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Protective effects of a benzoxazine derivative against oxidized LDL-induced apoptosis and the increases of integrin β4, ROS, NF-κB and P53 in human umbilical vein endothelial cells

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ABSTRACT

To investigate whether 6-amino-2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine (ABO) inhibits oxidized low-density lipoprotein (oxLDL)-induced human umbilical vein endothelial cell (HUVEC) apoptosis, we treated HUVECs with oxLDL in the absence or presence of ABO. The results showed that ABO could act as an effective inhibitor of oxLDL-elicited HUVEC apoptosis by inhibiting the levels of integrin β4, reactive oxygen species (ROS), NF-κB and P53, and suppressing NF-κB nuclear translocation.

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At present, it has been well accepted that vascular endothelium is critically involved in the maintenance of vascular homeostasis in health. Injury or dysfunction of vascular endothelial cell (VEC) is considered to be the key step in initiation and development of atherosclerosis.[1] VEC apoptosis elicited by oxidized low-density lipoprotein (oxLDL) has been assumed as a major cause of endothelial dysfunction associated with proatherogenic conditions.[2] Therefore, finding new chemical inhibitors of oxLDL-induced VEC apoptosis may help us to understand the mechanism of atherosclerosis and develop new drugs for its therapy.

The benzodioxine system has been widely used as a substructure of several biologically interesting agents.[3] In our laboratory, to discover novel agents that can modulate VEC survival and apoptosis, we synthesized and screened a series of 2,3-dihydro-3-substituted-1,4-benzoxazine derivatives. Surprisingly, contrary to other benzoxazine derivatives, 6-amino-2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine (ABO) could elevate the viability of human umbilical vein endothelial cells (HUVECs) in the absence of FGF-2 and serum.[4] Therefore, we suppose that ABO may inhibit HUVEC apoptosis. To address this question, we investigated its effects on oxLDL-induced apoptosis and the possible mechanism of its action in HUVECs.

According to the documents on oxLDL roles in VEC apoptosis, in this study, we firstly observed the effects of ABO on HUVEC apoptosis induced by 45 μg/ml of oxLDL. The anti-apoptotic effect of ABO was determined by Hoechst 33258 staining. Comparing with control, the ratio of cell apoptosis did not change in HUVECs treated with ABO alone ($p > 0.05$). Incubation of HUVECs with oxLDL enhanced the ratio significantly, whereas ABO and the antioxidant agent *N*-acetyl-cysteine (NAC) (positive control) abolished the effects ($p < 0.01$) (Fig. 1B). The result also showed that treatment of ABO caused a significant and dose-dependent decrease in the apoptotic ratio ($p < 0.01$) (Fig. 1B). Then we selected 50 μM of ABO as the concentration representative in subsequent experiments.

When treated with oxLDL for 24 h, HUVECs gradually detached from the dish and apoptosis occurred. And after treated with oxLDL and ABO 50 μM simultaneously, the cells were partly repaired in morphology compared with control group (Fig. 2A). The results from MTT assay showed that ABO prevented the reduction of cell viability induced by oxLDL efficiently ($p < 0.01$) (Fig. 2B). Further-

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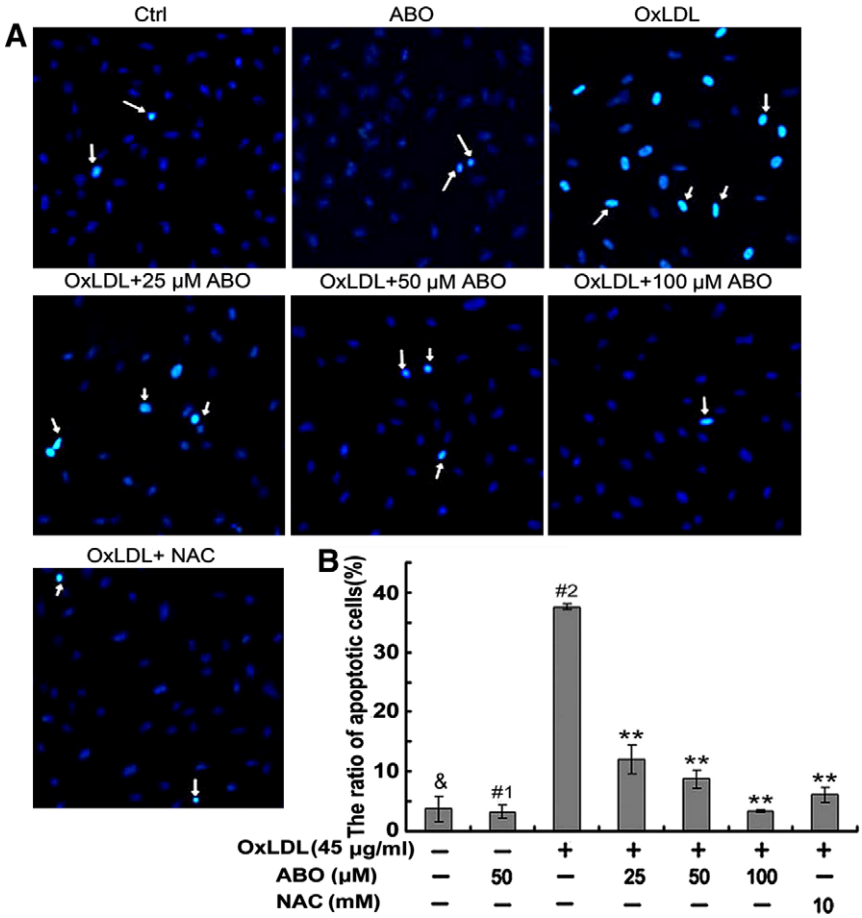

Figure 1. ABO inhibited oxLDL-induced HUVECs apoptosis in a dose-dependent manner. (A) Fluorescent micrographs of Hoechst 33258 staining (300×). The arrows indicate apoptotic cells. (B) Quantity of apoptotic cells (#1, $p > 0.05$ vs &; #2, $p < 0.01$ vs &; $^{**}p < 0.01$, $n = 3$).

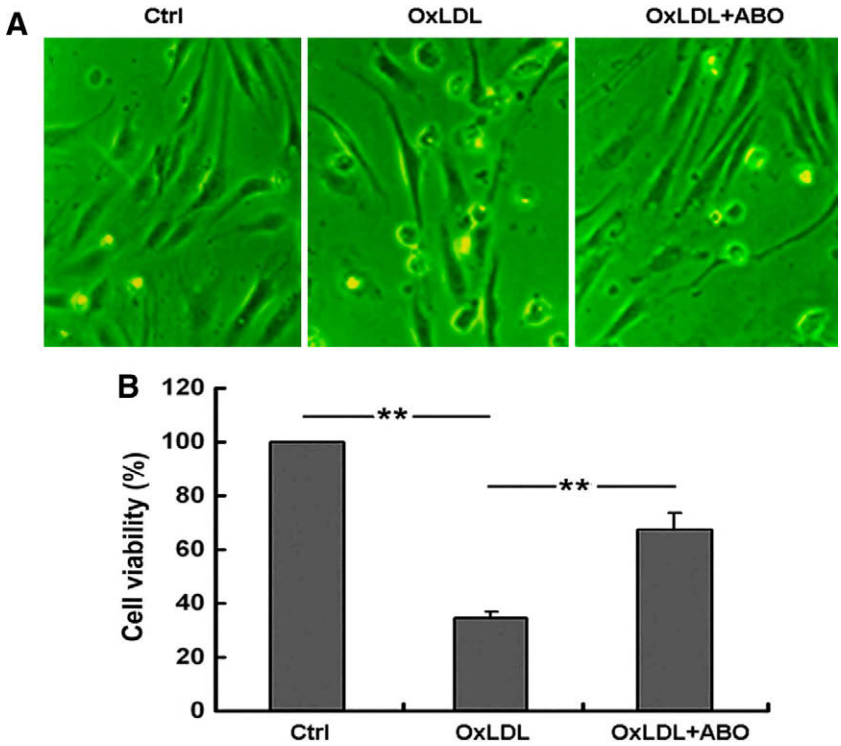

Figure 2. Effects of ABO 50 μM on cell morphology and viability. (A) Morphological micrographs of cells with various treatments (100×). (B) Cell viability measured by MTT assay ($^{**}p < 0.01$, $n = 3$).

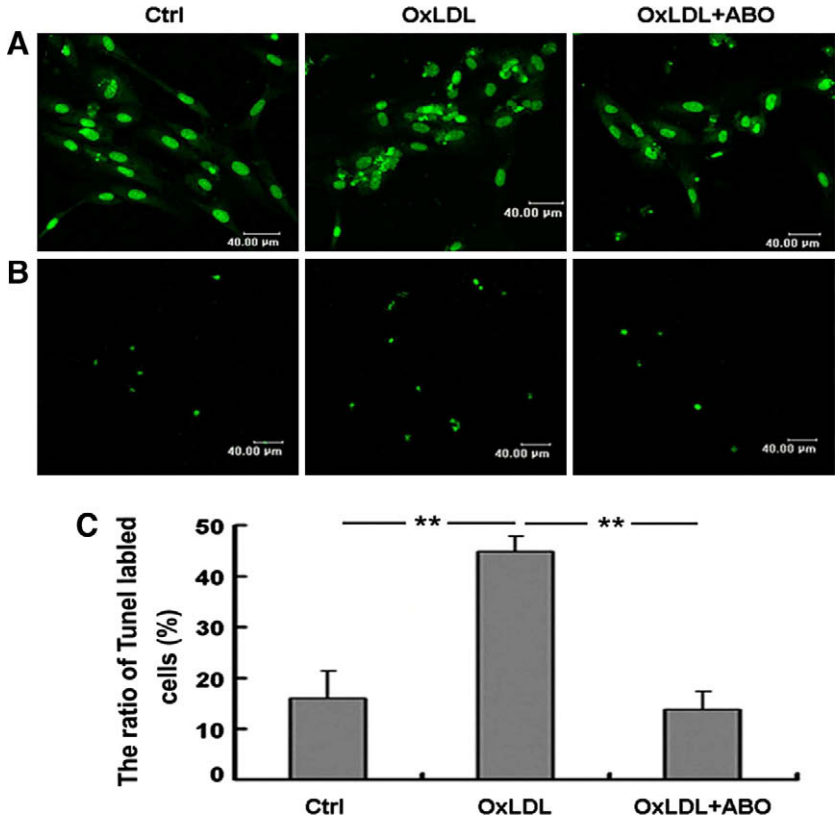

Figure 3. Effects of ABO 50 μM on nuclear fragmentation and apoptotic ratio of HUVECs. (A) Nuclear condensation and fragmentation detected by AO staining. (B) Quantification of apoptotic cells by TUNEL assay. (C) Quantity of apoptotic cells ($^{**}p < 0.01$, $n = 3$).

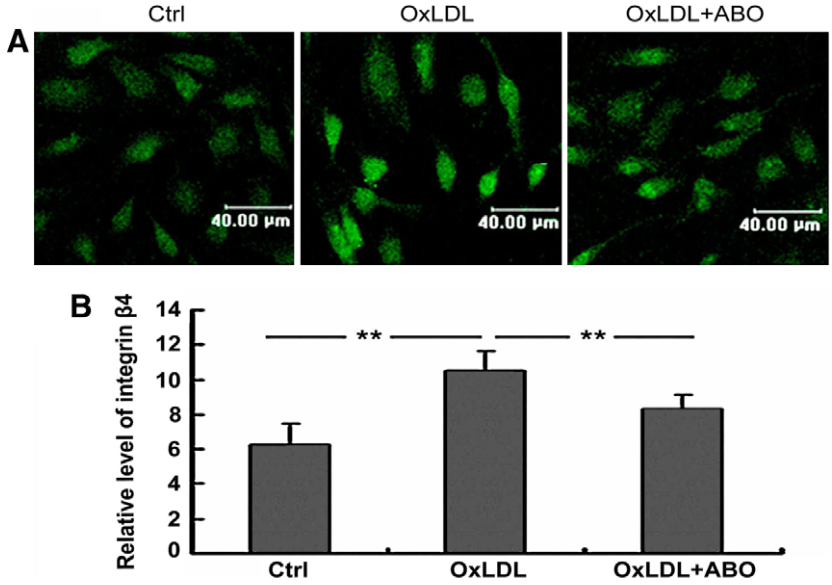

Figure 4. Effect of ABO 50 μM on the level of integrin β4. (A) Fluorescent micrographs show the relative level of integrin β4. (B) Quantity of integrin β4 in HUVECs ($^{**}p < 0.01$, $n = 3$).

more, the results from acridine orange (AO) staining and TUNEL assay showed that ABO obviously inhibited nuclear DNA condensation and fragmentation (Fig. 3A), as well as the percentage of TUNEL-positive (apoptotic) cells induced by oxLDL ($p < 0.01$) (Fig. 3B).

Integrin β4 has been implicated in VEC apoptosis in response to diverse stimuli.[5,6] Thus, to understand the mechanism underlying the anti-apoptotic effect of ABO on oxLDL-induced HUVEC apoptosis, we further explored the effects of ABO on integrin β4 level. The immunofluorescence results showed that ABO significantly inhibited the elevated level of integrin β4, which was elicited by oxLDL ($p < 0.01$) (Fig. 4B). Recent studies have indicated the implications of integrin β4 in oxLDL-triggered signalling transductions.[7] However, there is no report on the association between integrin β4 and oxLDL in HUVECs. Here, we provided the new evidence that the level of integrin β4 was increased in oxLDL-induced HUVEC apoptosis and ABO inhibited the apoptosis and the alteration of

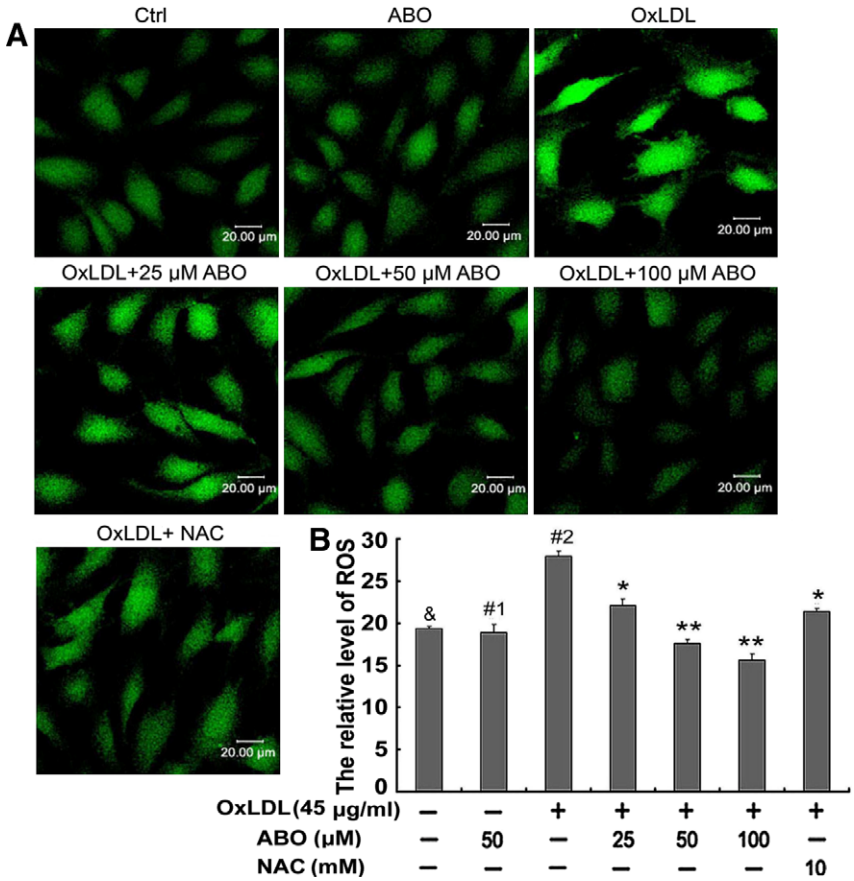

Figure 5. Effect of ABO on ROS level. (A) Fluorescent micrographs show the relative level of ROS level. (B) Quantity of ROS level (#1, $p > 0.05$ vs &; #2, $p < 0.01$ vs &; $^{*}p < 0.05$; $^{**}p < 0.01$, $n = 3$).

integrin β4 level. The data suggested that integrin β4 might play an important role in HUVEC apoptosis elicited by oxLDL.

OxLDL has been reported to elicit vascular cell apoptosis through increasing reactive oxygen species (ROS) level and the subsequent activation of NF-κB.[8,9] Once activation, NF-κB translocates from the cytosol to the nucleus of cells and binds to specific DNA sequences and initiates transcription, resulting in changes of gene expression, which is believed to be responsible for apoptosis.[9] Thus, we wondered whether ABO exerts its anti-apoptotic effect through modulating ROS and NF-κB. To address these questions, the levels of intercellular ROS and the level/distribution changes of NF-κB were detected. The results showed that the elevated ROS levels, which was elicited by oxLDL, were depressed significantly by ABO in a dose-dependent manner, and the anti-oxidant property of ABO was similar to NAC, one of ROS scavengers ($p < 0.01$) (Fig. 5A and B). Importantly, ABO obviously depressed the level of NF-κB induced by oxLDL and partly blocked NF-κB nuclear translocation ($p < 0.01$) (Fig. 6A, B and C).

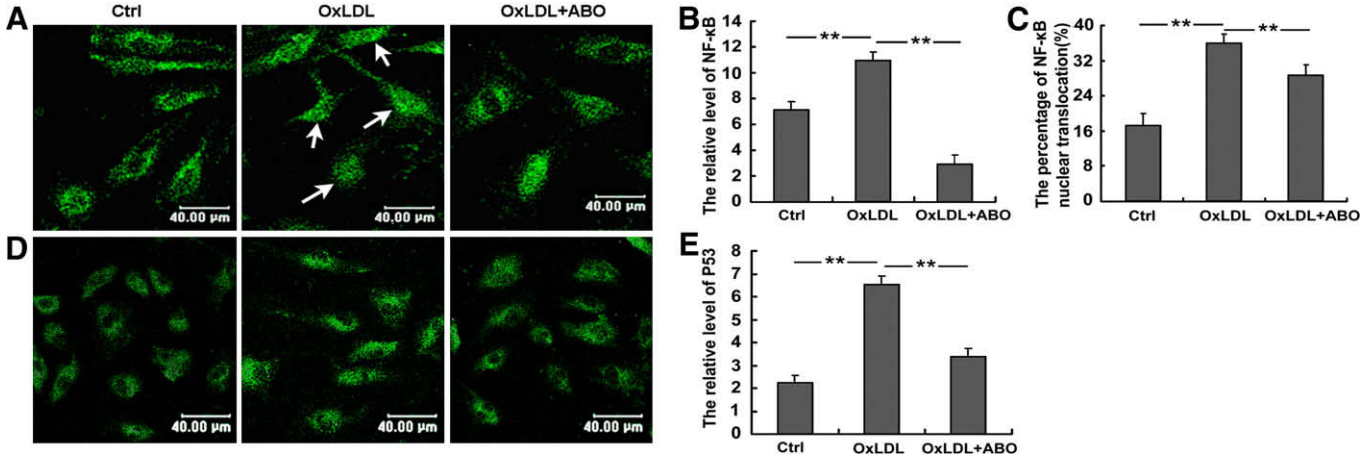

Figure 6. Effect of ABO 50 μM on NF-κB and p53: (A) and (D) fluorescent micrographs show the relative level and the location of NF-κB and p53 in VEC, respectively; (B) relative quantity of NF-κB level in HUVECs; (C) the percentage of NF-κB nuclear translocation; (E) relative quantity of P53. The arrowheads indicate the NF-κB nuclear translocation- positive cells ($^{**}p < 0.01$, $n = 3$).

NF-κB has been shown to be implicated in integrin β4 signaling in tumor cells.[10] However, most previous studies are focused on tumor cells and the HUVECs has not been well defined. Here, we reported that integrin β4 and NF-κB might be associated in oxLDL-elicited HUVEC apoptosis.

It's noteworthy that, recent evidence has demonstrated a key role of P53 tumor suppressor protein in oxLDL-triggered VEC apoptotic signal transduction pathways.[11]

Furthermore, our studies also showed that integrin β4 was associated with P53 in regulating HUVEC apoptosis.[5,6] Thus, in order to understand the mechanism of ABO action, we further explored the effects of ABO on P53 level. The results showed that ABO protected HUVECs against oxLDL-elicited apoptosis through depressing the level of P53 ($p < 0.01$) (Fig. 6D and E).

Our previous study demonstrated that knockdown of integrin β4 depressed the levels of P53 and ROS in senescent HUVECs.[12] Recent evidence supported the notion that NF-κB activation was accompanied by elevated p53 level and NF-κB might play a critical role in regulating p53.[13] In VECs, it has been reported that functional links between p53 and endothelial apoptosis induced by oxidative stress is through activating NF-κB.[14] Based on these findings, in this study, we speculate that ABO may inhibit oxLDL-induced HUVEC apoptosis through an integrin β4/ROS/NF-κB/p53 pathway. We will confirm this hypothesis in next studies.

In summary, this study provided the new evidence as follows. First, the level of integrin β4 was increased in oxLDL-triggered HUVEC apoptosis and ABO inhibited the apoptosis and the elevation of integrin β4. Second, ABO inhibited the oxLDL-induced increases of ROS, NF-κB levels, the subsequent NF-κB activation, and P53 level. Taken together, the data suggested that ABO could act as an effective inhibitor of oxLDL-induced apoptosis through depressing the elevated levels of integrin β4, ROS, NF-κB and P53, and probably inhibiting NF-κB activation in HUVECs.

Our findings provided new clues that ABO could be served as a useful tool for illustrating the molecular mechanism underlying endothelial dysfunction associated with oxLDL and ABO may have potential applications in prevention of atherosclerotic vascular diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.070.

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