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A novel butyrolactone derivative inhibited apoptosis and depressed integrin β4 expression in vascular endothelial cells

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Abstract—To understand the effects of a novel butyrolactone derivative, 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3*H*)-one (3BDO), on the apoptosis of vascular endothelial cells (VECs), we exposed 3BDO (20–60 μg/ml) to VECs deprived of serum and FGF-2 for 24 and 48 h, respectively. The results showed that 3BDO (20–60 μg/ml) increased VEC viability and inhibited VEC apoptosis induced by deprivation of serum and FGF-2 in a very weak dose-dependent manner. During this process, integrin β4 expression was depressed, but the level of reactive oxygen species (ROS) was not changed. The data suggested that 3BDO (20–60 μg/ml) could inhibit VEC apoptosis and suppress integrin β4 expression, but it could not depress the ROS level induced by deprivation of serum and FGF-2.

Vascular endothelial cells (VECs) form the inner lining of all blood vessels and function to maintain vascular tone and anticoagulant properties of vessels. Vascular endothelial cells (VECs) have been implicated in angiogenesis, which is critical for normal physiological processes, such as embryonic development, wound repair, and also promoting tumor growth.[1–3] VEC apoptosis or dysfunction could be an important mechanism of vascular injury, resulting in vascular leak, inflammation, and coagulation.[4] Therefore, the regulation of apoptosis of VECs is of great importance in various vascular diseases.

Butyrolactone derivatives have attracted much attention over the years, since the butyrolactone ring is an important functional structure in a wide range of natural products.[5,6] Butyrolactone-I, originally isolated from the cultured medium of Aspergillus species F-25799,[7] is a competitive inhibitor of ATP for binding and a potent inhibitor of cell cycle progression.[8] Recently, two synthetic α-methylene-γ-butyrolactone derivatives, CYL-19s and CYL-26z, were shown to inhibit ICAM-1 gene expression, monocyte adhesion, and cancer cell invasion by targeting IKK complex in human A549 alveolar epithelial cells and the adhesion of U937 cells to these cells.[9] It was apparent that butyrolactone affects cell growth in various cells. However, those researches were mostly carried out on cancer cells, the effects of butyrolactone and its derivatives on VEC apoptosis are still not well known.

Recently, chemical genetics, the use of small, cell-permeable molecules as probes of biological function, offers a significant value in diverse areas of biology.[10] In light of chemical genetics, a series of butyrolactone derivatives

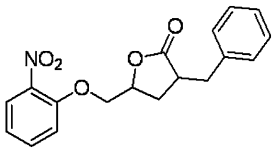

Figure 1. The chemical structure of 3BDO.

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were synthesized and screened in our laboratory to discover novel agents affecting cell growth and apoptosis. 3-Benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO) (Fig. 1) was found to inhibit the growth of A549 lung cancer cells.[11] But its effects on VEC survival and apoptosis are not known. To address these questions, we investigated the effects of 3BDO on VEC viability and apoptosis in this study. When deprived of serum and FGF-2, the cells gradually detached from the dish and underwent apoptosis (Fig. 2B and E).[12] After the treatment with 3BDO for 24 or 48 h, the morphological changes associated with apoptosis were impaired compared with the control (Fig. 2C and F). MTT assay showed that 3BDO prevented the reduction of cell viability induced by deprivation of serum and FGF-2 in a very weak dose-dependent manner (Fig. 2G).

In order to confirm the anti-apoptotic effect of 3BDO, its effects on nuclear DNA condensation and fragmentation in VECs were analyzed by acridine orange (AO) staining. The results showed that nuclear DNA condensation and fragmentation were inhibited obviously when the cells were exposed to 3BDO 40 μg/ml for 24 h (Fig. 3). The proportion of apoptotic cells was quantified by TUNEL

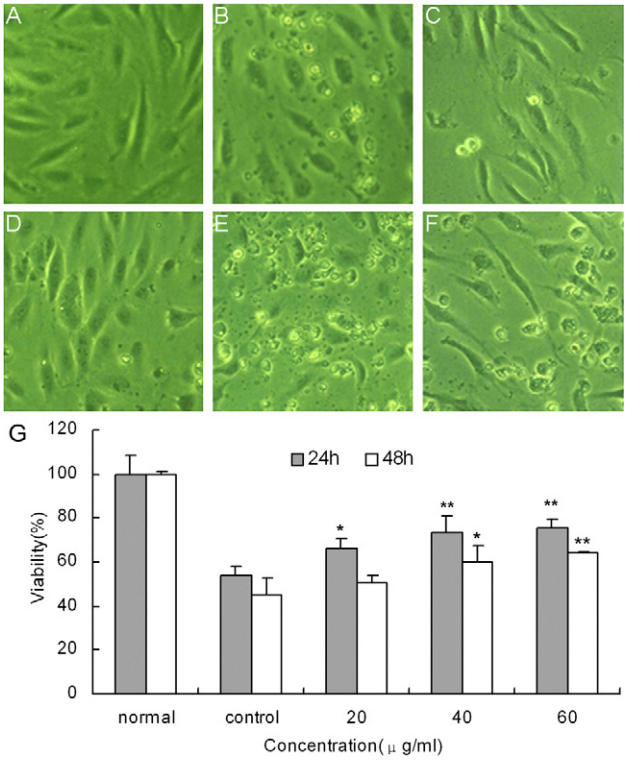

Figure 2. Effects of 3BDO on cell morphology and viability of VECs. (A) and (D), cells cultured in M199 medium with serum and FGF-2 for 24 or 48 h, respectively. (B) and (E), cells cultured in basal M199 for 24 or 48 h, respectively. (C) and (F), cells treated with 3BDO 40 μg/ml for 24 h or 48 h, respectively. (G) the reduction of cell viability induced by deprivation of serum and FGF-2 was inhibited by 3BDO in a very weak dose-dependent manner. Normal, cells cultured in M199 medium with serum and FGF-2. Control, cells cultured in basal M199 medium (without serum and FGF-2). 20, 40, 60, cells treated with 3BDO 20, 40, 60 μg/ml, respectively. (*$P < 0.05$, **$P < 0.01$ vs control group, $n = 3$).

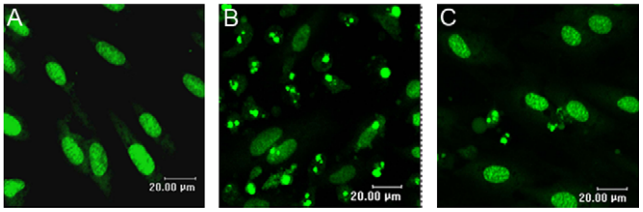

Figure 3. The effect of 3BDO on nuclear DNA fragmentation. (A) Nuclei of normal cells cultured in M199 medium with serum and FGF-2. (B) Nuclei of control cells deprived of serum and FGF-2 for 24 h. (C) Nuclei of cells treated with 3BDO 40 μg/ml for 24 h.

assay. When the cells were deprived of serum and FGF-2 for 24 h, the proportion of apoptotic cells was 30.89 ± 9.29%, which was much higher than that in the normal group ($P < 0.01$) (Fig. 4A, B, and D). After VECs were treated with 3BDO 40 μg/ml for 24 h, the proportion of apoptotic cells was reduced to 9.89 ± 5.66% ($P < 0.05$) (Fig. 4C and D). The result showed that 3BDO could inhibit VEC apoptosis induced by deprivation of serum and FGF-2.

Integrin β4 is found in hemidesmosomes, providing firm mechanical links between the basal lamina and the intermediate filament cytoskeleton system.[13,14] It is a key membrane protein in cellular signal transduction and involved in apoptotic cell death of VECs.[15,16] Thus, in order to understand the mechanism of 3BDO action, we further explored the effects of 3BDO on integrin β4 expression. The immunofluorescence results showed that the relative level of this integrin in the cells deprived of serum and FGF-2 was much higher than that in the normal cells ($P < 0.05$) (Fig. 5A, B, and D). But in the 3BDO treated cells, the relative level of integrin β4 was depressed significantly ($P < 0.05$) (Fig. 5C and D). This result showed that the increase of integrin β4 expression induced by deprivation of serum and FGF-2 could be

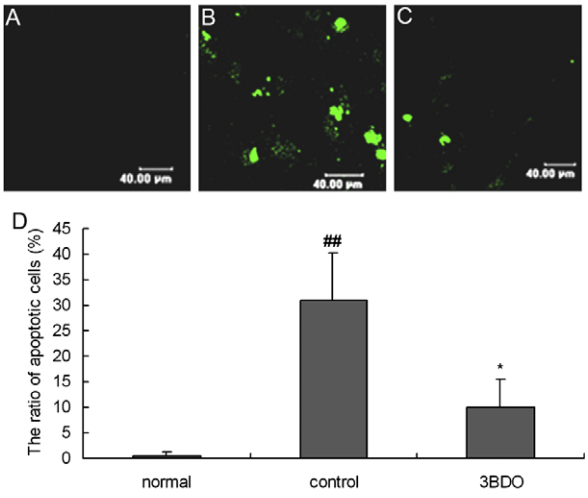

Figure 4. Quantification of apoptotic cells by TUNEL assay. Fluorescent micrographs show the TUNEL staining of VECs. (A) Cells cultured in M199 medium with serum and FGF-2 for 24 h. (B) Cells cultured in basal M199 medium for 24 h. (C) Cells treated with 3BDO 40 μg/ml for 24 h. (D) The quantity of apoptotic cells. ($^{\#\#}P < 0.01$ vs normal group, *$P < 0.05$ vs control group, $n = 3$).

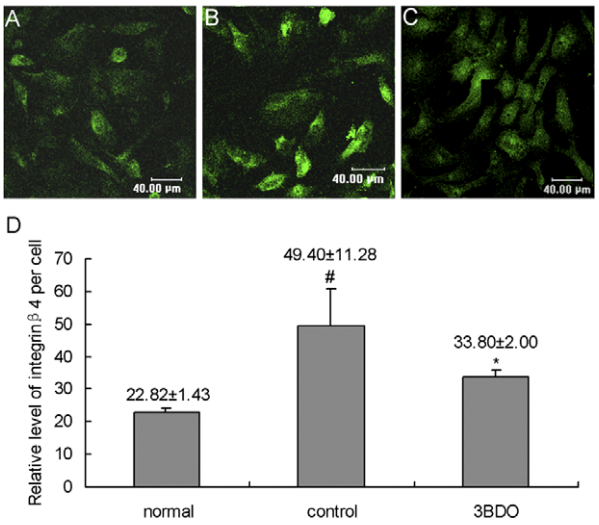

Figure 5. The effect of 3BDO on the expression of integrin β4 in VECs. (A)–(C) Fluorescent micrographs show the relative intensity of integrin β4. (A) cells cultured in M199 medium with serum and FGF-2 for 24 h. (B) Cells deprived of serum and FGF-2 for 24 h. (C) Cells treated with 3BDO 40 μg/ml for 24 h. (D) The relative quantity of integrin β4 expression in the three groups mentioned above. ($^{\#}P < 0.05$ vs normal group, $^{*}P < 0.05$ vs control group, $n = 3$).

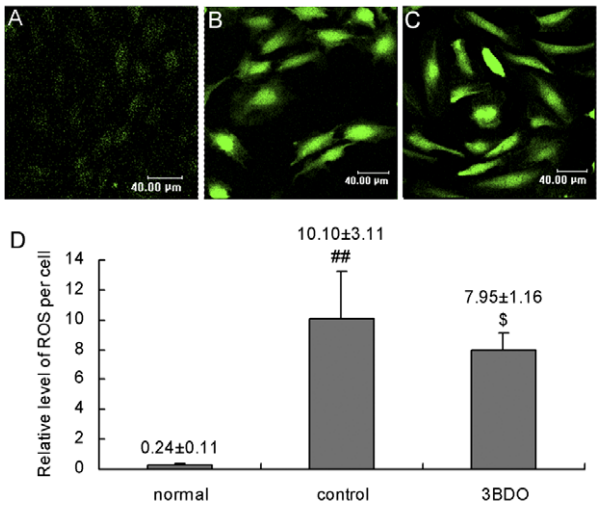

Figure 6. Effects of 3BDO on the level of ROS in VECs. (A)–(C) Fluorescent micrographs show the relative intensity of ROS. (A) Cells cultured in M199 medium with serum and FGF-2 for 24 h. (B) Cells deprived of serum and FGF-2. (C) Cells treated with 3BDO 40 μg/ml. (D) The relative quantity of ROS level in VECs. ($^{\#\#}P < 0.01$ vs normal group, $^{\$}P > 0.05$ vs control group, $n = 3$).

inhibited by 3BDO. This finding provided new evidence for our notion that integrin β4 had important roles in VEC apoptosis.

It was demonstrated that reactive oxygen species (ROS) play important roles in vascular endothelial cell activation and dysfunction as well as in the normal function of endothelial cells.[17,18] A large body of evidence indicates that ROS may also be involved in apoptosis or in a special form of programmed cell death associated with cell detachment from the extracellular matrix.[19,20] Our previous reports showed that, in the apoptosis induced by deprivation of serum and FGF-2, intracellular ROS were elevated.[21] Thus we wondered whether 3BDO inhibited VEC apoptosis by depressing ROS level. To address this question, a fluorescent probe, 2′,7′-dichlorodihydrofluorescein (DCHF), which could be oxidized into DCF by the intracellular ROS while entering into the cell, was used to detect the accumulation of intercellular ROS. In normal group, the relative fluorescent intensity of DCF was very low (Fig. 6A and D). When the serum and FGF-2 were deprived, the relative fluorescent intensity of DCF increased greatly in VECs ($P < 0.01$) (Fig. 6B and D). After the treatment with 3BDO 40 μg/ml for 24 h, there was no obvious difference in ROS level between the treatment group and control group ($P > 0.05$) (Fig. 6C and D). These results showed that 3BDO could not depress the intracellular ROS accumulation induced by deprivation of serum and FGF-2 although it could inhibit the apoptosis. The data suggested that, though ROS play important roles in VEC apoptosis, there might be ROS-independent pathways in VEC apoptosis.

In summary, we found that 3BDO 20–60 μg/ml inhibited the apoptosis induced by deprivation of serum and FGF-2. Our results showed that 3BDO remarkably suppressed the expression of integrin β4, but had no effect on ROS level in the cells deprived of the growth factors. The data provided new evidence for understanding the mechanism of apoptosis in VECs. Cell-permeable 3BDO with anti-apoptotic effects in VECs offered us an exciting tool to study the mechanism of apoptosis. Taken together, the data provided new evidence for the pivotal role of integrin β4 in apoptosis, and that there might be ROS-independent pathways in VEC apoptosis.

Acknowledgments

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