption of $\Delta\psi m$ and caused mitochondrial dysfunction, by the mitochondrial apoptosis pathway.

Autophagy assay

To investigate the role of $CoCl_2$-induced hypoxia in autophagy, we measured effects of $CoCl_2$ on the following autophagy markers: autophagic vesicles (AVs)/autophagosomal compartments, amphisomes and autolysosomes (26). Lysosomal mass/pH dye is a weak base that accumulates in lysosomes (acidic organelles) and determines changes to lysosomal physiology. We used the ArrayScan HCS System to detect locations of lysosomal mass/pH dye. Significant fluorescence intensity enhancement indicated increase in vesicular acidification after $CoCl_2$ treatment. When pre-treated with NAC, fluorescence intensity was lower (Fig. 3a,b). These results indicate that $CoCl_2$ caused lysosome dysfunction in the cells.

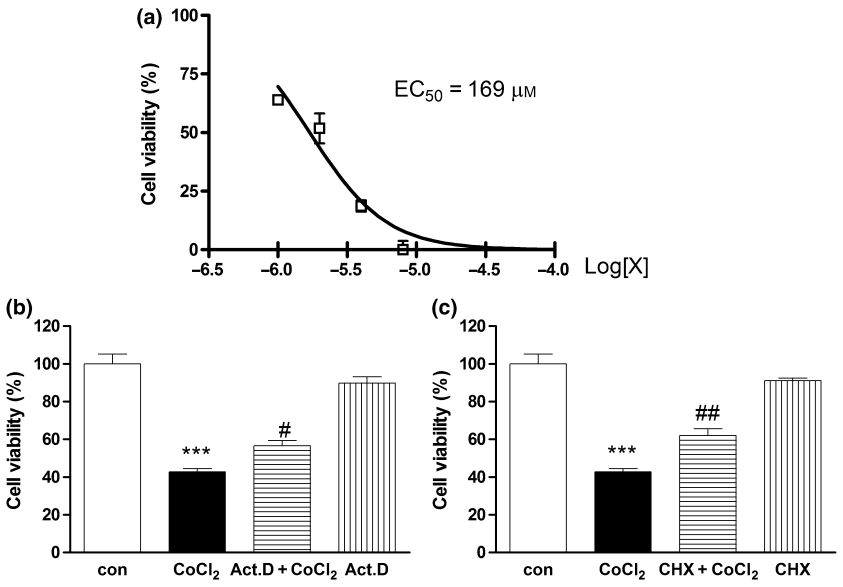

Figure 1. Effects of hypoxia induced by $CoCl_2$ on human PDL cell viability. PDLCs were cultured in complete media, with various concentration of $CoCl_2$, for 48 h. Cell viability was determined using the CCK-8 kit. Half maximal inhibitory concentration (EC50) of hypoxia induced by $CoCl_2$, on cell viability of human PDLCs (X, concentration of $CoCl_2$) (a). Viability of control cells without $CoCl_2$ treatment defined as 100% versus Control group. Protective effects of actinomycin D (b) and cycloheximide (c) against hypoxia-induced cytotoxicity (Act. D, actinomycin D; CHX, cycloheximide). Data presented as mean ± SEM. ***$P < 0.001$ versus control, #$P < 0.05$ versus $CoCl_2$ group, ##P versus $CoCl_2$ group.

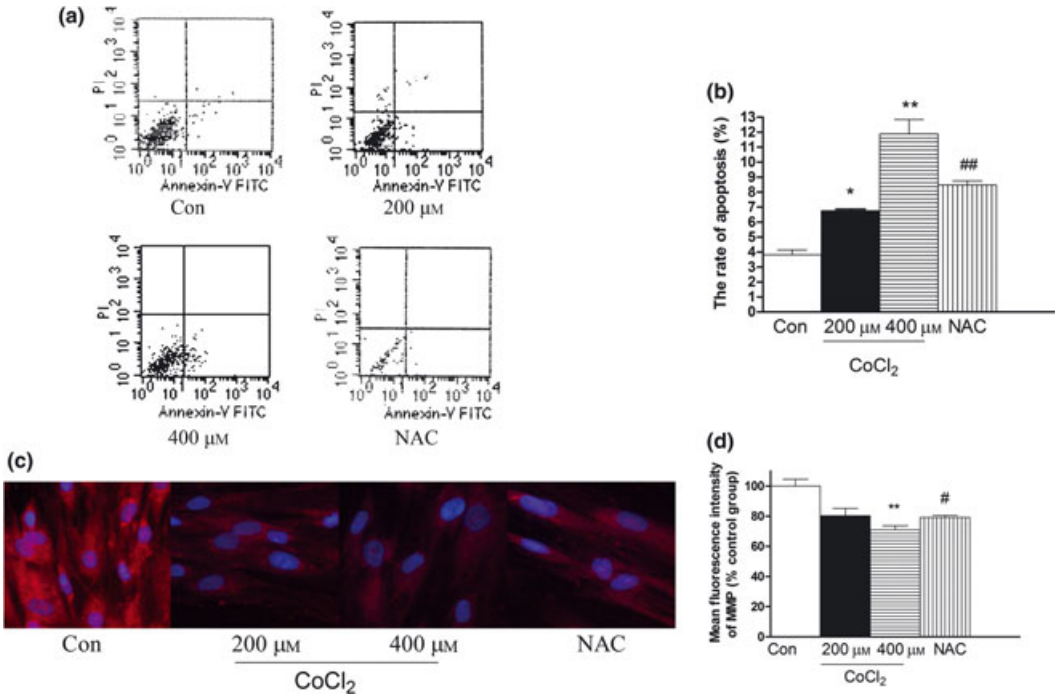

Figure 2. Effect of $CoCl_2$-induced hypoxia on apoptosis and MMP (mitochondrial membrane potential) in human PDLCs. Apoptosis was detected by flow cytometry using Annexin V-FITC Apoptosis Detection Kit. (a) Photomicrograph representative of three different sets of experiments. After pre-treatment with NAC for 2 h, cultures were exposed to 400 μM $CoCl_2$ for 48 h. $CoCl_2$ increased level of apoptosis in a concentration-and time-dependent manner. (b) Data represent mean ± SEM of three experiments. $^{***}P < 0.001$ versus control, $^{\#\#}P < 0.05$ versus $CoCl_2$ group. (c) By application of MitoTracker Red, red fluorescence occurred surrounding mitochondrial membranes; using ArrayScan HCS System (×20). (d) Data represent mean ± SEM of four individual variations. $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus 400 μM $CoCl_2$ group.

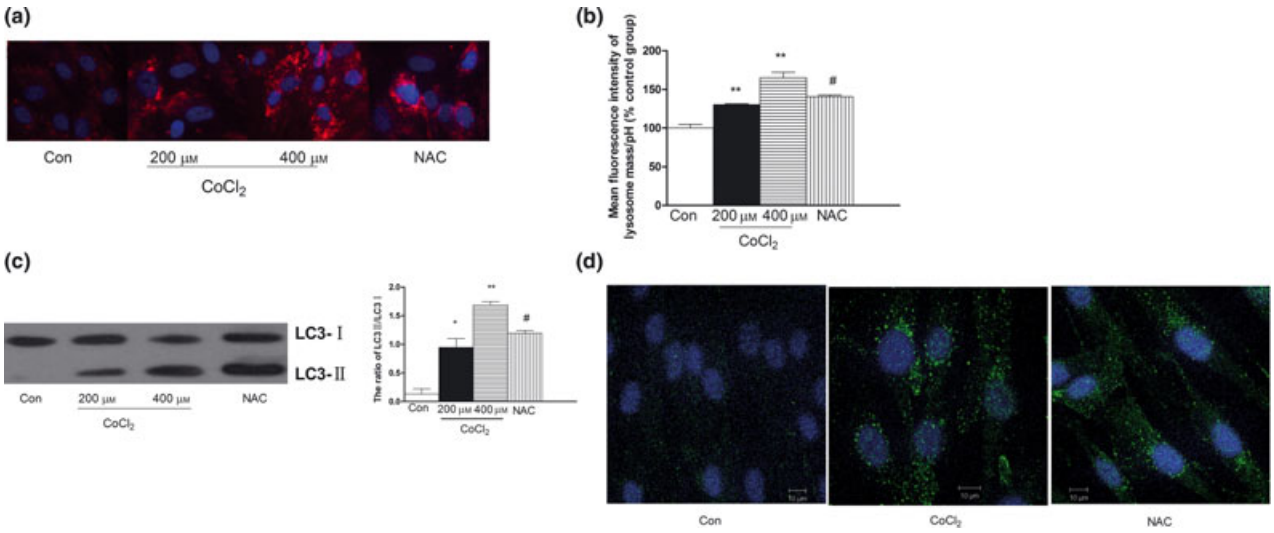

Figure 3. Effect of $CoCl_2$-induced hypoxia on lysosome function and expression of LC3 in human PDLCs. After pre-treatment with NAC for 2 h, cultures were exposed to 400 μM $CoCl_2$ for 48 h. (a) Lysosomal mass/pH dye was used to determine the changes in lysosomal physiology using the ArrayScan HCS System (×20). (b) Data represent mean ± SEM of four individual procedures. Significant fluorescence intensity increase indicated increase in vesicular acidification after $CoCl_2$ treatment. (c) Detection of LC3 by western blotting and densitometric quantification ratio of LC3 normalized to β-actin expression. There is increase in ratio of LC3-II to LC3-I. (d) Detection of LC3 was measured by confocal microscopy. Cells were stained with DAPI (blue) and induction of autophagy was visualized by LC3-punctuated cells (green) under confocal microscopy. Scale bar, 5 μM. Con, control. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus $CoCl_2$ group.

Synthesis and processing of LC3 (also called ATG8) increase during autophagy, making it a key readout of autophagy levels (27). Induction of autophagy was visualized as LC3-punctuated cells (green), by confocal microscopy. When PDLCs were treated with $CoCl_2$, protein redistributed into distinct puncta, a characteristic of cells undergoing autophagy. In addition, to obtain additional confirmation of $CoCl_2$-induced autophagic cell death, we examined conversion of LC3-I to LC3-II using western blot analysis. Accumulation of LC3-II, which is associated with autophagosome membranes, can occur due to increased autophagosome formation. As shown in Fig. 6, following $CoCl_2$ treatment, there was increase in ratio of LC3-II to LC3-I and reduction in this ratio after NAC pre-treatment (Fig. 3c,d).

To confirm induction of autophagy by molecular means, we measured expression of Beclin 1, an autophagy-related protein (28). Beclin 1 expression increased significantly in 200 and 400 μM $CoCl_2$-treated cells (Fig. 4) and could be attenuated by NAC. These data suggest that hypoxia had induced autophagy by upregulating Beclin1 expression.

HIF-1α and BNIP3 expression

HIF-1α, a key transcription factor, plays pivotal roles in hypoxia. Expressions of HIF-1α and its related gene were observed using RT-PCR and western blot analysis. As shown in Fig. 5, $CoCl_2$ did not promote levels of HIF-1α mRNA expression, but did increased levels of HIF-1α protein expression. However, expression of Bnip3 mRNA, and protein levels were significantly higher after $CoCl_2$ treatment compared to controls ($P < 0.01$). Furthermore, NAC attenuated upregulation of HIF-1α and downstream *BNIP3* gene after $CoCl_2$, which prevented apoptosis.

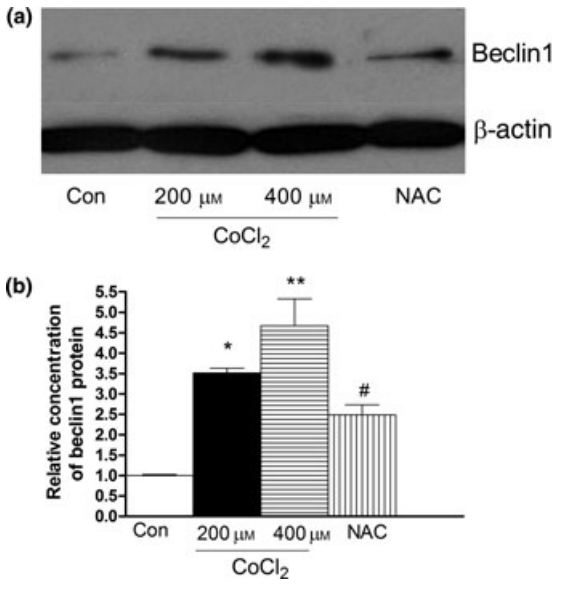

Figure 4. Effect of $CoCl_2$-induced hypoxia on expression of Beclin in human PDLCs. After pre-treatment with NAC for 2 h, cultures were exposed to 400 μM $CoCl_2$ for 48 h. (a) Expression of Beclin was examined by western blot analysis. At 48 h, treatment with 400 μM $CoCl_2$ increased expression of Beclin. (b) Beclin expression increased significantly in $CoCl_2$ treated cells at 48 h. $^*P < 0.05$ versus control, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus $CoCl_2$ group.

IL-1β and MMP-8 expression

We evaluated effects of hypoxia on production of IL-1β and MMP8 cytokines by PDLCs, by quantification of their extracellular concentrations using QPCR and ELISA. Significantly higher concentrations of IL-1β and MMP-8 were detected in hypoxia groups than in controls, at 48 h (Fig. 6). Expression of IL-1β and MMP-8 recovered with NAC treatment.

Discussion

To our knowledge, this is the first study to report effects of $CoCl_2$-induced hypoxia, on apoptosis and autophagic cell death in primary human PDLCs. The periodontal ligament is a dense soft connective tissue, situated between tooth-root cementum and its adjacent alveolar bone, which provides physiological tooth mobility under functional loading (29). Compression of the PDL induces changes in blood vessel morphology characterized by ischaemia and reduced blood flow. These changes cause local hypoxia, which may have an effect on PDLCs and cause release of chemical mediators, resulting in adaptive responses to maintain homoeostasis of periodontal tissue. Hypoxia-induced cell death is a major concern in various clinical settings such as hypoxic/ischaemic disease, organ transplantation, and others (21,30–32). To elucidate the mechanisms by which human PDLCs respond to hypoxia, we used treating them with $CoCl_2$ as a model, and demonstrated that $CoCl_2$ triggered both apoptosis and autophagy in PDLCs involving the HIF-1α pathway.

Goldberg *et al.* (33) have reported that 1% O_2/5% CO_2/94% N_2, or in the presence of $CoCl_2$, can mimic hypoxic conditions experimentally. $CoCl_2$ has been widely used as a hypoxia mimic both *in vitro* and *in vivo* studies (34,35). $CoCl_2$ can mimic hypoxic responses of cultured cells (36), including transcriptional changes of some genes, such as that of HIF-1α (37,38), which are potent transcription factors activating target genes that initiate cell death or cause population growth arrest, in response to stress or DNA damage. In this study, viability of human PDLCs after $CoCl_2$ treatment

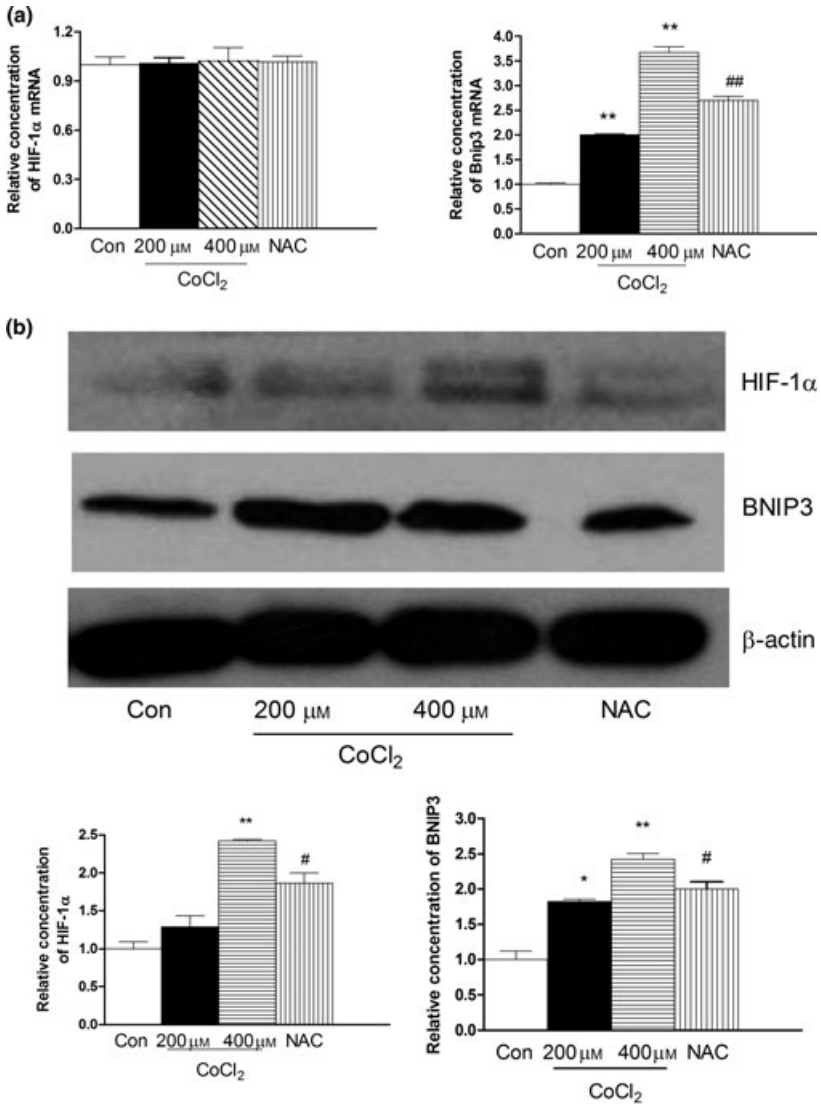

Figure 5. Effect of $CoCl_2$-induced hypoxia on expression of HIF-1α, Bnip3 in human PDLCs. After pre-treatment with NAC for 2 h, cultures were exposed to 400 μM $CoCl_2$ for 48 h. (a) Expressions of HIF-1α mRNA and BNIP3 mRNA were examined by real-time PCR in cells incubated with 200 and 400 μM $CoCl_2$ for 48 h. $CoCl_2$ did not promote level of HIF-1α mRNA expression. However, $CoCl_2$ increased level of Bnip3 mRNA expression. (b) Expression of HIF-1α and BNIP3 protein were detected by western blotting. β-actin was used as a control. Levels of HIF-1α and BNIP3 protein were significantly increased by $CoCl_2$ at 48 h. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus $CoCl_2$ group.

reduced with concentrations of $CoCl_2$, consistent with amounts of $CoCl_2$-induced expression of HIF-1α protein. The results strongly indicate that $CoCl_2$-induced hypoxia may result in cytotoxicity of human PDLCs.

When cells are exposed to chronic or extreme hypoxia, initiation by HIF-1α results in apoptosis. As a transcription factor, HIF-1α can participate in cell death by activating pro-death genes, including *Bnip3* (39). The gene for Bnip3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) is a primary pro-death gene of hypoxia. Bnip3 expression can be upregulated under hypoxia in cell lines derived from carcinomas, fibroblasts and macrophages (40). We have also found that expression of Bnip3 in human PDLCs after $CoCl_2$ treatment was significantly elevated both in mRNA and protein levels.

Previous studies have shown that overexpression of Bnip3 results in its integration into the outer mitochondrial membrane with specific orientation – its N-terminus in the cytoplasm and C-terminus in the membrane (41). After integration into the mitochondrial membrane, Bnip3 can interact with components of MPT pores to induce their rapid opening and dissipation of $\Delta\Psi m$, followed by apoptotic cell death (42). We have also proven that $CoCl_2$ promoted activation of HIF-1α, upregulated expression of HIF-1α and Bnip3, and ultimately resulted in apoptosis of the cells; this could be attenuated by use of NAC, an inhibitor of HIF-1 α. This indicated that $CoCl_2$-induced hypoxic insults can be mediated in the HIF-1α pathway.

It has been illustrated that autophagy resulting the total devastation of the cell is one of several types of programmed cell death (PCD). Autophagy plays key physiological cellular functions and also has pathophysiological roles. Too much autophagy or too little autophagy is

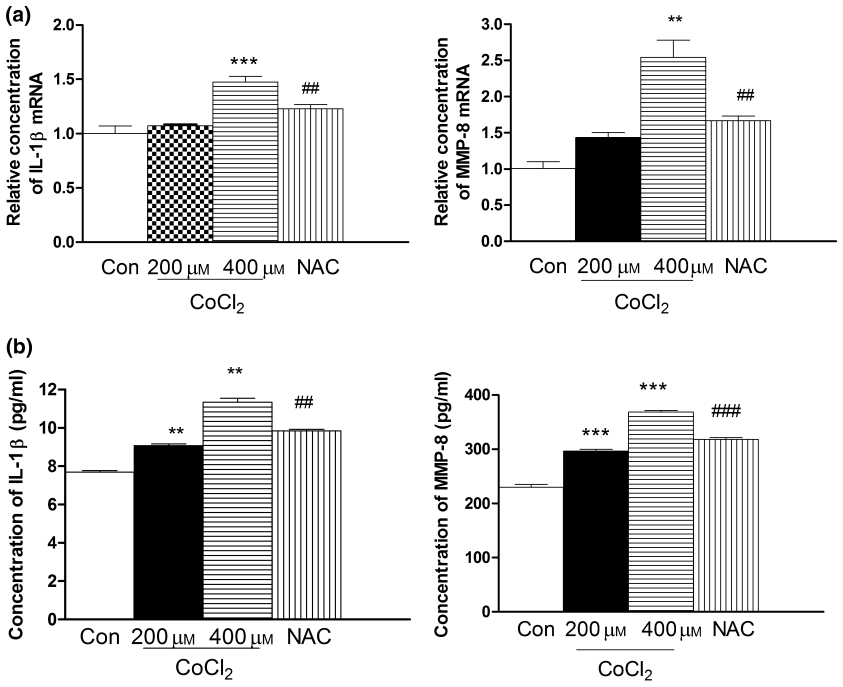

Figure 6. Effect of $CoCl_2$-induced hypoxia on expression of IL-1β and MMP8 in human PDLCs. After pre-treatment with NAC for 2 h, cultures were exposed to 400 μM $CoCl_2$ for 48 h. (a) Expressions of IL-1β mRNA and MMP8 mRNA were examined by real-time PCR in cells treated with 200 and 400 μM $CoCl_2$ for 48 h. (b) Effect of hypoxia on production of IL-1β and MMP8 was evaluated by ELISA. Significantly higher concentrations of IL-1β and MMP-8 were detected in the hypoxia group than in controls at 48 h. $^{**}P < 0.05$ versus control, $^{***}P < 0.01$ versus control, $^{\#\#}P < 0.01$ versus 400 μM $CoCl_2$ group, $^{\#\#\#}P < 0.01$ versus 400 μM $CoCl_2$ group.

linked to pathogen infection, neurodegeneration, muscular disorders, liver disease and cancer (43). Accumulating evidence has suggested that HIF-1 plays an important role in induction of autophagy *via* enhanced expression of its target gene *Bnip3* (44,45) or by inhibition of the mammalian target of rapamycin (mTOR). In our study, we found $CoCl_2$ caused lysosome dysfunction in human PDLCs. Following $CoCl_2$ treatment, there was increase in ratio of LC3-II to LC3-I, an indicator of autophagy. Expression of Beclin 1, an autophagy-related protein, also is upregulated in human PDLCs treated with $CoCl_2$. When pre-treated with NAC, $CoCl_2$-induced autophagy was attenuated. Here, we report for the first time that $CoCl_2$ can induce autophagy of human PDLCs through enhanced protein levels of HIF-1α.

Hypoxia and inflammation often occur simultaneously in numerous diseases, including in ischaemic diseases, chronic inflammatory diseases and growing tumours (46,47). Recent studies have demonstrated that hypoxia can activate macrophages, dendritic cells and monocytes, which are involved in the inflammatory response, by altering gene expression and cytokine secretion (48,49). HIF-1α is considered important in inflammatory processes as it also regulates phagocytosis of mononuclear cells (50). Hypoxia stimulates expression of several cytokines, from various cells, such as human PDLCs, gingival fibroblasts and synovial fibroblasts (5,51). Pro-inflammatory cytokines including IL-β and MMP-8 are pivotal in pathogensis of periodontal disease. One important effect of IL-1β is to stimulate fibroblasts to produce destructive metalloproteinases, such as collagenase and stromelysin, which degrade components of the extracellular matrix in periodontitis. IL-1β has been confirmed to be a potent inducer of bone resorption and of connective tissue degradation *via* induction of MMPs (52,53). In addition, pathologically excessive MMP plays a significant role in periodontal destruction (54). MMP-8 (collagenase 2) is a collagenolytic enzyme that can initiate digestion of type I collagen, the most dominant interstitial collagen type, in periodontal tissues. Collagen degradation is regarded to be one of the key factors in uncontrolled tissue destruction in periodontitis (55). Our data show that expression and release of IL-1β and MMP-8 in $CoCl_2$ treated groups were statistically higher than in control groups. However, NAC can abolish these changes. The results also suggest that hypoxia and HIF-1α may play a role in periodontal diseases.

In conclusion, exposure of human PDLCs to $CoCl_2$-induced hypoxia results in hypoxic cell death with apoptosis and autophagy, due to activation of the HIF-1α pathway. Further investigation to elucidate mechanisms of hypoxia-induced apoptosis and autophagic cell death will be useful in developing methods for prevention of periodontal diseases.

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