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A Novel Butyrolactone Derivative Inhibited Smooth Muscle Cell Migration and Proliferation and Maintained Endothelial Cell Functions Through Selectively Affecting Na, K-ATPase Activity and Mitochondria Membrane Potential During In Vitro Angiogenesis

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Abstract We have found that 3-benzyl-5-((2-nitrophenoxy) methyl)–dihydrofuran -2(3H)-one (3BDO), could effectively suppress human umbilical vascular endothelial cell (HUVEC) apoptosis induced by deprivation of fibroblast growth factor-2 and serum. Here, our purpose was to investigate whether 3BDO could modulate angiogenesis and its possible acting mechanism. The effect of 3BDO on angiogenesis was investigated by capillary-like tubule formation and rat aortic ring assay. Proliferation and migration of cells were detected by counting living cell number and scraping cell monolayer, respectively. Na, K-ATPase activity was measured spectrophotometrically. Mitochondrial membrane potential was analyzed using tetramethylrhodamine methylester fluorescence by confocal microscopy. Our results showed that 3BDO inhibited migration and proliferation of vascular smooth muscle cells (VSMCs), but maintained migration and tubule formation of HUVECs. In HUVECs, 3BDO inhibited Na, K-ATPase activity, but had no effect on mitochondria membrane potential. In VSMCs, it did not affect Na, K-ATPase activity, but depressed mitochondria membrane potential obviously. The data showed that 3BDO had selective effects on HUVECs and VSMCs, it might perform its role through the selective effects on the activity of Na, K-ATPase and the mitochondria membrane potential in HUVECs and VSMCs. J. Cell. Biochem. 104: 2123–2130, 2008.

Key words: angiogenesis; vascular endothelial cell; vascular smooth muscle cell; Na, K-ATPase; mitochondrial membrane potential

Angiogenesis is strongly dependent on the suppression of vascular endothelial cell (VEC) apoptosis. Therefore, the events that induce survival or apoptosis of VECs affect angiogenesis. In our previous study, it was found that a novel butyrolactone derivative, 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO), could effectively suppress human umbilical vascular endothelial cell (HUVEC) apoptosis induced by deprivation of fibroblast growth factor-2 (FGF-2) and serum [Wang et al., 2007]. The finding led us to investigate whether 3BDO could modulate angiogenesis. To answer this question, we first checked the effect of 3BDO on the angiogenesis of rat aortic rings. The results showed that 3BDO may have a distinct effect on vascular smooth muscle cells (VSMCs) and VECs. These interesting findings encourage us to investigate the effects and the possible

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mechanism of 3BDO acting in VECs and VSMCs.

Na, K-ATPase actively transports Na out and K into the myocyte. A most recent report showed that inhibiting the activity of Na, K-ATPase by ouabain results in activation of the proliferation and survival pathways involving PI3K, Akt activation, stimulation of eNOS (Endothelial Nitric Oxide Synthase), and production of nitric oxide (NO) in human umbilical cord endothelial cells (HUAECs) [Eva et al., 2006]. Not only in vascular endothelial cells, but in VSMCs, inhibition of Na, K-ATPase blocks the development of apoptosis, and also promotes activation of a signaling pathway that blocks apoptosis [Orlov et al., 1999]. Moreover, it has been shown that the apoptosis-associated Na, K-ATPase inactivation is related to mitochondria membrane potential changes in ATP- and reactive-oxygen-species-dependent manners in neural cells [Wang et al., 2003]. But in vascular cells, the association between Na, K-ATPase and mitochondria membrane potential in angiogenesis is not well known. Based on these reports, we supposed that 3BDO might perform its selective effects on VECs and VSMCs in angiogenesis through affecting Na, K-ATPase activity and/or mitochondria membrane potential. To test this hypothesis, we examined the effects of 3BDO on Na, K-ATPase activity and mitochondria membrane potential in VECs and VSMCs, respectively.

Here, we report that the novel butyrolactone derivative 3BDO inhibited the migration and proliferation of VSMCs, but maintained migration and tubule formation of HUVECs. In HUVECs, 3BDO inhibited the activity of Na, K-ATPase, but had no effect on the mitochondria membrane potential. In VSMCs, it did not affect Na, K-ATPase activity, but depressed the mitochondria membrane potential obviously.

MATERIALS AND METHODS

Reagents

MCDB131 medium was purchased from Sigma Co. Fetal bovine serum (FBS) and M199 medium was obtained from Hycolon Lab, Inc. FGF-2 was purchased form EssexBio Group, China. Matrigel was purchased from BD Biosciences Co. Na, K-ATPase detection kit was obtained from Nanjing Jiancheng Biotechnology Institute, China. Tetramethylrhodamine methylester (TMRM) was purchased from Promega Co. 3BDO (3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one) was synthesized as described by Sha et al. [2006]. 3BDO was dissolved in dimethyl sulfoxide (DMSO) and used at the final concentration of DMSO below 0.01% in culture medium (v/v) (DMSO at these final concentrations did not affect the viability of the cells). In this study, we treated cells with 120 μM of 3BDO. All other reagents were ultrapure grade.

Rat Aortic Ring Assay

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Rat aortic rings in the presence or deprived of VECs were cultured as described previously in MCDB131 medium supplemented with 10% (v/v) FBS and 2 ng/ml of FGF-2 [Nicosia and Ottinetti, 1990]. For the rat aortic ring assay, the rat aortic rings were cultured and treated with 120 μM of 3BDO at day 0 or day 6 after the start of culture. The rings cultured in the medium without 3BDO were as the control. Curves of microvascular growth were observed under a phase contrast microscope at day 7.

Culture of VSMCs and HUVECs

The investigation conforms to the principles outlined in the Declaration of Helsinki. VSMCs and HUVECs origin from the human umbilical vein were cultured as described previously [Jaffe et al., 1973; Leik et al., 2004]. Cells were grown in M199 medium with 10% FBS and 2 ng/ml of FGF-2. The 5–8 population doubling level (PDL) VSMCs and10–20 PDL HUVECs were used for all experiments.

Cell Migration Analysis

Cells were seeded on 24-well plates at a density of 1.5×10^5 cells/well. At postconfluent state, wounds of 1-mm width were created by scraping cell monolayers with a sterile pipette tip. Cell migration was documented by photos taken immediately after scraping, as well as 12 and 18 or 24 h later at a 100× magnification. Cell migration was quantified by measuring the distance between the wound edges before and after injury using the AnalySIS software [Vasvari et al., 2007]. Data are means of 3 independent experiments with three replica platings each.

Capillary-Like Tube Formation Assay

The formation of capillary-like structures by HUVECs on matrigel was studied as previously described [Nagata et al., 2003]. Twenty-four-well culture plates were coated with matrigel according to the manufacturer's instructions. HUVECs were seeded 5×10^4 cells/well in the fresh assay medium deprived of FGF-2, followed by 120 μM of 3BDO treatment 48 h after the start of culture. Tubule formation images were captured at a magnification of 100× with a digital microscope camera system (Olympus, Tokyo, Japan).

Nuclear Fragmentation Assay

Nuclear fragmentation was observed under a fluorescence microscope as described by Dobrucki and Darzynkiewicz [2001]. Briefly, after the cells were cultured in fresh medium with or without 3BDO for 24 h, they were stained with 5 μg/ml of acridine orange (AO) at room temperature. Then cells were observed under an Olympus fluorescence microscope.

Cell Proliferation Assay

Cells treated with or without 3BDO for 24 h were trypsinized (0.1% (w/v) trypsin in PBS) after washed twice with PBS. Then, the reaction of enzyme was ended in M199 medium with 10% serum. Cells detached from the dishes were washed away before the treatment with trypsin. The cells which remained attached to dishes after washing were regarded as living cells [Araki et al., 1990]. Trypsinized cells were counted with a leukocytometer [Zhao et al., 2003].

Na, K-ATPase Assay

After treating the cells with or without 3BDO for 24 h, we prepared the enzyme as described before [Zhao et al., 2005]. Protein concentration of each enzyme sample was determined by the Bradford protein assay [Bradford, 1976]. The enzyme samples obtained from all treatments in an experiment were added to tubes contained Na,K-ATPase assay buffer. The tubes were incubated for 10 min at 37°C after the reaction was started by the addition of ATP, the tube without ATP was as a control. Then the reaction was terminated by addition of Stop solution. The tubes were centrifuged at 3,000*g* for 10 min at 4°C and the supernatants were used to determine the content of Pi after addition of the Color Reagent. The tubes in which distilled water or Pi standard solution instead of the supernatants were as the blank control and the standard control, respectively. The optical density was measured at 636 nm (wave length). The enzyme activity was expressed as U per mg of protein (U/mgprot; 1U = 1 μmol Pi/mgprot/h). The experiment was repeated four times independently.

Mitochondrial Membrane Potential Measurement

Tetramethylrhodamine methylester (TMRM) was used as an indicator of mitochondria membrane potential as well as to visualize mitochondria as described [Falchi et al., 2005]. Cells treated with or without 3BDO for 24 h were incubated with 200 nM TMRM for 30 min at 37°C in humidified incubator. Then the samples were evaluated by laser scanning confocal microscope (Leica, Germany). We randomly selected the region of interest, and then zoomed in the same frames. The value of relative fluorescent intensity per cell was quantified by the software of Leica and the photographs were representatives in the scan rooms.

Statistical Analysis

Data were expressed as mean ± SE and accompanied by the number of experiments performed independently, and analyzed by *t*-test. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

3BDO Had Selective Effects on VSMCs and VECs in Angiogenesis

To investigate whether 3BDO could modulate angiogenesis, we first checked the effect of 3BDO on angiogenesis of rat aortic rings. At day 7, many microvessels grew from the rat aortic rings, so we added 3BDO at day 0 or day 6 after the start of culture to study the effects of 3BDO on microvessel growth and the newly formed microvessels. The results showed that after the rat aortic rings in the presence of VECs were cultured for 7 days, more microvessels grew from the rings (Fig. 1A), while in the rings treated with 3BDO immediately after the start of culture, there were only several cells migrating from the rings at day 7 (Fig. 1B). Furthermore, in the rings treated with 3BDO at day 6 after the start of culture, newly formed microvessels were broken at day 7, some cells became

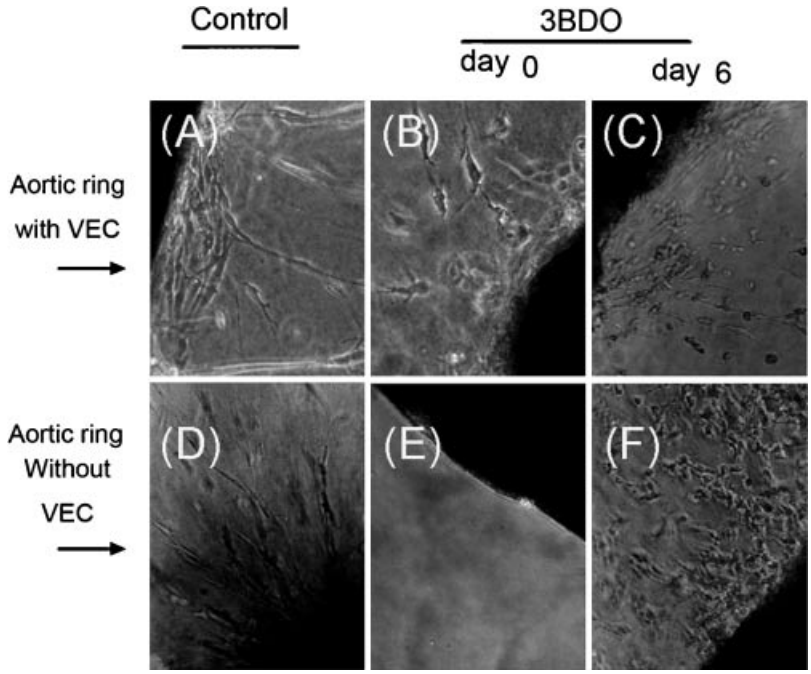

Fig. 1. Effects of 3BDO on angiogenesis of artery rings. Rat aortic rings with (**A**–**C**) or without VECs (**D**–**F**) were cultured for 7 days. Curves of microvascular growth in rat aortic rings treated with or without 3BDO were observed under a phase contrast microscope at day 7. A,D: Control, the rings cultured in the medium without 3BDO. 3BDO, the rings treated with 120 μM of 3BDO added at day 0 (B,E) or day 6 (C,F) after the start of culture. Magnification 200×.

round and dispersed, but some cells showed spreading shapes (Fig. 1C). The data suggested that 3BDO might have distinct effects on VSMCs and VECs in angiogenesis.

To prove this hypothesis, we investigated the effect of 3BDO on the angiogenesis of rat aortic rings deprived of VECs. The results showed that after the rings deprived of VECs were cultured for 7 days, many cells migrated from the rings (Fig. 1D).When the rings were treated with 3BDO immediately after the start of culture, no cells migrated from the rings at day 7 (Fig. 1E). When they were treated with 3BDO at day 6 after the start of culture, the cells became round at day 7 (Fig. 1F). The results showed that 3BDO inhibited the migration of VSMC in angiogenesis.

Effects of 3BDO on Migration and Capillary-Like Structure Formation of HUVECs on Matrigel

To demonstrate the effect of 3BDO on VECs, we investigated the migration and angiogenic property of HUVECs treated with 3BDO. As shown in Figure 2A, 3BDO maintained the ability of migration in HUVECs ($P < 0.01$). The results in Matrigel assay showed that HUVECs deprived of FGF-2 showed wedge-like morphology, however, after exposed to 3BDO for 48 h, HUVECs formed capillary-like structures on matrigel (Fig. 2C).

Effects of 3BDO on Proliferation and Migration of VSMCs

In order to confirm the effect of 3BDO on VSMCs, we examined the living cell number and migration of VSMC cultures treated with 3BDO. As shown in Figure 3, the nuclei remained complete as that in control group at 24 h after the treatment of 3BDO, the number of cells treated with 3BDO was 71.3% compared with control group, suggesting that 3BDO inhibited the growth of VSMCs ($P < 0.01$). On the other hand, the inhibitory effect of 3BDO on VSMC migration was observed at 24 h (Fig. 3D,E, $P < 0.05$).

3BDO Selectively Affected Na, K-ATPase Activity in HUVECs and VSMCs

To understand the possible mechanism of 3BDO acting, we examined the changes of Na, K-ATPase activity in the cells treated with this small molecule. As shown in Figure 4, in HUVECs treated with 3BDO, the enzyme activity decreased obviously compared with

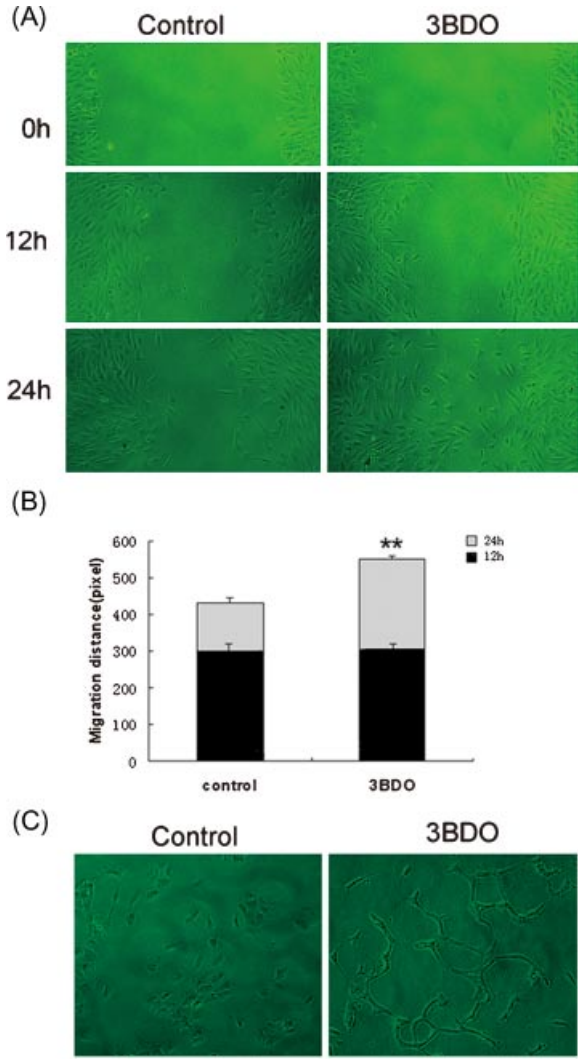

Fig. 2. Effects of 3BDO on migration and tubule formation of HUVECs. Control, cells cultured in the medium without 3BDO. 3BDO, cells treated with 120 μM of 3BDO. **A**: Photomicrographs of HUVECs migrating at time points 0, 12, and 24 h in the presence and absence of 3BDO. **B**: Cell migration of HUVECs is promoted after addition of 3BDO at 24 h as measured by the migration distance on 100× photomicrographs. $^{**}P < 0.01$ versus control, n = 3. **C**: Photomicrographs of tubule formation of HUVECs on Matrigel in the absence or presence of 3BDO at 48 h. Treatment with 3BDO promoted tubule formation of HUVECs deprived of FGF-2. Magnification 100×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that in the control HUVECs ($P < 0.05$), but in VSMCs treated with 3BDO, the activity of Na, K-ATPase was not changed.

3BDO Had a Selective Effect on Mitochondrial Membrane Potential in HUVECs and VSMCs

To understand the effects of 3BDO on mitochondrial function, we examined the changes of MMP. As shown in Figure 5, in HUVECs treated with 3BDO, the relative fluorescent intensity of TMRM was not changed compared with control group, but in VSMCs treated with 3BDO, the fluorescent intensity decreased obviously ($P < 0.05$). These data suggested 3BDO had a selective effect on mitochondrial function in HUVECs and VSMCs.

DISCUSSION

VEC apoptosis has been shown to be very critical for the destabilization of atherosclerotic plaque. Therefore, suppression VEC apoptosis and maintaining the function of endothelium possess therapeutic potential in atherosclerosis. On the other hand, the pathogenesis of atherosclerosis involves the proliferation and migration of medial VSMCs into the vessel intima. So, suppression VSMC proliferation and migration is another therapeutic strategy for atherosclerosis. In this study, our results showed that 3BDO could block the migration and growth of VSMCs, and maintain the migration and tubule formation functions of HUVECs. Our previous report showed that 3BDO suppressed HUVEC apoptosis induced by withdrawal of FGF-2 and serum [Wang et al., 2007]. These data suggested that 3BDO may be a promising compound in the prevention of atherosclerosis. Furthermore, in light of chemical biology, it may be a useful tool for finding new factors and pathways involved in VEC apoptosis and VSMC migration and proliferation.

Na, K-ATPase is well known for its role as a maintainer of electrolyte and fluid balance in cells, organs and whole body. Now, Na, K-ATPase has been noted as an interesting drug target [Aperia, 2007]. A recent report has shown that, in VECs, suppressing activity of Na, K-ATPase results in activation of the proliferation and survival pathways and inhibits apoptosis [Orlov et al., 2004; Eva et al., 2006]. Consistent with this report, our results showed that 3BDO inhibited HUVEC apoptosis and maintained their ability in angiogenesis through suppressing Na, K-ATPase activity. In our previous study, it was showed that 3BDO depressed the level of integrin β4, a very important membrane protein that has been shown to be involved in HUVEC apoptosis signaling [Miao et al., 1997; Zhao et al., 2005], when it inhibited HUVEC apoptosis induced by deprivation of FGF-2 and serum [Wang et al., 2007]. However, integrin β4 is not expressed in VSMCs [Hiran et al., 2003].

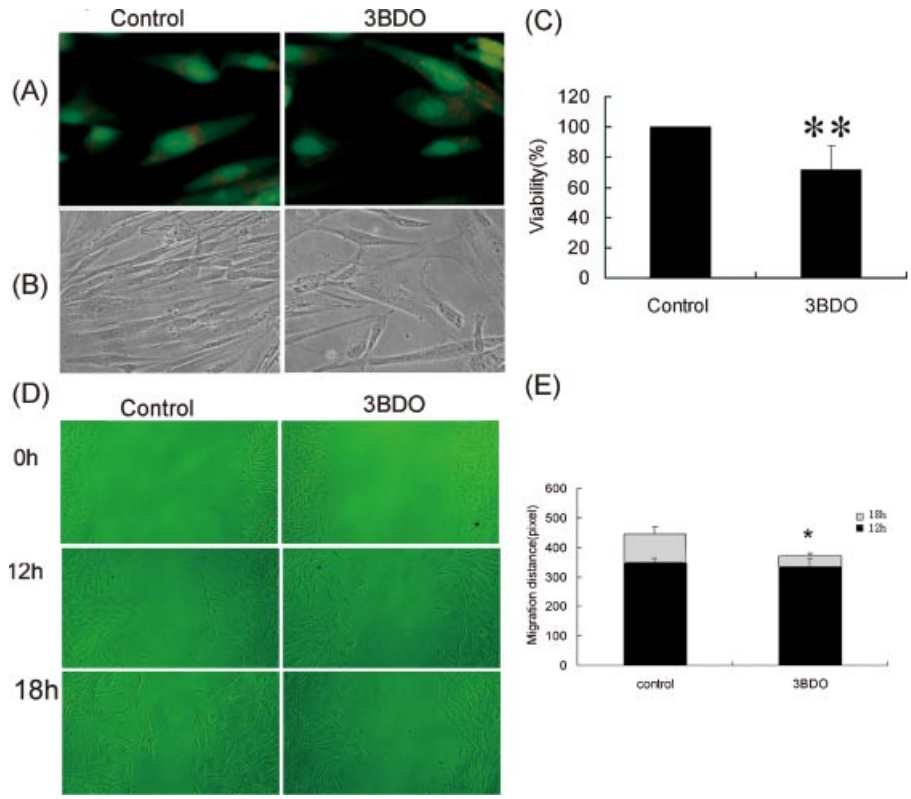

Fig. 3. Effects of 3BDO on proliferation and migration of VSMCs. Control, cell cultured in the medium without 3BDO. 3BDO, cells treated with 120 μM of 3BDO. **A**: Effect of 3BDO on nuclear morphology of VSMCs was examined by acridine orange staining. **B**: Effect of 3BDO on VSMC morphology. Magnification 200×. **C**: Effect of 3BDO on VSMC proliferation. Values are the ratio of the living cell number in 3BDO treatment group to that in the control group. 3BDO inhibited the proliferation of VSMCs. $^{**}P < 0.01$ versus control, n = 3. **D**: Photomicrographs of VSMC migration at time points 0, 12, and 18 h in the presence and absence of 3BDO. **E**: Cell migration of VSMCs is inhibited after addition of 3BDO at 18 h as measured by the migration distance on 100× photomicrographs. $^{*}P < 0.05$ versus control, n = 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

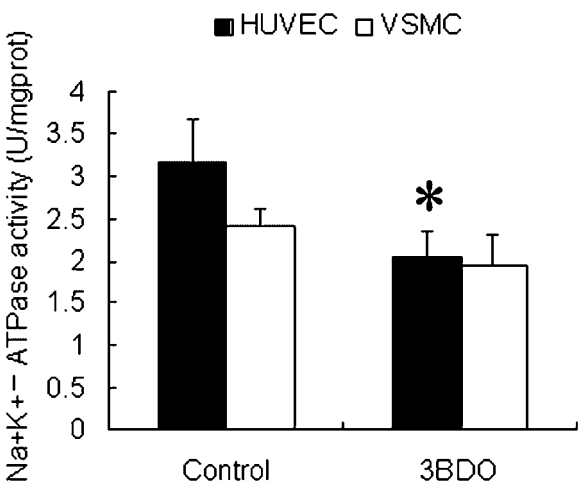

Fig. 4. Effects of 3BDO on Na, K-ATPase activity in HUVECs and VSMCs. Control, the enzyme activity of cells cultured in the medium without 3BDO. 3BDO, the enzyme activity of cells treated with 120 μM of 3BDO for 24 h. In VECs treated with 3BDO, the enzyme activity decreased obviously compared with that in control VECs, but in VSMCs treated with 3BDO, the activity of Na, K-ATPase was not changed. $^{*}P < 0.05$ versus control, n = 4.

Taken our previous reports together with the present result, it was suggested that Na, K-ATPase and integrin β4 might cooperate in regulating HUVEC apoptosis, although the relationship between these two membrane proteins needs to be demonstrated.

In VSMCs, 3BDO inhibited cell growth, but did not affect the activity of Na, K-ATPase. It was reported that, in rat VSMC, inhibition of Na, K-ATPase blocks the development of apoptosis [Orlov et al., 1999], but a 2–4-day treatment with ouabain (Inhibitor of Na,K-ATPase) did not affect the survival of VSMCs from rat aorta [Orlov et al., 2001], suggesting that Na, K-ATPase might not a very important factor for VSMC survival. Our result is consistent with this report. The distinct effects of 3BDO on the activity of Na, K-ATPase in vascular endothelial cells and smooth muscle cells suggested its future application to inhibiting the development of atherosclerosis.

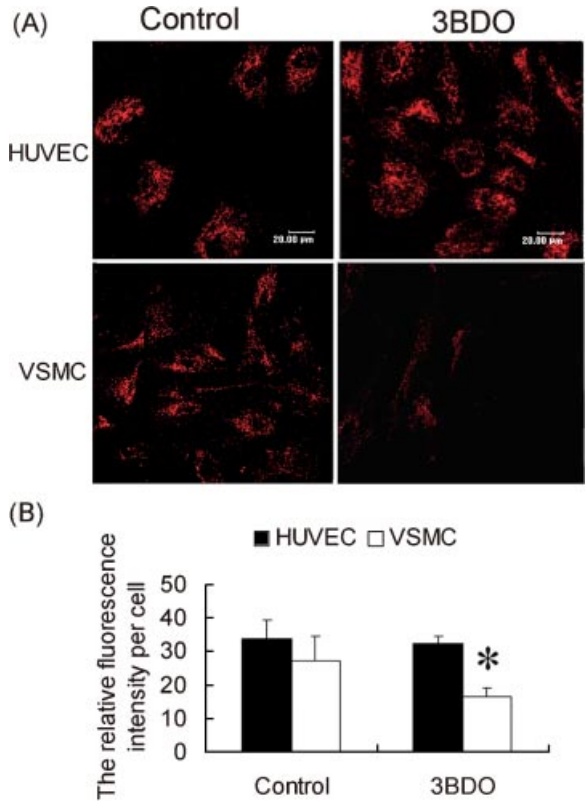

Fig. 5. The effect of 3BDO on membrane potential of mitochondrial. **A**: The images of the cells loaded with 200 nM TMRM under the laser scanning confocal microscope. Control, cells cultured in the medium without 3BDO. 3BDO, cells treated with 120 μM of 3BDO for 24 h. **B**: The changes of mitochondrial membrane potential. In VECs treated with 3BDO, the mitochondrial membrane potential was not changed compared with that in control VECs, but in VSMCs treated with 3BDO, the mitochondrial membrane potential decreased obviously. The relative quantity of TMRM staining depicted as a bar chart. $*P < 0.05$ versus control, $n = 3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The association between Na, K-ATPase and mitochondria membrane potential in neural cell apoptosis has been noted [Wang et al., 2003], but it is not well known in angiogenesis and VEC apoptosis. Here, the results showed that in HUVECs, 3BDO inhibited Na, K-ATPase activity, but had no effect on mitochondria membrane potential. In VSMCs, it did not affect Na, K-ATPase activity, but depressed mitochondria membrane potential obviously. The data suggested that 3BDO might perform its action through modulating the function of mitochondria in VSMCs, but in HUVECs, through inhibiting Na, K-ATPase activity. We deduced that the roles of Na, K-ATPase and mitochondria membrane potential in regulating vascular cell survival were cell-type specific. The association between Na, K-ATPase and mitochondria membrane potential in angiogenesis and VEC apoptosis still needs to be investigated.

In summary, the results of this study showed that 3BDO inhibited the migration and proliferation of VSMCs, and maintained the migration and tubule formation functions of VECs. 3BDO inhibited the activity of Na, K-ATPase, but had no effect on the mitochondria membrane potential in HUVECs. On the other hand, it did not affect Na, K-ATPase activity, but depressed mitochondria membrane potential obviously in VSMCs. Taken together, the anti-apoptotic effect of 3BDO on endothelial cells, and its antimigration and antiproliferation roles in VSMCs might be of great benefit to endothelium functional integrity and control of atherosclerosis development.

ACKNOWLEDGMENTS

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