Original Article

PARP-1 Involvement in Autophagy and Their Roles in Apoptosis of Vascular Smooth Muscle Cells under Oxidative Stress

(PARP-1 / autophagy / AMPK-mTOR / oxidative stress / apoptosis)

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Abstract. Autophagy and poly(ADP-ribose) polymerase 1 (PARP-1) are activated and involved in a series of cell processes under oxidative stress, which is associated with pathogenesis of atherosclerosis. Research on their relationship under oxidative stress has been limited. In this study, we aimed to investigate the activation, relationship, and role of autophagy and PARP-1 in vascular smooth muscle cell (VSMC) death under oxidative stress. This study explored the signal molecule PARP-1 and autophagy in VSMCs using gene silencing and the hydrogen peroxide (H_2O_2)-stimulated oxidative stress model. We observed that H_2O_2 could induce autophagy in VSMCs, and the inhibition of autophagy could protect VSMCs against oxidative stress-mediated cell death. Meanwhile, PARP-1 could also be activated by H_2O_2. Additionally, we analysed the regulatory role of PARP-1 in oxidative stress-mediated autophagy and found that PARP-1 was a novel factor involved in the H_2O_2-induced autophagy via the AMPK-mTOR pathway. Finally, PARP-1 inhibition protected VSMCs against caspase-dependent apoptosis. These data suggested that PARP-1 played a critical role in H_2O_2-mediated autophagy and both of them were involved in apoptosis of VSMCs.

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Abbreviations: 3-MA – 3-methyladenine, 4-HNE – 4-hydroxynonenal, 7-KC – 7-ketocholesterol, AMPK – adenosine 5'monophosphate (AMP)-activated protein kinase, AS – atherosclerosis, ATP – adenosine triphosphate, α-SMA – α-smooth muscle actin, DMSO – dimethyl sulphoxide, ERK – extracellular regulated protein kinases, H_2O_2 – hydrogen peroxide, HCQ – hydroxychloroquine, JNK – c-Jun N-terminal kinase, LC3 – light chain 3, mTOR –mammalian target of rapamycin, PAR – poly(ADP-ribose), PARP-1 – poly(ADP-ribose)polymerases 1, PBS – phosphate-buffered saline, p-P70S6K – phospho-ribosomal protein s6 kinase, ROS – reactive oxygen species, RT-PCR – real-time polymerase chain reaction, SD – standard deviation, VSMC(s) – vascular smooth muscle cell(s).

Introduction

Autophagy is a catabolic pathway for the bulk destruction of long-lived proteins and damaged organelles via lysosomes, which generates amino acids and substrates for cellular metabolism to maintain cellular homeostasis (Kroemer et al., 2010; Mizushima and Komatsu, 2011). Autophagy is activated under nutrient deprivation and/or oxidative stress (Mizushima et al., 2004; Lee et al., 2012) and associated with atherosclerosis (Martinet and De Meyer, 2009). It is well known that oxidative stress, especially reactive oxygen species (ROS), is involved in the pathogenesis of atherosclerosis (AS), serving as an important promoting factor of AS, as well as an important inducer and mediator of autophagy (Stocker and Keaney, 2004; Scherz-Shouval et al., 2007).

At present, accumulating evidence suggests that autophagy is a double-edged sword in determining cell fate (Shintani and Klionsky, 2004). Autophagy is involved in the maintenance of normal cellular homeostasis and generally serves as a pro-survival mechanism, whereas excessive autophagy triggers cell death (Gomez-Santos et al., 2003; Liao et al., 2012). In some studies,

autophagy promotes vascular smooth muscle cell (VSMC) survival in response to reactive species, such as 7-keto-cholesterol (7-KC) (He et al., 2013), 4-hydroxynonenal (4-HNE) (Hill et al., 2008), and free cholesterol (Xu et al., 2010). A recent study demonstrated that autophagy inhibition by gene deletion prevented oxidative stress-induced cell death in VSMCs (Grootaert et al., 2015). Therefore, the role of autophagy in the viability of VSMCs under oxidative stress is uncertain and remains to be elucidated.

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme that is activated by DNA injury and implicated in a wide range of essential cellular processes, including DNA repair, gene transcription, metabolism, and cell death (Langelier et al., 2012; Xu et al., 2014). However, the hyperactivation of PARP-1 induced by DNA damage results in various biological consequences, including cellular dysfunction and cell death (Szabo et al., 1996; Radovits et al., 2007). The ROS-DNA injury-PARP pathway has recently been established as a major downstream intracellular pathway of oxidative stress and implicated in the pathogenesis of various cardiovascular diseases (Pacher and Szabo, 2007).

It has been demonstrated that PARP-1 participates in the regulation of autophagy. Some chemotherapeutic agents such as alkylating agents (Zhou et al., 2013), doxorubicin (Munoz-Gamez et al., 2009) and radiotherapy (Chen et al., 2015) could induce autophagy through PARP-1 activation, but these studies are more concentrated on the field of cancer. A recent report found that PARP-1 was involved in autophagy induction in umbilical vein endothelial cells (Li et al., 2015). In addition, PARP-1 hyperactivation not only led to necrotic cell death (Ha and Snyder, 1999), but was also involved in apoptosis (Wang et al., 2011). Recent studies have revealed that PARP-1 may mediate a new non-caspase dependent cell death type (Wang et al., 2011).

PARP-1 may be involved in multiple signal pathways, but the specific mechanism by which PARP-1 affects cell fate remains unclear. The activation of autophagy and PARP-1 is closely related to oxidative stress and plays an important role in atherosclerosis. However, their precise role and relationship in VSMCs under oxidative stress has yet to be elucidated. Therefore, we sought to explore the role of PARP-1 in autophagy and their effects on cell death in VSMCs through a H_2O_2-stimulated oxidative stress model. In this study, we showed that inhibition of autophagy and PARP-1 deficiency could decrease cell death in VSMCs, and PARP-1 might contribute to the induction of autophagy via the AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathway.

Material and Methods

Reagents

We used the following antibodies from rabbits: antibodies against Beclin1, LC3, GAPDH, p-AMPK, p-P70S6K (Cell Signaling Inc, Beverly, MA), antibody against PARP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), antibody against PAR (Enzo Life Sciences, Farmingdale, NY). Chemicals: H_2O_2 was obtained from Sigma-Aldrich (St. Louis, MO); 3-methyladenine(3-MA) and hydroxychloroquine sulphate (HCQ) were purchased from Selleck (Houston, TX).

Cell culture

VSMCs were isolated from the thoracic aorta of C57BL/6J mice by tissue explantation and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in air supplemented with 5% CO_2 at 37 °C. Cultures were confirmed to consist primarily (> 95%) of VSMCs both morphologically, by their classical "hill and valley" appearance, and immunohistochemically, by α-smooth muscle actin (α -SMA) immunoreactivity. VSMCs at passages four to six and grown to 70% to 80% confluence were used for the experiments.

MTT assay

The MTT assay procedure was used to determine cell viability of the drug-treated VSMCs and untreated cells. MTT assays were performed by adding 20 µl MTT (5 mg/ml) to wells in a 96-well plate according to manufacturer's instructions (Sigma-Aldrich). After incubating 4 h in the dark, the medium was removed and the cells were dissolved in 150 µl dimethyl sulphoxide (DMSO). The absorbance value was measured for each well using a microplate reader (Bio-Tek, Winooski, VT) at 490 nm (A490).

Detection of cell apoptosis

1) Hoechst 33258 staining: VSMCs were fixed and stained with Hoechst 33258. Subsequently, the cells were examined and photographed under a fluorescence microscope (Olympus, Tokyo, Japan). Apoptotic cells were stained brighter due to chromatin condensation.

2) Flow cytometry: Apoptosis was analysed by using an Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, according to the instructions). VSMCs were collected together, washed twice with cold phosphate-buffered saline (PBS) and resuspended in 500 µl of binding buffer. Cells were then labelled for 15 min with annexin V-FITC (5 µl) and propidium iodide (5 µl) at room temperature in the dark. Cells were analysed with Accuri C6 flow cytometry (BD Biosciences, La Jolla, CA).

Western blot analysis

After the treatments, cells were collected and lysed in a lysis buffer. Cell samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF) (Millipore, Bedford, MA). Membranes were subsequently blocked with 5% skimmed milk powder for 2 h and probed with primary antibodies overnight at 4 °C. Thereafter, membranes were incubated with HRP-conjugated secondary antibodies for 2 h with shaking.

The optical density of bands was quantified by QuantityOne software (Bio-Rad, Hercules, CA).

PARP1 gene silencing

The small interfering RNA (siRNA) targeting PARP-1 (5'-GAGCGACGCTTATTACTGT-3') and the negative control siRNA were purchased from GeneChem Biotechnology (Shanghai City, China). VSMCs were transfected with PARP-1-specific siRNA or siRNA control according to the manufacturer's instructions. The efficiency of siRNA-silenced genes was evaluated by real-time polymerase chain reaction (RT-PCR) and western blotting of the targeted proteins.

Quantitative RT-PCR analysis

Total mRNA was extracted from the cultured cells by the Trizol extraction method (BioTeke, Beijing, China). An equal amount of RNA was reverse-transcribed to cDNA by reverse transcription and amplified by PCR using primers. The expression of PARP-1 and housekeeping β-actin were measured by RT-PCR analysis using SYBR Green (Solarbio, Beijing, China) and the Exicycler 96 Real-Time PCR System (Bioneer, Daejeon, Korea) according to the manufacturer's protocol. Data analysis was performed by the comparative method ($2^{-\Delta\Delta CT}$) using β-actin transcripts as an internal control.

Caspase-3 activity assay

The activity of caspase-3 like protease in the lysate was measured using a colorimetric caspase-3 assay kit (Sigma Aldrich) according to the manufacturer's protocol. VSMCs from differently treated groups were harvested, lysed, and centrifuged. Then, aliquots of supernatants were collected and incubated with caspase-3 substrates at 37 °C overnight. The absorbance was read at 405 nm and the results were calculated using a calibration curve prepared using a stock solution of p-nitroaniline (at the concentration range of 0–200 μM).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). SPSS19.0 was used to analyse the differences between experimental groups. Statistical analyses were performed by using *t*-tests (2 groups) or one-way ANOVA with Bonferroni's procedure for multiple comparison tests (3 groups). P value < 0.05 was considered statistically significant.

Results

The effect of autophagy on VSMC survival under oxidative stress

In the present study, we first investigated VSMC death after the treatment with different concentrations of H_2O_2 and at different time points. H_2O_2 induced cell death in a dose-dependent manner (Fig. 1A-a) and generally, a time-dependent manner (Fig. 1A-b). In addition, we measured the autophagy response in VSMCs treated with H_2O_2 at different time points. Our results showed that autophagy marker protein LC3-I to LC3-II conversion and beclin-1 expression significantly increased in VSMCs in a time-dependent manner after the treatment with H_2O_2 (Fig. 1B-a). Next, we examined the autophagy flux by lysosomal inhibitor HCQ. The presence of HCQ further increased the LC3-II/LC3-I ratio (Fig. 1B-b), suggesting that H_2O_2 is capable of promoting the autophagy flux.

To determine the role of autophagy in VSMC survival under oxidative stress, we studied the role of autophagy in H_2O_2-mediated cell death by inhibition of autophagy. The results showed that autophagy inhibition by 3-MA significantly decreased the expression of LC3-II (Fig. 1B-b). Compared to the H_2O_2-treated group, the inhibition of autophagy markedly increased cell viability as determined by MTT (Fig. 1C-a). To clarify the features of the decreased cell viability induced by H_2O_2, we further examined morphologic changes of VSMCs. As shown in Fig. 1C-b, apoptotic cells were observed to have condensed or segmented nuclei accompanied by bright blue fluorescence in H_2O_2-treated cells. The apoptotic cells were reduced in H_2O_2+3-MA-treated cells. In addition, apoptosis was further evaluated by flow cytometry.

H_2O_2 treatment led to a dramatic increase in the percentages of early apoptotic (annexin V^+/PI^-) cells, end-stage apoptosis and dead cells (annexin V^+/PI^+) ($P < 0.05$), and to a lesser extent, in the percentage of damaged cells (annexin V^-/PI^+) ($P > 0.05$). Inhibition of autophagy attenuated H_2O_2-induced cell death mainly through reducing early apoptosis ($P < 0.05$) (Fig. 1C-c). Taken together, these data indicated that autophagy suppression protected VSMCs against oxidative stress-mediated cell death.

The activation of PARP-1 and its modulation of VSMC autophagy under oxidative stress

PARP-1 is activated in response to oxidative stress and implicated in the cell death and autophagy process under certain conditions. In this study, we first examined the activation of PARP-1 under oxidative stress by detecting PAR formation at different time points. H_2O_2 rapidly induced massive PAR polymer formation and peaked after 0.5 h (Fig. 2A).

Based on the previous research, we hypothesize that PARP-1 activation, AMPK activation, and subsequent mTOR inhibition might contribute to the induction of autophagy under oxidative stress. To determine whether PARP-1 activation contributes to the cell autophagy induced by H_2O_2 and its potential downstream signalling molecules, we used the gene silencing technology to block PARP-1 function in order to detect changes of PARP-1, AMPK and mTOR activity of VSMCs treated with H_2O_2.

Compared with the untreated group, H_2O_2 treatment markedly increased the PAR formation, the expression of p-AMPK, decreased the expression of p-P70S6K,

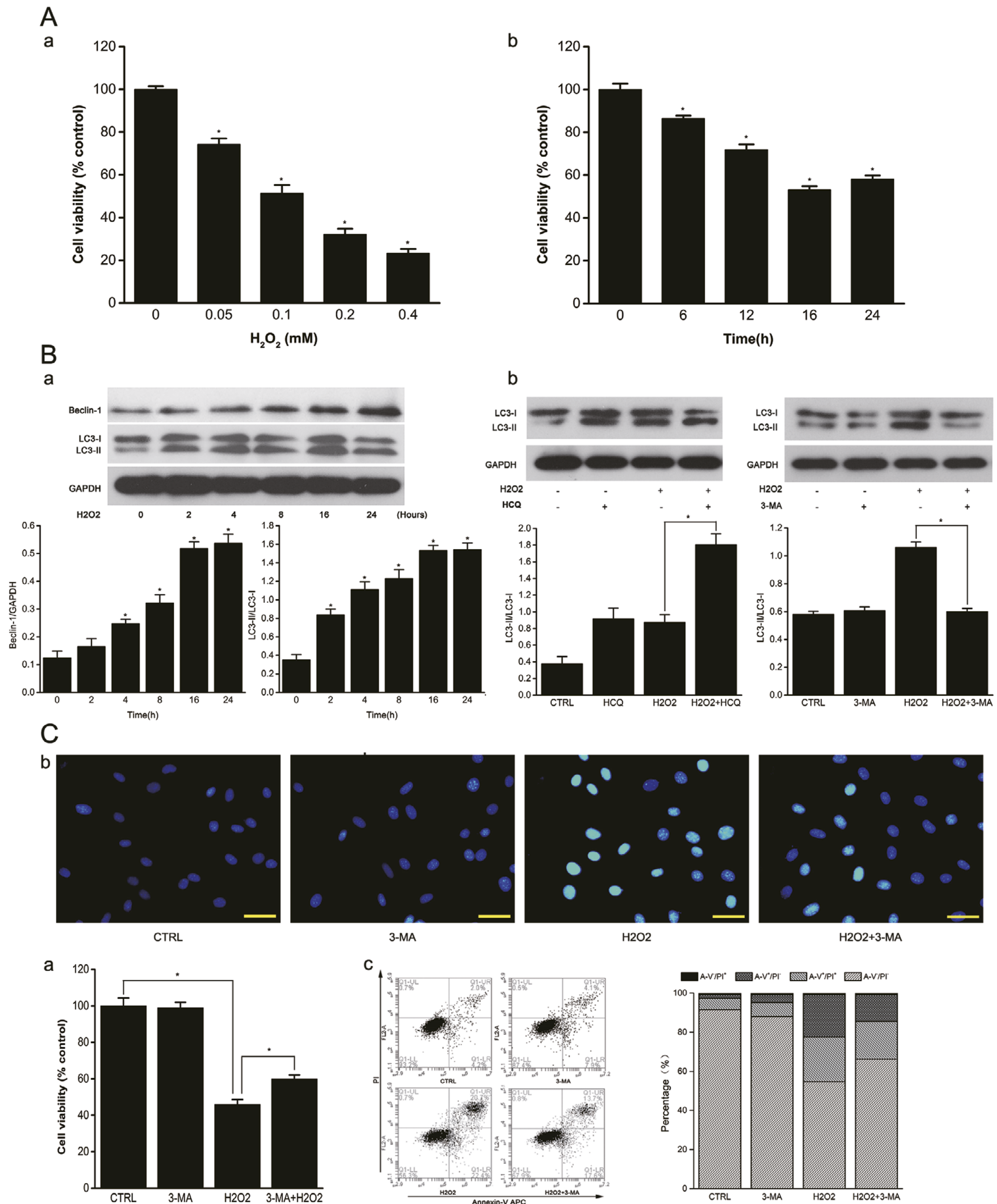

Fig. 1. H_2O_2 induces cell death and autophagy, and the effect of autophagy on VSMC survival
(**A**) VSMCs were treated with different concentrations of H_2O_2 or for different time periods. (A-a) Treatment of VSMCs with H_2O_2 at various concentrations for 16 h. (A-b) Treatment of VSMCs with H_2O_2 (0.1 mmol/l) for different time periods. Cell viability was assessed using the MTT assay. *P < 0.05 versus control. (**B**) Detection of autophagy and autophagy flux. (B-a) Treatment of VSMCs (0.1 mmol/l) with H_2O_2 for different time periods. (B-b) VSMCs were pre-treated with HCQ (10 µM) or 3-MA (5 mM) for 1 h and then incubated with H_2O_2 (0.1 mmol/l) for 16 h. The protein expression of LC3 and beclin-1 was determined by western blot. GAPDH served as loading controls. (**C**) The effect of autophagy on VSMC survival under oxidative stress. (C-a) Cell viability was measured using the MTT assay after the same treatment as in (B-b). (C-b) Hoechst 33258 staining was used to visualize cell death. Bars, 50 µm. (C-c) Cell death was further analysed using annexin V/propidium iodide (PI) staining and flow cytometry. Data were expressed as mean ± S.D. of three independent experiments. *P < 0.05

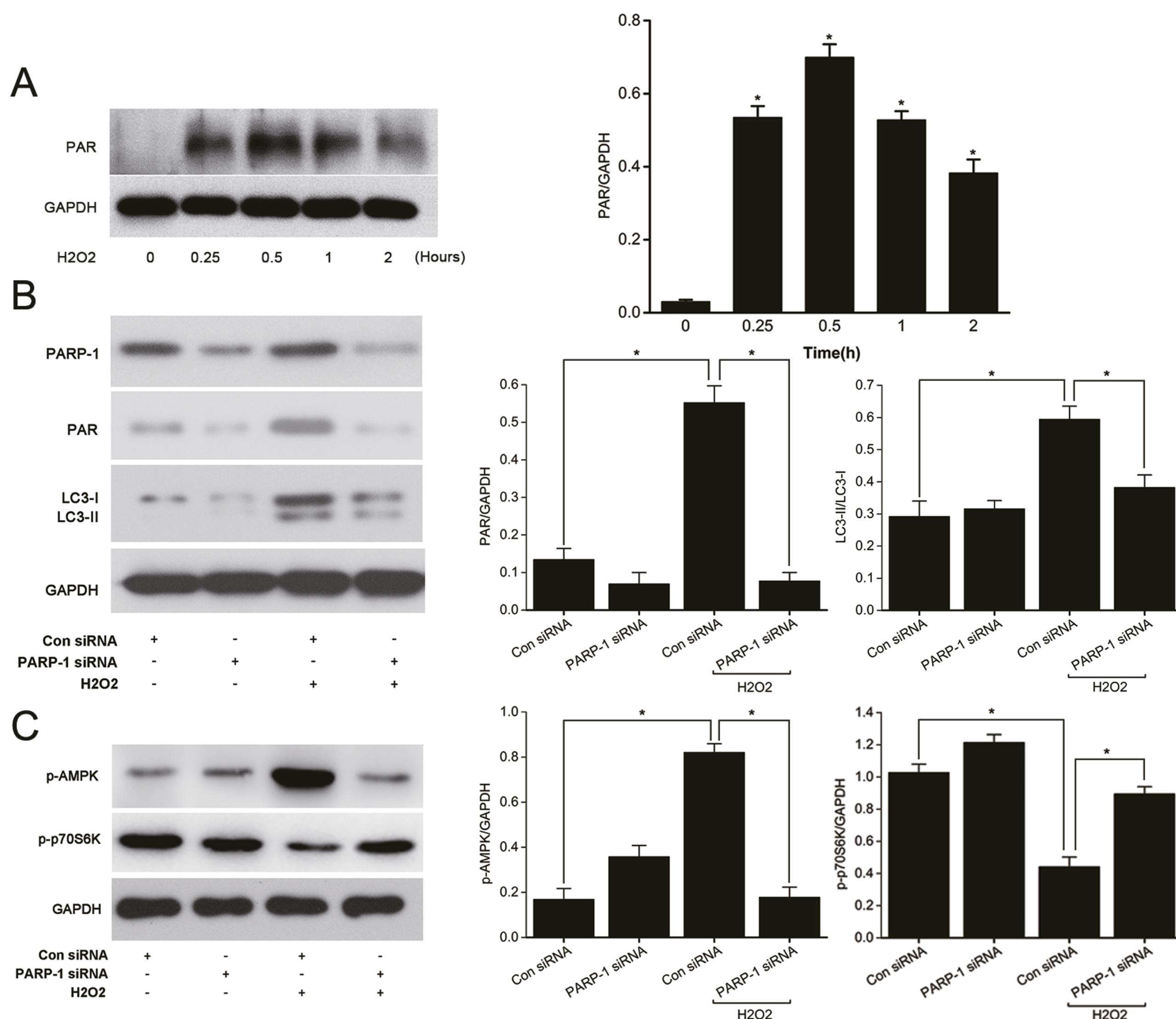

Fig. 2. PARP-1 activation contributed to H_2O_2-induced autophagy.
(**A**) H_2O_2 induces PARP-1 activation. Treatment of VSMCs with H_2O_2 (0.1 mmol/l) for different time periods. PAR expression was determined by western blot. *$P < 0.05$ versus control. (**B**) VSMCs were pre-treated with PARP-1 siRNA and then treated with or without H_2O_2 (0.1 mmol/l), PAR was measured after 2 h, and LC3 was measured after 16 h by western blot. (**C**) VSMCs were treated as (B) and the expression of p-AMPK, p-p70S6K proteins was analysed by western blot. Data were expressed as mean ± S.D. of three independent experiments. *$P < 0.05$

and finally enhanced LC3-I to LC3-II conversion (Fig. 2B, Fig. 2C). The silencing of PARP-1 significantly inhibited H_2O_2-induced PAR formation, the expression of p-AMPK, restored the expression of p-P70S6K and subsequently attenuated LC3-I to LC3-II conversion (Fig. 2B, Fig. 2C). These observations suggest that the PARP-1-AMPK-mTOR axis seems to be involved in the induction of autophagy in response to oxidative stress.

The effect of PARP-1 on VSMC survival under oxidative stress

To investigate the role of PARP-1 in the cell death induced by H_2O_2, we used a genetic approach to silence PARP-1 with siRNA and measured cell viability by MTT. We observed that PARP-1 knockdown significantly attenuated H_2O_2-induced cell death (Fig. 3A).

We further examined the nature of death by Hoechst 33258 staining, as seen in Fig. 3B-a. Condensed bright apoptotic nuclei were readily observed in H_2O_2-treated cells, and PARP-1 inhibition reduced cell apoptosis. The presence of apoptotic cells was further confirmed by flow cytometry. H_2O_2 treatment of VSMCs mostly resulted in a dramatic increase in the percentages of early apoptotic (annexin V^+/PI^-) cells, end-stage apoptosis and dead cells (annexin V^+/PI^+) ($P < 0.05$). PARP-1 inhibition significantly decreased early apoptosis, as well as end-stage apoptosis and dead cells ($P < 0.05$). There was no significant difference between damaged cells (Fig. 3B). Taken together, these results suggested that PARP-1

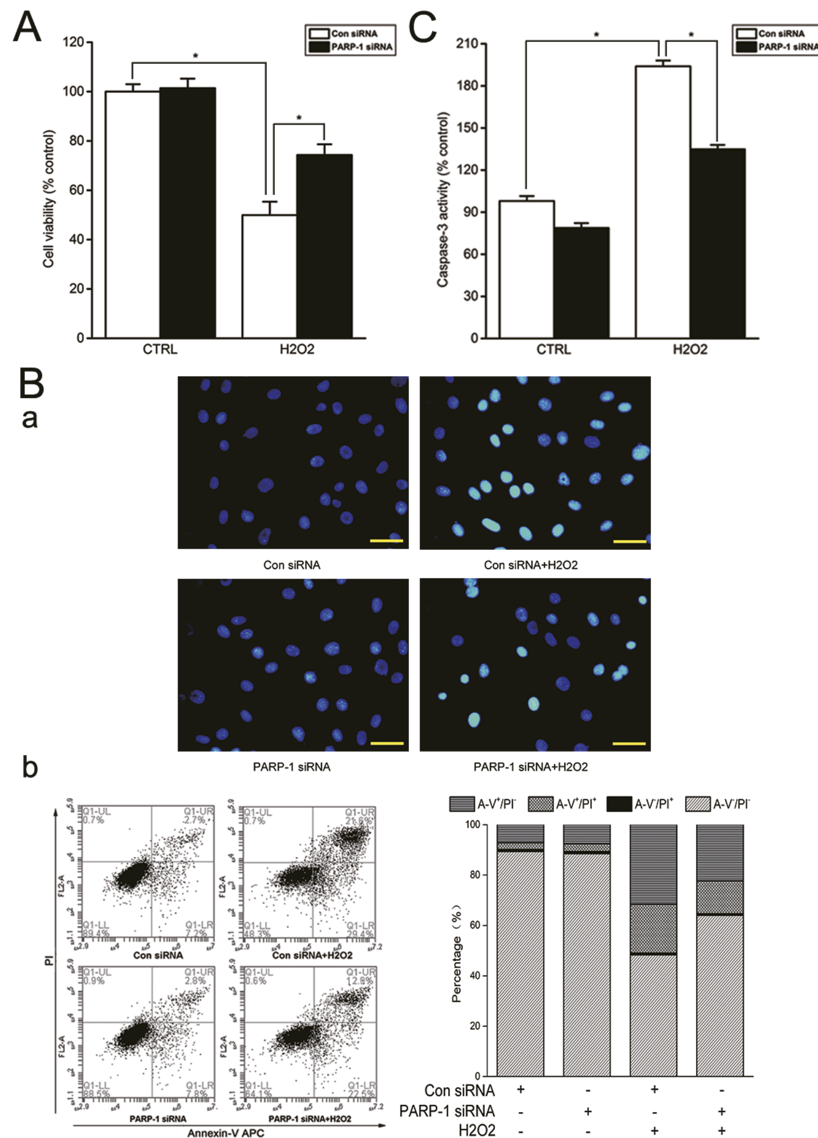

Fig. 3. PARP-1 effect on apoptosis in VSMCs through a caspase-dependent way
(**A**) VSMCs were pre-treated with PARP-1 siRNA and then treated with or without H_2O_2 (0.1 mmol/l) for 16 h, and then cell viability was measured using MTT. (**B**) The effect of PARP-1 on VSMC survival under oxidative stress. (B-a) Cells were treated with vehicle control or H_2O_2 (0.1 mmol/l) for indicated time periods, and the nuclear morphology was detected using fluorescent microscopy. Bar = 50 µm. (B-b) Cell death was analysed using flow cytometry after the same treatment as in A. (**C**) VSMCs were pre-treated with PARP-1 siRNA and then treated with or without H_2O_2 (0.1 mmol/l) for 6 h, and caspase-3 activity was analysed by the colorimetric method. Data were expressed as mean ± S.D. of three independent experiments. *$P < 0.05$

inhibition protected VSMCs from cell death in response to H_2O_2.

Parthanatos, also known as PARP-1-dependent cell death, is a new form of programmed cell death based on DNA damage and PARP-1 activation, but independent of caspase (David et al., 2009). To examine whether the effect of PARP-1 was associated with the activation of caspase – a key factor of apoptosis, we measured the activity of caspase-3. We observed that *PARP1* gene silencing significantly reduced caspase-3 activation in VSMCs treated with H_2O_2 (Fig. 3C). Thus, the effect of PARP-1 on VSMC apoptosis was associated with caspase activation.

Discussion

Autophagy, which is a fundamental cellular process and links to several cellular pathways, impacts VSMC survival and function. Emerging evidence demonstrates that autophagy in VSMCs would contribute to atherosclerosis. Some stimuli can directly or indirectly activate autophagy in VSMCs through ROS, such as H_2O_2, 7-KC, 4-HNE, ox-LDL (Hill et al., 2008; Huang et al., 2009; Ding et al., 2013; He et al., 2013). The role of autophagy has been investigated in many studies, suggesting that autophagy may exert different roles depending on the cell type and the autophagic trigger. Some

studies have found that autophagy induced by hypoxia, 7-KC, 4-HNE protected VSMCs against cell death (Hill et al., 2008; He et al., 2013; Ibe et al., 2013), while in some cases, autophagy played an opposite effect, such as the autophagy triggered by osteopontin leading to VSMC death (Zheng et al., 2012). Moreover, a recent study demonstrated that the inhibition of autophagy by gene deletion reduced oxidative stress-induced cell death (Grootaert et al., 2015).

Whether induction of autophagy in VSMCs exposed to H_2O_2 is the cause of death or actually an attempt to support survival in response to cellular stress remains to be determined. In this study, we found that autophagy was activated by H_2O_2 in VSMCs, and autophagy suppression by chemical inhibitor protected VSMCs against oxidative stress-mediated cell death. Grootaert et al. (2015) found that the effect of autophagy gene deletion, which protected VSMCs against oxidative stress-mediated cell death, was attributed to nuclear translocation of nuclear factor erythroid 2-like 2 (NFE2L2) resulting in up-regulation of several anti-oxidative enzymes. Another explanation is that autophagy might lead to autophagic cell death. Due to the lack of methods and accurate molecular hallmarks for measuring autophagic cell death specifically and quantitatively, the mechanistic study of autophagic cell death has been difficult. However, we failed to explore this point in the current study, and further investigation should be conducted.

PARP-1 is a group of nuclear enzymes that participate in DNA damage repair by poly(ADP-ribosyl)ation, which is activated by oxidative/nitrosative stress and critically involved in the pathogenesis of atherosclerosis through numerous cellular processes. This study explored the activation of PARP-1 and its regulatory function in VSMCs under oxidative stress. So far, the autophagy signalling pathway under oxidative stress has not been fully understood. Studies suggest that autophagy might be activated by up-regulation of NADPH oxidase 4 (Nox4) leading to inhibition of Atg4B activity, or be triggered by a JNK-dependent mechanism (Scherz-Shouval et al., 2007; Haberzettl and Hill, 2013). Recent studies have found that PARP-1 may be involved in the autophagy, but were mainly focused on the field of oncology, such as effects of alkylating agents, radiation therapy, and doxorubicin (Huang et al., 2009; Munoz-Gamez et al., 2009; Zhou et al., 2013; Chen et al., 2015). These reports revealed a novel signalling pathway linking PARP-1 to autophagy through AMPK-mTOR.

In this study, we found that PARP-1 was activated by H_2O_2 and PARP-1 suppression drastically inhibited PARP-1 activation, AMPK activation, mTOR phosphorylation and subsequently autophagy activation. Our data are found to be consistent with a very recent report that ox-Lp(a) induced human umbilical vein endothelial cells autophagy via PAPR-1-LKB1-AMPK-mTOR pathway (Li et al., 2015). Our observations suggest that the PARP-1-AMPK-mTOR axis appears to be an alternative signalling pathway of autophagy during oxidative stress. We speculate the molecular mechanism to be that ATP depletion caused by PARP-1 activation following oxidative DNA damage leads to AMPK activation. AMPK, an AMP-dependent protein kinase, is a sensor of the cellular energy level, which is activated during energy stress, particularly reduction in ATP levels (Jeon, 2016). There is now accumulating evidence indicating that AMPK is a key upstream regulator of autophagy via mTOR. AMPK negatively regulates mTOR activity executed on downstream effectors, such as ribosomal S6 kinase, thereby regulating autophagy (Alers et al., 2012).

Besides autophagy, PARP-1 also plays an important role in determining cell fate. We further explored the role of PARP-1 in the death of VSMCs under oxidative stress. This study found that H_2O_2 mainly induced VSMC apoptosis, and apoptosis decreased after the *PARP1* gene was silenced, indicating that PARP-1 inhibition has a protective effect. These results are consistent with the report that PARP-1 deletion conferred protection to VSMCs by blocking apoptosis in response to H_2O_2 and 7-KC (Hans et al., 2008). Parthanatos is a new form of programmed cell death, also known as PARP-1-dependent cell death. PARP-1 mediates parthanatos when it is over-activated in response to extreme genomic stress and synthesizes PAR, which causes nuclear translocation of AIF from mitochondria to the nucleus, where it induces DNA fragmentation and ultimately cell death (David et al., 2009). Based on the fact that apoptosis is dependent on the caspase pathway activated by cytochrome c release, while the parthanatos pathway can act independently of caspase (Fatokun et al., 2014), we could distinguish them through caspase activation. To further clarify the mechanism of PARP-1-mediated apoptosis, we examined the activity of caspase-3, a key apoptotic enzyme. The result showed that caspase-3 activity in the PARP-1 silencing group was significantly reduced compared to the control group after H_2O_2 exposure, indicating that PARP-1-mediated apoptosis is caspase-dependent. A previous study showed that the involvement of PARP-1 in apoptosis might be associated with extracellular regulated protein kinases (ERK) and c-Jun N-terminal kinase (JNK) signalling pathway (Hans et al., 2008).

Overall, our results indicated that PARP-1 contributed to H_2O_2-induced autophagy through the AMPK-mTOR signalling pathway. Moreover, the inhibition of PARP-1 and autophagy conferred protection to VSMCs against cell apoptosis under oxidative stress. These findings provided novel insights into the function of PARP-1, which might be a promising therapeutic target for cardiovascular diseases.

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