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Contradictory Effects of Superoxide and Hydrogen Peroxide on $K_{Ca}3.1$ in Human Endothelial Cells

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Reactive oxygen species (ROS) are generated in various cells, including vascular smooth muscle and endothelial cells, and regulate ion channel functions. $K_{Ca}3.1$ plays an important role in endothelial functions. However, the effects of superoxide and hydrogen peroxide radicals on the expression of this ion channel in the endothelium remain unclear. In this study, we examined the effects of ROS donors on $K_{Ca}3.1$ expression and the K^+ current in primary cultured human umbilical vein endothelial cells (HUVECs). The hydrogen peroxide donor, tert-butyl hydroperoxide (TBHP), upregulated $K_{Ca}3.1$ expression, while the superoxide donors, xanthine/xanthine oxidase mixture (X/XO) and lysophosphatidylcholine (LPC), downregulated its expression, in a concentration-dependent manner. These ROS donor effects were prevented by antioxidants or superoxide dismustase. Phosphorylated extracellular signal-regulated kinase (pERK) was upregulated by TBHP and downregulated by X/XO. In addition, repressor element-1-silencing transcription factor (REST) was downregulated by TBHP, and upregulated by X/XO. Furthermore, $K_{Ca}3.1$ current, which was activated by clamping cells with 1 μM Ca^{2+} and applying the $K_{Ca}3.1$ activator 1-ethyl-2-benzimidazolinone, was further augmented by TBHP, and inhibited by X/XO. These effects were prevented by antioxidants. The results suggest that hydrogen peroxide increases $K_{Ca}3.1$ expression by upregulating pERK and downregulating REST, and augments the K^+ current. On the other hand, superoxide reduces $K_{Ca}3.1$ expression by downregulating pERK and upregulating REST, and inhibits the K^+ current. ROS thereby play a key role in both physiological and pathological processes in endothelial cells by regulating $K_{Ca}3.1$ and endothelial function.

Key Words: Ca^{2+}-activated K^+ channel, Endothelial cells, Hydrogen peroxide, Superoxide

INTRODUCTION

Vascular endothelial cells are in contact with blood cells and vascular smooth muscle cells, and are therefore constantly exposed to reactive oxygen species (ROS) that are released from these cell types on activation [1,2]. Moreover, endothelial cells themselves generate ROS by stimulation with various substances in plasma [1,2]. ROS play a key role in the physiological and pathological processes in endothelial cells; hydrogen peroxide upregulates endothelial NO synthase (eNOS) [3], and serves as an endothelium-derived hyperpolarizing factor (EDHF) that mediates vascular relaxation [4,5]. Conversely, superoxide impacts endothelial function by downregulating the expression of eNOS [3], thus mediating vascular contraction [6,7]. In addition, excessive ROS production damages endothelial cells, leading to endothelial dysfunction [1].

ROS may regulate cellular function by affecting ion channels. ROS were shown to regulate various types of ion channels, such as Ca^{2+}-dependent K^+ channels [8,9], ATP-sensitive K^+ channel [9,10], HERG channels [11], and Ca^{2+} channels [12,13], and also affected Ca^{2+} release-activated Ca^{2+} current [14]. In addition, ROS exert complex effects on voltage-dependent K^+ channels (Kvs). Hydrogen peroxide significantly accelerated the activation kinetics of Kv1.4 and Kv3.4, whereas did not alter Kv1.3, Kv2.1 and Kv2.2 [15]. In addition, hydrogen peroxide negatively shifted the activation curve of Kv1.5 [16]. Conversely, superoxide decreased the strength of the current through this channel [17]. Ca^{2+}-dependent K^+ channels were activated by hydrogen peroxide [8] or superoxide [9] radicals, resulting in the hyperpolarization of the membrane, or dilation

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ABBREVIATIONS: 1-EBIO, 1-ethyl-2-benzimidazolinone; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NO synthase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; Kv, voltage-dependent K^+ channel; LPC, lysophosphatidylcholine; NAC, N-acetyl-L-cysteine; pERK, phosphorylated extracellular signal-regulated kinase; ROS, reactive oxygen species; REST, repressor element-1-silencing transcription factor; SOD, superoxide dismutase; TBHP, tert-butyl hydroperoxide; X/XO, xanthine/xanthine oxidase mixture.

of cerebral arterioles. Both superoxide and hydrogen peroxide radicals enhanced the activity of large-conductance Ca^{2+}-dependent K^+ channel in rat and cat cerebral arterioles; in contrast, the peroxynitrite radical decreased this activity in rat cerebral arteries [18].

Evidence indicates that $K_{Ca}3.1$ profoundly regulates endothelial function. It mediates a part of the endothelium-derived hyperpolarization response, and contributes to endothelium-dependent relaxation of blood vessels. In addition, $K_{Ca}3.1$ modulates Ca^{2+} influx in endothelial cells. On inhibition of $K_{Ca}3.1$, Ca^{2+} influx and endothelium-dependent relaxation effects were abrogated [19]. Thus, $K_{Ca}3.1$ dysregulation causes endothelial dysfunction, and thereby may contribute to vascular diseases such as preeclampsia [20] and Fabry disease [21]. In $K_{Ca}3.1$-deficient mice, the endothelial $K_{Ca}3.1$ current was abolished, leading to a considerable increase in arterial blood pressure and to a mild left ventricular hypertrophy [22]. We previously reported that in α-galactosidase A-knockout mice (an animal model of Fabry disease), endothelial dysfunction is caused by $K_{Ca}3.1$ downregulation and dysfunction [21]. Furthermore, we suggested that $K_{Ca}3.1$ downregulation contributes to the endothelial dysfunction seen in preeclampsia [20]. However, the role of ROS in the regulation of endothelial $K_{Ca}3.1$ has received little attention.

In the present study, we compared the effects of hydrogen peroxide and superoxide radicals on $K_{Ca}3.1$ expression in human umbilical vein endothelial cells (HUVECs). We found that the hydrogen peroxide radical donor TBHP increased $K_{Ca}3.1$ expression by upregulating pERK expression and downregulating repressor element-1 silencing transcription factor (REST) expression, and augmented $K_{Ca}3.1$ current. On the other hand, the superoxide donor, xanthine/xanthine oxidase mixture (X/XO), or L-α-lysophosphatidylcholine (LPC) decreased $K_{Ca}3.1$ expression by downregulating pERK expression and upregulating REST expression, and inhibited $K_{Ca}3.1$ current.

METHODS

Cell culture

Endothelial cells were isolated from human umbilical veins by collagenase treatment, as previously described [23]. HUVECs in suspension were plated into 6 well cell culture plates and grown in complete M199 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Life Technologies Corp., Carlsbad, CA), 100 units/ml penicillin, 100 μg/ml streptomycin, 15 μg/ml endothelial cell growth supplement (BD Biosciences, Rockville, MD), 0.1 mM MEM non-essential amino acids (Life Technologies Corp., Carlsbad, CA) and 10 unit/ml heparin. Cultured cells were identified as endothelial cells in origin by their cobble stone appearance at confluence and positive staining with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labelled acetylated low density lipoprotein (Biomedical Technologies Inc., Stoughton, MA). HUVECs were used up to the second or third passage.

The investigation was approved by local ethics committee, the Institutional Review Board of the Ewha Womans University, and was in accordance with the Declaration of Helsinki.

Real-time PCR and immunoblot analysis

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA), and RNA was then reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCRs were performed on an ABI 7000 sequence detection system (Applied Biosystems) using a SYBR Green PCR Master Mix (Applied Biosystems). Primers for REST were 5'-GTGCGAACTCACACA-GGAGA-3' (sense) and 5'-AAGAGGTTTAGGCCCGTTGT-3' (antisense). mRNA expression was normalized to the house-keeping gene, human *Gapdh* (5-GGCCTCCAAGGA-GTAAGACC-3' (sense) and 5'-AGGGGTCTACATGGCAA-CTG-3' (antisense)).

For immunoblot analysis, 30 μg of protein from cell homogenates was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5～12% gels), and proteins were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour with TBST (10 mM Tris-HCl, 150 mM NaCl, and 1% Tween 20, pH 7.6) containing 5% bovine serum albumin at room temperature. The blots were incubated for 3 hours with primary antibody against primary $K_{Ca}3.1$ antibody (IK1; Santa Cruz Biotechnology, Santa Cruz, CA), or p-ERK (Cell Signaling Technology, Beverly, MA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Bands were visualized by chemiluminescence. Data collection and processing were performed using a luminescent image analyzer LAS-3000 and Image Gauge software (Fuji-Film, Tokyo, Japan).

Immunocytochemistry

HUVECs were grown on glass coverslips precoated with 1% gelatin. Cells were incubated overnight at 4°C with a diluted (1 : 50) primary $K_{Ca}3.1$ antibody, washed and incubated for 1 hour at room temperature with a secondary antibody, Alexa Flour 488 donkey anti-goat IgG (1 : 1,500; Molecular probes, Eugene, OR). After that, the cells were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The mounted coverslips were viewed under a confocal microscope (Carl Zeiss, Gottingen, Germany) and photographed as described previously [24].

Electrophysiology

The patch-clamp technique was used in whole-cell configurations at room temperature. Whole-cell currents were measured using ruptured patches and monitored in voltage-clamp modes with an EPC-9 (HEKA Elektronik, Lambrecht, Germany). The holding potential was 0 mV and currents were monitored by the repetitive application of voltage ramps from −100 to +100 mV with a 10-second interval (sampling interval 0.5 milliseconds, 650 millisecond duration). The standard external solution contained (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH. The pipette solution for whole-cell recording contained (in mM): 40 KCl, 100 K-aspartate, 2 $MgCl_2$, 0.1 EGTA, 4 Na_2ATP, 10 HEPES, pH adjusted to 7.2 with KOH. For buffering free Ca^{2+}, the appropriate amount of Ca^{2+} (calculated using CaBuf software; G. Droogmans, Leuven, Belgium; ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip) was added in the presence of 5 mM EGTA.

$K_{Ca}3.1$ current was activated by loading 1 μM Ca^{2+} via a patch pipette in whole-cell clamped HUVECs and adding the $K_{Ca}3.1$ activator 1-ethyl-2-benzimidazolinone (1-EBIO, 100 μM) to the external solution. $K_{Ca}3.1$ current was normalized to cell capacitance and the selective $K_{Ca}3.1$ blocker TRAM-34-sensitive current was measured as $K_{Ca}3.1$ current.

Chemicals

LPC from egg yolk, N-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), TBHP, tempol, tiron and TRAM-34 were purchased from Sigma-Aldrich (St. Louis, MO); DAPI from Molecular Probes (Eugene, OR), X/XO from Calbiochem (Gibbstown, NJ), 1-EBIO from Tocris Bioscience (Ellisville, MO). TRAM-34 and 1-EBIO were applied to the bath solution at 10 μM and 100 μM, respectively. LPC, NAC, tempol, tiron, TRAM-34 and 1-EBIO were dissolved in DMSO. The final concentration of DMSO was less than 0.05%.

Statistical analysis

Pooled data are given as mean±SEM. Statistical evaluation of data was performed by Student *t* test. Values of $p < 0.05$ were considered significant.

RESULTS

Effect of ROS donors on endothelial $K_{Ca}3.1$ expression

We examined whether the ROS donors, TBHP or X/XO, can modulate $K_{Ca}3.1$ expression in endothelial cells. Primary HUVECs were incubated with ROS donors or vehicle for 24 h, followed by western blot analysis of protein expression (Fig. 1). Treatment with the hydrogen peroxide donor TBHP, but not the vehicle, unregulated $K_{Ca}3.1$ expression in a concentration-dependent manner (Fig. 1A). In contrast, treatment with the superoxide donor X/XO, but not the vehicle, downregulated $K_{Ca}3.1$ expression in a concentration-dependent manner (Fig. 1B). In addition, we examined the effects of LPC, a molecule involved in superoxide generation in endothelial cells [3], on $K_{Ca}3.1$ expression (Fig. 1C). Similar to the X/XO effect, treatment with LPC decreased $K_{Ca}3.1$ expression in a concentration-dependent manner. To corroborate these findings we next examined whether ROS donors affected $K_{Ca}3.1$ expression in HUVECs by using immunocytochemical staining to detect expression of $K_{Ca}3.1$ protein (Fig. 2). $K_{Ca}3.1$ expression (indicated by green florescence) was markedly increased in HUVECs treated with TBHP compared to those treated with vehicle (Fig. 2A). In contrast, $K_{Ca}3.1$ expression was markedly lower in HUVECs treated with X/XO than in those treated with vehicle (Fig. 2B). These results, which indicate that $K_{Ca}3.1$ expression is upregulated or downregulated following treatment with TBHP and X/XO, respectively, suggest that the hydrogen peroxide and superoxide radicals exert converse effects on the expression of this ion channel.

Antioxidants prevent ROS donor-induced modulation of $K_{Ca}3.1$ expression

We then examined whether treatment with antioxidants or SOD could prevent the ROS donor- or LPC-induced modulation of $K_{Ca}3.1$ expression. Pre-treatment of HUVECs with the antioxidants tempol, tiron, or NAC prevented TBHP-induced upregulation (Fig. 3A), as well as X/XO-induced downregulation (Fig. 3B) of $K_{Ca}3.1$ expression. Furthermore, LPC-induced $K_{Ca}3.1$ downregulation was abrogated by pre-treatment with SOD (Fig. 3B). In combination, these findings reinforce that $K_{Ca}3.1$ is upregulated by the

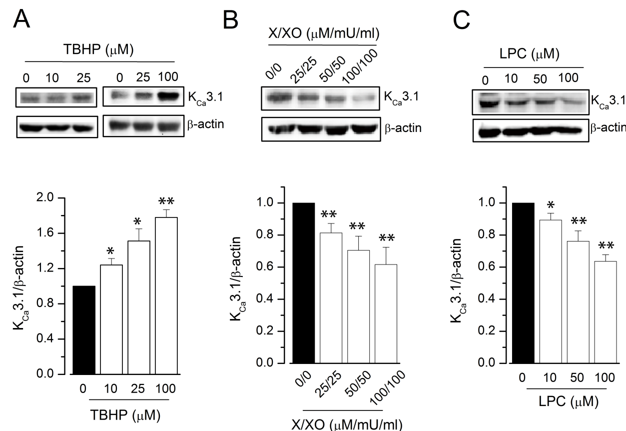

Fig. 1. $K_{Ca}3.1$ expression in ROS donor-treated HUVECs. $K_{Ca}3.1$ expression was measured using immunoblot, and relative protein expression was represented as a ratio of the levels in the vehicle-treated group to that in the test group. HUVECs were treated with TBHP (A), X/XO (B), or LPC (C), for 24 h; n=4~7. *$p < 0.05$, **$p < 0.01$ versus vehicle-treated control.

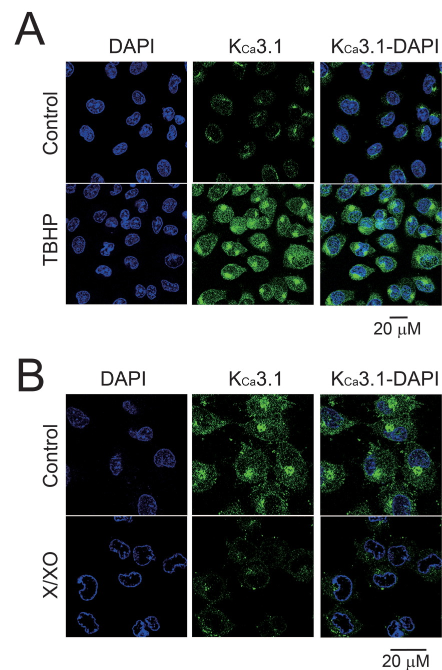

Fig. 2. $K_{Ca}3.1$ expression in ROS donor-treated HUVECs. $K_{Ca}3.1$ expressions were measured using immunocytochemistry. HUVECs were treated with 50 μM TBHP (A), or 50 μM/50 mU/ml X/XO (B), for 24 h. Immunocytochemistry images show $K_{Ca}3.1$ staining in green, and nuclear staining with DAPI in blue; combined images are also presented.

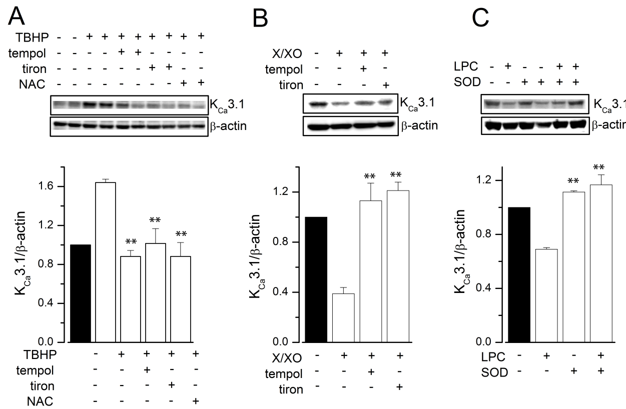

Fig. 3. Effects of antioxidants on ROS donor-induced $K_{Ca}3.1$ regulation. HUVECs were treated with vehicle or 100 μM TBHP (A), 100 μM/100 mU/ml X/XO (B), or 100 μM LPC (C), for 24 h, with or without pre-treatment with antioxidants (5 μM tempol, 50 μM tiron, or 10 mM NAC), or SOD (1,000 U/ml). The results are mean±SEM of three independent experiments. **p<0.01 versus TBHP, X/XO or LPC alone.

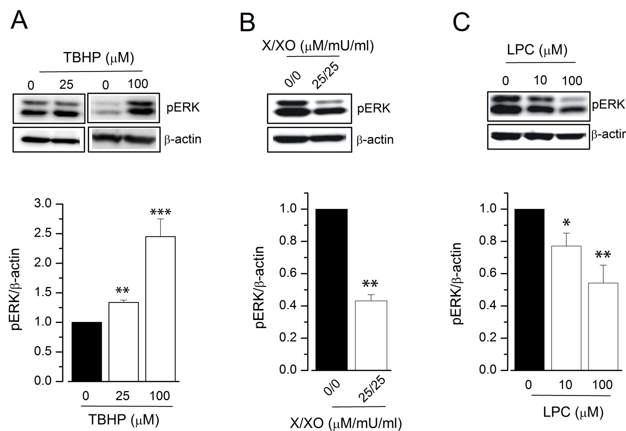

Fig. 4. Effect of ROS donors on pERK expression in HUVECs. pERK expression was measured using immunoblot, and relative protein expression was expressed as a ratio of the levels in the vehicle-treated group to that in the test group. HUVECs were treated with TBHP (A), X/XO (B), or LPC (C), for 24 h; n=4~7. *p<0.05, **p<0.01, ***p<0.005 versus vehicle-treated control.

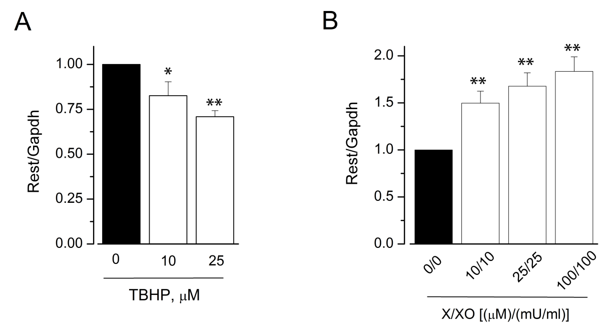

Fig. 5. Effect of ROS donors on REST expression in HUVECs. REST expression was measured using real-time PCR and the relative expression of mRNA was expressed as a ratio of the levels in the vehicle-treated group to that in the test group. HUVECs were treated with TBHP (A) or X/XO (B) for 24 h; n=4~7. *p<0.05, **p<0.01 versus control.

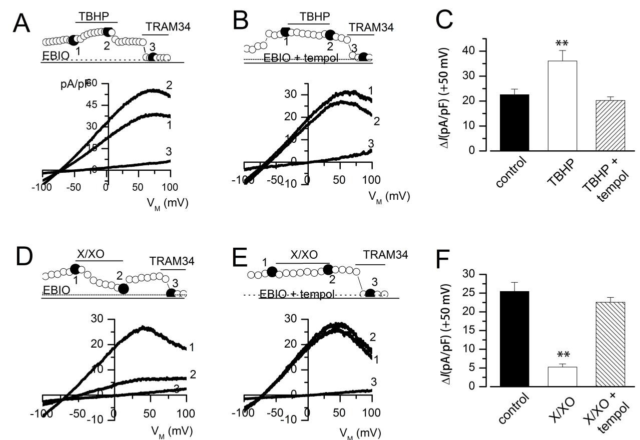

Fig. 6. Effect of ROS donors on $K_{Ca}3.1$ current in HUVECs. $K_{Ca}3.1$ current was activated by loading cells with 1 μM Ca^{2+} by using a patch pipette, and treating the cells with 1-EBIO (100 μM). The $K_{Ca}3.1$ currents were normalized to cell capacitance, and the TRAM-34-sensitive current was measured as the $K_{Ca}3.1$ current. (A, B, D, F) Current densities are shown at a membrane potential of +50 mV, and marked by open circles, while I/V relationships were obtained at the points marked by closed circles. (C, F) $K_{Ca}3.1$ current densities at +50 mV; n=6~8 (right panel). *p<0.05 versus control.

hydrogen peroxide radical, and downregulated by the superoxide radical.

Effect of ROS donors on pERK and REST

To further evaluate the molecular mechanisms of ROS donor-induced modulation of $K_{Ca}3.1$ expression, we examined whether ROS donors affected the signal transduction pathways involved in $K_{Ca}3.1$ expression, the ERK pathway [25] and REST [26]. We thus assessed the phosphorylation of ERK (pERK) by immunoblot analysis (Fig. 4). TBHP treatment for 24 h significantly increased the levels of pERK in HUVECs (34±4% and 145±30% increase by 25 and 100 μM TBHP, respectively; Fig. 4A). In contrast, pERK levels were significantly reduced in HUVECs treated with X/XO (25 μM/25 mU/ml) for 24 h (57±4% inhibition; Fig. 4B). In addition, LPC reduced the level of pERK in a concentration-dependent manner in HUVECs (Fig. 4C). We then examined whether ROS donors modulate $K_{Ca}3.1$ expression by regulating the REST pathway. The expression of this transcription factor in HUVECs was significantly downregulated after incubation with TBHP in a concentration-dependent manner (29±3% decrease by 25 μM TBHP; Fig. 5A). In contrast, X/XO significantly upregulated REST expression in HUVECs (83±16% increase by 100 μM/100 mU/ml X/XO; Fig. 5B). These data suggest that TBHP upregulates $K_{Ca}3.1$ by activating the ERK pathway and downregulating REST. Conversely, X/XO downregulates $K_{Ca}3.1$ by inhibiting the ERK pathway and increasing REST expression.

ROS donors modulate $K_{Ca}3.1$ currents in endothelial cells

We next examined whether the ROS donors could modulate $K_{Ca}3.1$ current, which was activated by loading whole cell-clamped HUVECs with 1 μM Ca^{2+}, via a patch pipette, and supplementing the medium with the $K_{Ca}3.1$ activator 1-EBIO (100 μM). When the $K_{Ca}3.1$ current reached a steady state, TBHP or X/XO was applied in the external solution. The activated $K_{Ca}3.1$ current was further enhanced by TBHP (Fig. 6A, C), but it was inhibited by X/XO (Fig. 6D, F). The effect of TBHP (Fig. 6B, C), or X/XO (Fig. 6E, F) was inhibited by tempol. These data suggest that $K_{Ca}3.1$ current is augmented by the hydrogen peroxide radical, and inhibited by the superoxide radical.

DISCUSSION

In this study, we show that ROS regulate $K_{Ca}3.1$ expression through the modulation of the ERK and REST pathways, and influence $K_{Ca}3.1$ current production in human endothelial cells. The hydrogen peroxide donor TBHP increases $K_{Ca}3.1$ expression through pERK upregulation and REST downregulation. In contrast, the superoxide donors X/XO and LPC decrease $K_{Ca}3.1$ expression through pERK downregulation and REST upregulation. In addition, $K_{Ca}3.1$ current is augmented by TBHP, and inhibited by X/XO. These findings shed light on the mechanisms underlying ROS-mediated regulation of this ion channel, a process that may be implicated in ROS-induced modulation of endothelial function.

Furthermore, the effects of the ROS donors, TBHP, XXO, or LPC were nullified by pre-treatment with antioxidants. The upregulation in $K_{Ca}3.1$ expression by the hydrogen peroxide donor TBHP was abrogated by antioxidants, suggesting that this effect is mediated by the hydrogen peroxide radical. In parallel, antioxidant treatment also nullified the ion-channel downregulating effects of the superoxide radical donors X/XO or LPC. LPC suppressed SOD1 and increased catalase expression [3], indicating that LPC increases the production of the superoxide, but not the hydrogen peroxide radical. These results suggest that X/XO- or LPC-induced downregulation of $K_{Ca}3.1$ is mediated by the superoxide radical. Since the ion channel $K_{Ca}3.1$ is an important physiological modulator in the endothelium, these findings suggest that ROS may affect endothelial function by regulating $K_{Ca}3.1$ expression and function.

Superoxide and hydrogen peroxide radicals are generated in vascular endothelial cells by several cellular enzymes, including eNOS, cytochrome P-450, and NADPH oxidases [27]. eNOS was suggested to contribute to ROS production in the endothelium, since acetylcholine-induced hydrogen peroxide radical production was markedly reduced in the blood vessels of eNOS-knockout mice [5], and eNOS activation generates superoxide radicals under the depletion of tetrahydrobiopterin [28,29]. In addition, cytochrome P-450 may be a source of ROS, since the hydrogen peroxide radical was produced in a cytochrome P-450-dependent manner in the coronary arteries of rats [30] and pigs [31]. NADPH oxidases play an important role in the generation of endothelial superoxide radicals. The enzymes are regulated by various stimuli such as shear stress, hypoxia, and hyperlipidemia [2]. Since the production of superoxide radicals promotes generation of hydrogen peroxide radicals through SOD, both free radicals may be produced in endothelial cells in response to various stimuli, and may thereby regulate $K_{Ca}3.1$ expression and its function.

$K_{Ca}3.1$ expression is upregulated by the ERK pathway [25] and downregulated by REST [26]. External stress and physiological stimuli can affect the MAPK pathway by generating ROS [32]. Blood flow regulates $K_{Ca}3.1$ expression; a laminar flow upregulates $K_{Ca}3.1$ by increasing hydrogen peroxide generation, whereas turbulent flow downregulates $K_{Ca}3.1$ by increasing superoxide generation [33,34]. ERK phosphorylation and eNOS expression were inhibited by superoxide donors, but stimulated by the hydrogen peroxide radical donor THBP [3]. Thus, an inhibition of ERK phosphorylation may suppress $K_{Ca}3.1$ expression, as suggested previously [25]. In addition, ROS can activate as well as inactivate transcription factors [35]. The apparent regulation of REST levels and activity by transcriptional and post-transcriptional mechanisms [36] suggest that ROS are likely to regulate REST by affecting transcription factors. In addition, REST can modulate the phosphoinositide-3 kinase-Akt/ERK pathway, since cells lacking REST exhibit increased phosphoinositide-3 kinase signalling [37].

There are many reports on the superoxide or hydrogen peroxide radical-induced modulation of K^+ currents, such as $K_{Ca}1.1$ current [38-40], the voltage-dependent K^+ current [16,17], and the ATP-sensitive K^+ current [10,38]. However, this is the first report of the superoxide or hydrogen peroxide radical-induced modulation of the current generated through the ion channel $K_{Ca}3.1$. We observed that the hydrogen peroxide radical stimulates $K_{Ca}3.1$ current in cells dialyzed with 5 mM EGTA. It is possible that a hydrogen peroxide radical-induced increase in cytosolic free Ca^{2+} [41,42] could lead to activation of K_{Ca} current. However, a high intracellular EGTA concentration may buffer the increase in Ca^{2+} induced by ROS. Since $K_{Ca}3.1$ current is activated in a voltage- and cytosolic free Ca^{2+}-dependent manner, the augmentation of $K_{Ca}3.1$ current by the hydrogen peroxide radical suggests that this radical increases the sensitivity of $K_{Ca}3.1$ channel to voltage, or cytosolic free Ca^{2+}.

In conclusion, the ROS donors TBHP, X/XO, and LPC regulate $K_{Ca}3.1$ expression in HUVECs. In addition, $K_{Ca}3.1$ current is modulated by the ROS donors TBHP, and X/XO. These ROS donor effects on $K_{Ca}3.1$ expression and the K^+ current are prevented by pre-treatment with antioxidants. $K_{Ca}3.1$ plays an important role in vasomotor regulation; therefore, modulation of this ion channel in the vascular endothelium may be useful in the treatment of atherosclerosis and endothelial damage.

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