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Original Article

Olecular mechanism underlying the myeloperoxidase induced apoptosis of HUVEC-12 cells

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Abstract: Objective: This study aimed to investigate the molecular mechanism underlying the myeloperoxidase (MPO) induced apoptosis of human umbilical vein endothelial cells (HUVECs). Methods: HUVEC-12 cells were treated with myeloperoxidase at different concentrations (0.1 μ/ml, 0.2 μ/ml, 0.4 μ/ml and 0.6 μ/ml) and apoptotic cells were detected by flow cytometry. Then, cells were harvested for the detection of mRNA and protein expression of caspase-3 and Bax by reverse transcription PCR and Western blot assay, respectively. Results: When compared with negative control group, the apoptosis index in 0.2 μ/ml MPO group, 0.4 μ/ml MPO group and 0.6 μ/ml MPO group increased markedly ($P<0.05$). When compared with negative control group, the mRNA expression of caspase-3 in 0.6 μ/ml MPO group and positive control group increased dramatically ($P<0.05$). When compared with negative control group, the protein expression of pre-caspase-3 and activated caspase-3 elevated significantly in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group ($P<0.05$). When compared with negative control group, the mRNA and protein expression of Bax elevated dramatically in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group ($P<0.05$). Conclusion: MPO at certain extents may induce the apoptosis of HUVEC-12. The MPO induced apoptosis of HUVEC-12 may be dependent on capase-3 signaling pathway, and Bax is also involved in the MPO induced apoptosis of HUVEC-12.

Keywords: Myeloperoxidase, human umbilical vein endothelial cells, apoptosis, caspase-3, Bax

Introduction

Cardiovascular diseases are major factors threatening the human health and causing death in modern society. Atherosclerosis (AS) is an important pathophysiological basis of cardiovascular diseases. Vascular endothelial cells are localized on the inner surface of arteries and play an important role in the maintenance of homeostasis of cardiovascuar system. Currently, the injury to the vascular endothelial cells is ascribed to an initiator of AS [1], can be observed in different stages during the development of AS and has been regarded a key step in the development of AS.

Myeloperoxidase (MPO) is a haematin protein and highly expressed in a majority of addicted granular leukocytes (such as neutrophils and monocytes) [2]. Under physiological conditions, MPO plays important roles in the natural defense and immune function and has a high expression in the atherosclerotic plaques [3, 4]. Increasing epidemiological studies [5-9] confirm that increased MPO expression is an independent risk factor of coronary AS and has predictive value for cardiovascular events [10-12]. Genetic studies [13, 14] also reveal that there is causal relationship between MPO and coronary diseases. In addition, studies also find that MPO is an independent predictor of endothelial dysfunction [15, 16]. To date, MPO has been indicated to involve in the occurrence and development of atherosclerotic plaque via causing oxidative modification of low-density lipoprotein (LDL) and functional defect of high-density lipoprotein (HDL), reducing the biological activity of nitric oxide (NO) and playing a role in the plaque instability. In addition, our previous study showed MPO at a certain concentration could induce the apoptosis of endothelial cells (ECV-304) which further cause damage to the endothelial cells promoting the occurrence and development of AS [17].

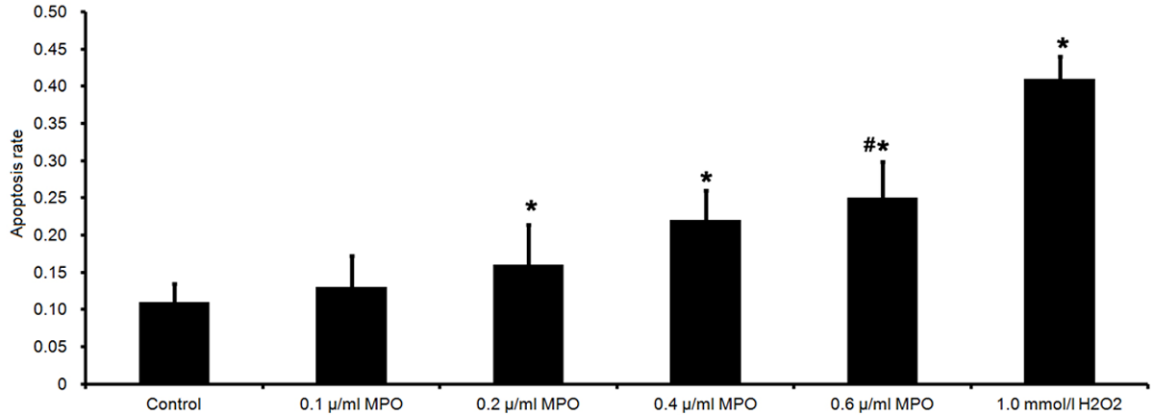

Figure 1. Apoptosis index of HUVEC-12 cells after treatment with MPO at different concentrations. Note: Data were expressed as mean ± SD. *$^{*}P<0.05$* vs negative control group; *$^{\#}P<0.05$* vs. 0.1 μ/ml MPO group and 0.2 μ/ml MPO group.

The present study aimed to investigate the molecular mechanisms underlying the MPO induced apoptosis of endothelial cells and to explore the key molecules involved in the apoptosis of endothelial cells. Our findings may provide targets for the protection of endothelium.

Materials and methods

Materials

MPO (Sigma), RPMI 1640 (Gibco), reverse transcription kit, protein ladder (Fermentas), rabbit anti-human caspase-3 antibody, rabbit anti-human Bax antibody (Cell Signaling Technology), thermal cycler for PCR (Applied Biosystems), Real-time PCR instrument, micropipettes (Eppendorf), electrophoresis instrument, and transferring instrument (Bio-RAD) were used in the present study.

Cell culture

Human umbilical vein endothelial cells (HUVEC-12) were purchased from the Institute of Clinical Oncology of Peking University (derived from ATCC in USA). HUVEC-12 cells were thawed and signal cell suspension was prepared with RPMI 1640 containing 10% fetal bovine serum (FBS). Then, these cells were seeded into a flask and maintained in an environment with 5% CO_2 at 37°C. The medium was refreshed once every 2-3 days. When the cell confluence reached about 80%, passaging was performed by digestion with 0.25% trypsin. The cell density was adjusted to 1×10^5 cells/ml, and cells were plated into 6-well plates. When the cell confluence reached about 80%, cells were maintained in RPMI 1640 containing 1% FBS for 24 h for synchronization. Then, different treatments were administered.

Grouping

Cells were divided into negative control group, positive control group and different MPO groups. In negative control group, cells were not treated; in different MPO group, cells were treated with MPO at different concentration (0.1 μ/ml, 0.2 μ/ml, 0.4 μ/ml and 0.6 μ/ml); in positive control group, cells were treated with 1.0 mmol/L hydrogen peroxide to induce cell apoptosis.

Detection of apoptotic cells by flow cytometry

Cells were harvested and washed in PBS thrice. Then, cells were suspended in 0.5 ml of 1x binding buffer. Following addition of 5 μl of annexin V-FITC and 5 μl of Propidium Iodide (PI), incubation was done at room temperature in dark for 5-15 min. Then, apoptotic cells were detected by flow cytometry and the apoptosis index was calculated.

Detection of mRNA expression of caspase-3 and Bax by reverse transcription PCR

Total RNA was extracted with one step extraction kit by using Trizol, and reverse transcription was performed according to the manufacturer's instructions. The mixture for reverse transcription included 1 μl of reverse transcriptase, 4 μl of buffer for reverse transcription, 2 μl of

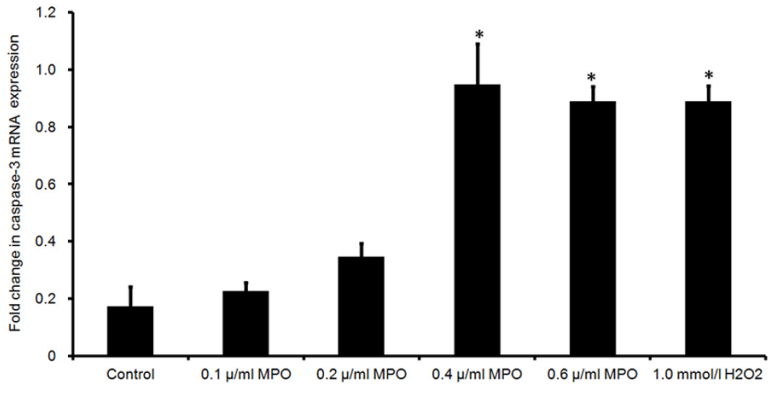

Figure 2. Caspase-3 mRNA expressions in different groups. Data were expressed as mean ± SD. *$P<0.05$ vs. negative control group.

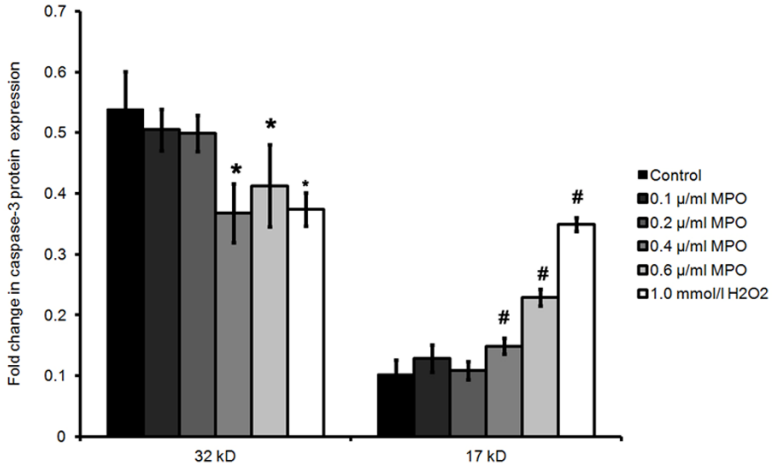

Figure 3. Protein expression of activated pre-caspase-3 (35 kD) and activated caspase-3 (17 kD) in different groups. Note: Data were expressed as mean ± SD. *$P<0.05$ vs. negative control group.

dNTP, 1 μl of Rnase inhibitor, 1 μl of Oligo (dT), 18 μl of primers, 1 μg of RNA and DEPC treated water. Reverse transcription was done at 65°C for 5 min, 42°C for 60 min and 70°C for 5 min. The PCR conditions were as follows: pre-denaturation at 95°C for 2 min, 31 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The primers were as follows: GAPDH: 5'-CAAGGTCATCCATGACAACTTTG-3' (forward), 5'-GTCCACCACCCTGTTGCTGTAG-3' (reverse), and the anticipated length was 496 bp; caspase-3: 5'-TTTTTCAGAGGGGATCGTTG-3' (forward), 5'-CGGCCTCCACTGGTATTTTA-3' (reverse), and the anticipated length was 151 bp; Bax: 5'-TGGCAGCTGACATGTTTTCTGAC-3' (forward), 5'-TCACCCAACCACCCTGGTCTT-3' (reverse), and the anticipated length was 195 bp. Primers were designed with Primer 5.0, and their specificity was examined in GeneBank.

Western blot assay

Cells were harvested and total protein was extracted with RIPA lysis buffer. After determination of protein concentration, western blot assay was performed according to instructions.

Results

Effects of MPO at different concentration on cell apoptosis

The apoptosis index in 0.1 μ/ml MPO group and 0.2 μ/ml MPO group was comparable to that in negative control group ($P>0.05$). However, when compared with negative control group, the apoptosis index increased markedly in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group ($P<0.05$). In addition, the apoptosis index increased gradually with the increase in MPO concentration, and significant difference was noted in the apoptosis index between 0.6 μ/ml MPO group and 0.1 μ/ml MPO group or 0.2 μ/ml MPO group ($P<0.05$), but there was no remarkable difference in remaining groups ($P>0.05$; **Figure 1**).

Effects of MPO on mRNA and protein expression of caspase-3 in endothelial cells

The capase-3 mRNA expression in 0.1 μ/ml MPO group and 0.2 μ/ml MPO group was comparable to that in negative control group ($P>0.05$). When compared with negative control group, the capase-3 mRNA expression increased markedly in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group ($P<0.05$; **Figure 2**).

The caspase-3 (activated pre-caspase-3 [32 kD] and activated caspase-3 [17 kD]) protein

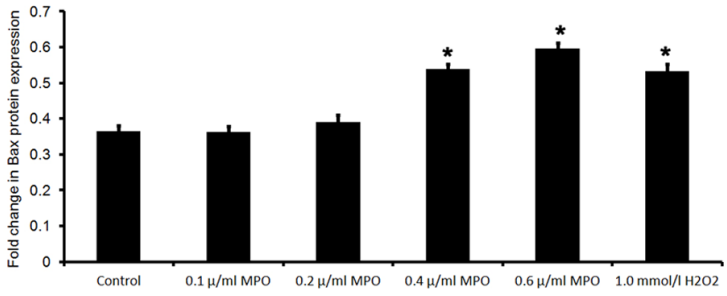

Figure 4. Bax mRNA expressions in different groups. Note: Data were expressed as mean ± SD. **P<0.05* vs. negative control group.

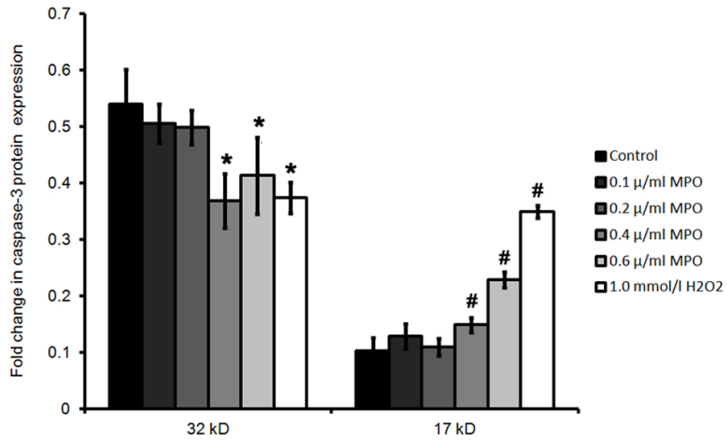

Figure 5. Bax protein expressions in different groups. Note: Data were expressed as mean ± SD. **P<0.05* vs. negative control group.

expression in 0.1 μ/ml MPO group and 0.2 μ/ml MPO group was comparable to that in negative control group (P>0.05). The protein expression of activated pre-caspase-3 reduced markedly and that of activated caspase-3 increased dramatically in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group when compared with negative control group (P<0.05; **Figure 3**).

Effects of MPO on mRNA and protein expression of Bax in endothelial cells

The Bax mRNA expression in 0.1 μ/ml MPO group and 0.2 μ/ml MPO group was comparable to that in negative control group (P>0.05). However, when compared with negative control group, the Bax mRNA expression increased dramatically in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group (P<0.05; **Figure 4**).

The Bax protein expression in 0.1 μ/ml MPO group and 0.2 μ/ml MPO group was comparable to that in negative control group (P>0.05). However, when compared with negative control group, the Bax protein expression increased markedly in 0.4 μ/ml MPO group, 0.6 μ/ml MPO group and positive control group (P<0.05; **Figure 5**).

Discussion

It has been confirmed that the injury to the endothelial cells is an initiator of AS [1] and can be observed in the whole stages of AS development. The integrity of endothelial cells is dependent on the dynamic balance between proliferation and apoptosis. When there is excessive apoptosis, following consequences may be present: 1) the reduction in endothelial cells may increase the endothelial permeability, which then promotes the migration and deposition of lipids and migration of monocytes and smooth muscle cells into the intima, further causing damage to the vessels and facilitating the formation of plaques. 2) The intact vascular endothelial cells may secret NO, thrombosis-modulating protein, heparan sulfate and tissue factor inhibitor to exert anticoagulation effect. When there is excessive apoptosis of endothelial cells, the anti-coagulants reduce, and the phosphorylated serine produced in cell apoptosis may promote the coagulation, resulting in a hypercoagulable state [18, 19]. 3) The pathophysiological change in endothelial cells secondary to apoptosis may activate platelets and white blood cells and increase the inflammation and immune response at the injured sites. In addition, the activated platelets may aggregate at the sites where the apoptosis of endothelial cells is active. Thus, cell apoptosis play crucial roles in the formation of AS and unstable plaque thrombosis.

Cell apoptosis is also known as programmed cell death following exogenous stimulations. In the present study, Annexin-V-FITC/PI double staining was performed to detect the apoptotic cells. Results showed the apoptosis index

increased markedly when the MPO concentration reached 0.2 μ/ml, and the apoptosis further increased with the elevation of MPO concentration. These findings were consistent with those published in our previous study [17].

In cells of vertebrates, apoptosis executes in two ways: death receptor pathway (also known as extrinsic apoptosis pathway) and mitochondrial pathway (also known as intrinsic apoptosis pathway). In the death receptor pathway, death ligand (such as Tumor Necrosis Factor) binds to corresponding receptor, resulting in trimerization of the receptor. Then, the cytoplasmic part of receptor binds to Fas-associated death domain (FADD) and pro-caspase-8. Pro-caspase-8 has a little bit activity of mature caspase-8. After aggregation, pro-caspase-8 undergoes cleavage to produce mature caspase-8 which further activates down-stream caspase-3. In the mitochondrial pathway, pro-apoptotic Bcl-2 family members (such as Bax) undergo self-oligomerization and enter the mitochondrial membrane, resulting in change in the permeability of the mitochondrial outer membrane, loss of transmembrane potential and release of cytochrome C (cytc) and other proteins. Cytc may form multimeric complexes with Apaf-1. The caspase-recruiting domain at the N terminal of Apaf-1 may recruit pre-caspase-9 which undergoes self-cleavage, resulting in caspase-9 activation and subsequent caspase cascade. The down-stream caspase-3 is activated and able to cleave the corresponding substrates, inducing apoptosis. There is crosslink between death receptor pathway and mitochondrial pathway. However, under a majority of conditions, this crosslink is limited, and two pathways exist independently [20, 21]. Thus, in two apoptosis signaling pathways, caspase family members are key elements for cell apoptosis and can regulate the change at cellular and biochemical levels. The activation and excessive expression of caspase family members may cause cell apoptosis [22]. Of 14 caspase family members, caspase-3 is an executive in the caspase cascade and the most important caspase member. Multiple apoptosis induced signals may converge at caspase-3, and the activation of caspase-3 is a marker of irreversible stage in apoptosis [23]. The pre-caspase-3 exists in cells in the form of dimer and may not induce apoptosis. Its molecular weight is 32 kD. A specific aspartic acid cleaving plasminogen may cleave pre-caspase-3 between the large subunit (containing active cysteine - histidine dimer) and small subunit (containing a domain determing the catalytic specificity), resulting in formation of mature and activated caspase-3 (molecular weight: 17 kD) [24]. The activated caspase-3 may induce the apoptosis. Bax plays an important role in the mitochondrial apoptosis pathway and its molecular weight is 20 kD.

Our results showed, after treatment with MPO at certain concentrations, the caspase-3 mRNA expression increased, the protein expression of pre-caspase-3 (32 kD) reduced and the protein expression of activated caspase-3 (17 kD) increased in the endothelial cells, which were dependent on the MPO concentrations. This suggests that MPO at certain concentrations may activate caspase-3 to induce the apoptosis of endothelial cells. In addition, after treatment with MPO at certain concentrations, the mRNA and protein expression of Bax increased. This suggests that Bax is also involved in the MPO induced apoptosis of HUVEC-12. On the basis of previous findings, Bax may exert pro-apoptotic effect on HUVEC-12 cells following MPO treatment.

Taken together, MPO is a key enzyme involved in the formation of AS and plaque instability and an independent predictor of endothelial dysfunction. Our findings confirmed that MPO at certain concentrations may induce the apoptosis of HUVEC-12 in a concentration dependent manner, which might be attributed to the activation of caspase-3 signaling pathway and increased Bax expression. In the present study, two key molecules in the apoptosis (caspase-3 and Bax) were investigated after MPO treatment. However, how MPO induced the activation of caspase-3 signaling pathway or the changes in molecules upstream and down-stream of caspase-3 signaling pathway are still unclear, and more studies are required.

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Disclosure of conflict of interest

None.

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