Poly(ADP-ribose) protects vascular smooth muscle cells from oxidative DNA damage

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Vascular smooth muscle cells (VSMCs) undergo death during atherosclerosis, a widespread cardiovascular disease. Recent studies suggest that oxidative damage occurs in VSMCs and induces atherosclerosis. Here, we analyzed oxidative damage repair in VSMCs and found that VSMCs are hypersensitive to oxidative damage. Further analysis showed that oxidative damage repair in VSMCs is suppressed by a low level of poly (ADP-ribosylation) (PARylation), a key post-translational modification in oxidative damage repair. The low level of PARylation is not caused by the lack of PARP-1, the major poly(ADP-ribosyl) polymerase activated by oxidative damage. Instead, the expression of poly(ADP-ribosyl) glycohydrolase, PARG, the enzyme hydrolyzing poly(ADP-ribosyl), is significantly higher in VSMCs than that in the control cells. Using PARG inhibitor to suppress PARG activity facilitates oxidative damage-induced PARylation as well as DNA damage repair. Thus, our study demonstrates a novel molecular mechanism for oxidative damage-induced VSMCs death. This study also identifies the use of PARG inhibitors as a potential treatment for atherosclerosis. [BMB Reports 2015; 48(6): 354-359]

INTRODUCTION

Atherosclerosis, the leading cause of cardiovascular disease, is formerly considered a chronic inflammatory disease. However, increasing evidence suggests that oxidative stress-induced DNA damage induces the apoptosis of VSMCs during the pathogenesis of atherosclerosis (1, 2). For example, the level of 8-oxoG, a DNA adduct from oxidative damage, is significantly higher in VSMCs of the aorta wall (3, 4). However, in response to DNA damage, cells usually activate DNA damage repair systems to repair DNA lesions. Thus, it is unclear why VSMCs are sensitive to oxidative damage.

Oxidative DNA damage is usually induced by reactive oxygen species (ROS) primarily generated from normal intracellular metabolism in mitochondria and peroxisomes. A number of external hazards such as ionizing radiation, chemicals and UVA solar light can also trigger ROS production (5, 6). These active free radicals attack double-stranded DNA, inducing various types of DNA lesions, including DNA single-stand breaks (SSBs) and double-strand breaks (DSBs), which may lead to genomic instability (7, 8). To cope with these threats, cells have evolved DNA damage response systems to detect and repair DNA lesions. As one of the earliest alarm systems and regulators in DNA damage response, poly(ADP-ribose) (PAR) participates in the repair of numerous types of DNA damage including SSBs and DSBs (9, 10). Thus, the cellular metabolism of PAR is critical for DNA damage response and genomic stability. The reaction of poly(ADP-ribosyl)ation (PARylation) is catalyzed by a group of PAR polymerases (PARPs). Using NAD⁺ as the substrate, PARPs covalently add ADP-ribose to proteins, other ADP-riboses can be covalently linked onto the proteins, other ADP-riboses can be covalently linked onto the proteins, other ADP-riboses can be covalently linked onto the proteins, other ADP-riboses can be covalently linked onto the proteins, other ADP-riboses can be covalently linked onto the proteins, other ADP-riboses can be covalently linked onto the proteins, other ADP-riboses can be covalently linked onto the proteins. After catalyzing the first ADP-ribose onto the proteins, other ADP-ribosylated proteins can be covalently linked and the continuous reactions produce both linear and branched polymers known as PAR (11, 12). The structure of PAR has been well characterized: the ADP-ribose unit in the polymer is linked by glycosidic ribose-ribose 1'-2' bonds. The chain length is heterogeneous and can reach around 200 units with 20-30 units in each branch (13). PARylation is regulated not only by PARPs but also by PARG, the major enzyme for hydrolyzing PAR. In response to DNA damage, PARG is recruited to DNA lesions and digest PAR within a few minutes. Although PARylation has been examined both in vivo and in vitro, the metabolism of PAR in VSMCs remains elusive. In this study, we examined PAR metabolism following oxidative DNA damage in mouse aortic VSMCs (MOVAS), and used mouse embryonic fibroblasts (MEFs) as the control cell line. Similar to MOVAS, MEFs can be used to study DNA damage (14, 15).
and originate from mesenchymal stem cells with the ability to differentiate into myocytes (16, 17). With mass spectrometry, we quantitatively measured the level of PAR in MOVAS, and found that that it was relatively low. Our study also suggests that the PARG level in MOVAS is relatively high, which suppresses PARylation following oxidative damage, and thus affect DNA damage repair. Suppression of PARG by the PARG inhibitor facilitates PARylation and DNA damage repair in MOVAS. Thus, PARG inhibitor treatment could be a potential therapeutic approach for arteriosclerosis.

RESULTS AND DISCUSSION

H2O2 induces DNA damage in MOVAS

ROS is one of the most common by-products during metabolism and induces SSBs (18). Under physiological conditions, ROS-induced SSBs can be repaired via the base excision repair pathway (19). However, when two SSBs happen in close proximity, or when the DNA-replication apparatus encounters a SSB, DSBs, the more deleterious genomic lesion, are formed by overwhelming ROS (20, 21). Excessive ROS imposes an oxidative stress condition on vascular cells especially VSMCs, triggering the apoptosis of VSMCs and arteriosclerosis (22, 23).

It is well known that ROS can be generated by externally adding H2O2 (24). Thus, to study the oxidative DNA damage in MOVAS, we treated MOVAS with H2O2, and employed alkaline comet assays (25) to detect SSBs and DSBs in the cells. Damaged genomic DNA fragments migrated from nuclei during electrophoresis (Fig. 1A). Shorter DNA fragments move faster in electrophoresis, therefore, by measuring the migrated length of DNA fragments, we can quantitatively examine the repair of oxidative damage. To our surprise, we found that the repair in MOVAS was much slower than that in MEFs since much shorter DNA fragments were found in MOVAS especially at 60 minutes (MOVAS: 7.18 ± 0.99, MEFs: 2.68 ± 0.44, P = 0.000) and 120 minutes (MOVAS: 2.87 ± 0.24, MEFs: 0.70 ± 0.16, P = 0.000) following H2O2 treatment (Fig. 1A, B).

The H2O2-induced DSBs in MOVAS and MEFs were examined by staining the cells with anti-γH2AX antibody. We observed γH2AX nuclear foci in MOVAS, suggesting that DSBs occurred in MOVAS following oxidative damage (the number of foci were 2.00 ± 1.75, 8.86 ± 2.21, 25.03 ± 7.93, 36.13 ± 7.06 per cell from the control group to each H2O2 group). Meanwhile, we found little γH2AX foci in MEFs damage (the number of foci were 2.20 ± 2.10, 3.20 ± 2.05, 3.30 ± 3.06, 2.80 ± 2.77 per cell from the control group to each H2O2 group) (Fig. 1C, D). Thus, these results suggest that oxidative damage-induced DSBs were not repaired in a timely manner in MOVAS unlike in MEFs, which may partly explain the mo-

![Fig. 1. H2O2-induced DSBs activate DNA damage repair in MOVAS.](http://bmbreports.org)

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Fig. 2. PAR synthesis is suppressed in MOVAS. (A) Structure of PAR. In response to oxidant DNA damage, PARP-1 hydrolyzes NAD+, releases nicotinamide and one proton (H+), and transfers the ADP-ribose moiety (blue) to protein acceptors. Subsequent digestion of PAR with phosphodiesterase (red scissors) and phosphotase (purple scissors) releases ribosyladenosine (green). (B) Ribosyladenosine levels in MOVAS and MEFs were measured by Q-TOF mass spectrometry, and arbitrary units are shown in the histogram (C), the error bars represent the standard deviation, *P < 0.05. (D) Following 20 min H2O2 treatment, PAR was purified from MOVAS, MEFs, MECs and MSCs, then examined by dot blot using anti-PAR antibody. β-actin was examined by western blot and used as the loading control. (E) Immunofluorescence staining of PAR in artery tissue (Scale bar = 100 μm). (F) The number of PAR positive cells are shown in the histogram. Error bars represent the standard deviation (n = 3), *P < 0.05.
duced a higher ribosyladenosine peak value in MEFs than in MOVAS before and after \( \text{H}_2\text{O}_2 \) treatment ratio of ribosyladenosine peak: MOVAS: 1.61 ± 0.03, MEFs: 3.31 ± 0.05 (Fig. 2B, C). This result suggests that the amount of PAR generated in MEFs in response to oxidative DNA damage is greater than in MOVAS. To validate the results, we introduced two other cell lines, mouse aortic endothelial cells (MECs) and skeletal muscle cells (MSCs), and used dot blot assays to determine the amount of PAR in the cells. We found that the amount of PAR in MOVAS was much lower than in the other three cell lines after \( \text{H}_2\text{O}_2 \) treatment (Fig. 2D). Meanwhile, we investigated PAR synthesis in the wire-injured mice model, which is reported to induce oxidative damage (26, 27). The PAR-positive percentage in MECs was consistently higher than in MOVAS after wire injury (Fig. 2E, F). Thus, a correlation between PAR synthesis and DNA damage repair in VSMCs was observed in both cell and animal experiments, further suggesting that PARylation plays an important role for oxidative DNA damage repair in VSMCs.

High expression of PARG decreases PARylation in MOVAS

We next examined why the level of PAR in MOVAS was significantly lower than in the other cells. Since DNA damage-induced PAR is mainly synthesized by PARP-1, the founding member in PARPs family (13), we first examined the expression of PARP-1 in MOVAS and MEFs by immunofluorescence staining. We co-stained PAR and PARP-1 in the nuclei of both MOVAS and MEFs. As shown in Fig. 3A, although the level of PAR was significantly higher in MEFs, the expression level of PARP-1 in MOVAS and MEFs did not show significant differences. To further examine the level of PARP-1, we analyzed PARP-1 in the above four cell lines by western blot (Fig. 3B). Again, we did not observe significant differences in PARP-1’s expression in the control and \( \text{H}_2\text{O}_2 \) treated cells. PARP-1 is the most important enzyme involved in oxidative DNA repair (28), which normally occurs in the cell nucleus. When DNA damage occurs, PARP-1 is activated and recruited to the damage sites, but the amount does not increase (29). Besides PARP-1, PARG, the enzyme that regulates PAR degradation, also plays an important role in PAR metabolism. To characterize PARG, we examined the expression of PARG following \( \text{H}_2\text{O}_2 \) treatment. Under physiological conditions, the expression level of PARG was relatively low, and there was no significant difference in PARG expression in the MOVAS, MEFs, MECs and MSCs. However, upon treatment with \( \text{H}_2\text{O}_2 \), we found that the expression of PARG increased in varying degrees in different cells, and oxidative stress induced higher expression of PARG in MOVAS (Fig. 3B). Thus, it is likely that the high level of PARG expression in MOVAS is responsible for the low level of PAR in response to DNA damage.

PARG inhibitor rescues MOVAS from oxidative stress-induced cell death

To further examine the role of PARG, we treated the cells with the PARG inhibitor GLTN. In the presence of the PARG inhibitor, the amount of PAR increased significantly in MOVAS.

**Fig. 3.** High expression of PARG level promotes PAR degradation in MOVAS. (A) Representative immunostaining images of PARP-1 (green) and PAR (red) in \( \text{H}_2\text{O}_2 \)-treated MOVAS and MEFs cells at 20 min. Nuclei were stained with Hoechst 33258 (blue), and outlined by white dashed lines in enlarged boxes to denote the localization of PARP-1 and PAR in nuclei. (B) The expression of PARP-1 and PARG in cells were examined at 1 h following \( \text{H}_2\text{O}_2 \) treatment, \( \beta \)-actin was used as the protein loading control. The error bars represent the standard deviation.
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Fig. 4. PARG inhibitor reduced oxidative DNA damage level and cell death. (A) Following PARG treatment, PAR synthesis in MOVAS was examined by dot blot. (B, C) MOVAS were pre-treated with or without PARG inhibitor followed by H$_2$O$_2$. DNA breaks were examined at 20 min following H$_2$O$_2$ treatment by alkaline comet assays. Tail moment was measured. (D, E) MOVAS were pre-treated with or without PARG inhibitor. H$_2$O$_2$-induced DSBs were examined and the foci of γH2AX in each cell were counted (n = 15). (F) MOVAS were pre-treated with or without PARG inhibitor and then exposed to 100 μM H$_2$O$_2$. Cell viability was evaluated using an MTT assay. The error bars represent the standard deviation, *P < 0.05.

In conclusion, our study suggests that a high level of endogenous PARG in VSMCs impairs oxidative DNA damage repair, inducing the death of VSMCs during atherosclerosis. PARG inhibitor treatment suppresses PARG activity and facilitates DNA damage repair in VSMCs by prolonging PARylation. Thus, PARG acts as a mediator of cardiovascular disease, and PARylation may play a pivotal protective role as an antioxidant defense mechanism by maintaining VSMCs function under pathologic conditions of oxidative stress. Hence, PARG inhibitors may provide a novel clinical treatment for suppressing arteriosclerosis caused by oxidative damage.

MATERIALS AND METHODS

Materials and Methods are described in the online data supplement, available at http://www.bmbreports.org/.

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