Hydrogen Peroxide Stimulates the Ca^{2+} -activated Big-Conductance K Channels (BK) Through cGMP Signaling Pathway in Cultured Human Endothelial Cells

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Key Words

Hydrogen peroxide • Nitric oxide • Endothelial cell • cGMP • K channel

Abstract

We used the whole cell patch-clamp technique to examine the effect of hydrogen peroxide (H_2O_2) on the Ca^{2+} -activated BK channels in human endothelial cells. We confirmed the previous finding that a 200 pS BK channel activity was detected when the cell membrane potential was clamped at 50 mV. Application of H_2O_2 or adding glucose oxidase (GO) stimulated BK channels. The stimulatory effect of H_2O_2 and GO was absent in cells treated with ebselen, a scavenger of reactive oxygen species (ROS). To determine whether the stimulatory effect of H_2O_2 and GO on BK channels is the result of increasing NO production in the endothelial cells, we examined the effect of H_2O_2 and GO on BK channels in the presence of 0.1 mM L-NAME which inhibits NO synthase (NOS). Inhibition of NOS completely abolished the stimulatory effect of H_2O_2 on BK channels. In contrast, treatment of endothelial cells with D-NAME did not block the effect of H_2O_2 on BK channels. Moreover, inhibiting soluble guanylate cyclase (sGC) with ODQ mimicked

the effect of L-NAME and abolished the effect of H_2O_2 . Addition of 8-bromo-cGMP stimulated BK channels and further application of H_2O_2 did not increase BK channel activity in the presence of cGMP analog. The notion that the effect of H_2O_2 on BK channels was the result of stimulating NO-cGMP pathway is further indicated by the observation that inhibition of PKG with KT5823 also abolished the stimulatory effect of H_2O_2 on BK channels. We conclude that H_2O_2 stimulates the Ca^{2+} BK channels through NO/sGC/cGMP pathway in cultured human endothelial cells.

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Introduction

Ca^{2+} -activated K channels including small-conductance, intermediate-conductance and big-conductance have been shown to be expressed in endothelial cells [1-5]. Although Ca^{2+} -activated small conductance and intermediate-conductance K channels are mainly responsible for determining the membrane potential under basal conditions [3], BK channels may

also be involved in regulating the membrane potential of endothelial cells because BK channels have a high channel conductance. It is conceivable that BK channels could play an important role in participating in generating the membrane potential in endothelial cells when they are activated [3]. Indeed, it has been shown that expression of BK channels in cultured endothelial cells caused a transient hyperpolarization induced by ATP [2]. The membrane potential of endothelial cells plays an important role in regulating Ca^{2+} influx via Ca^{2+} -permeable channels such that hyperpolarization is expected to increase electrochemical driving force of Ca^{2+} entry in endothelial cells [3]. Thus, activation of BK channels are expected to elevate intracellular Ca^{2+} which further enhances the release of endothelial-derived relaxing factor (EDRF) including nitric oxide (NO) and epoxyeicosatrienoic acid (EET) in endothelial cells [6]. A large body of evidence has shown that endogenous H_2O_2 plays an important role in mediating vasodilation [7-10]. Moreover, it has been suggested that H_2O_2 -mediated vasodilation is partially mediated by stimulating BK channels in smooth muscle cells [9, 10]. H_2O_2 has also been reported to increase Ca^{2+} -dependent BK channels in human endothelial cells [11]. However, the mechanism by which H_2O_2 activates Ca^{2+} activated BK channels is not completely clear. Thus, the aim of the present study is to examine whether H_2O_2 stimulates BK channels in the cultured human endothelial cells and to determine the mechanism by which H_2O_2 activates BK channels in the endothelial cells.

Materials and Methods

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science Inc (Walkersville, MD) and were cultured with 5% CO_2 at 37°C in EGM-2 medium obtained from the same company. Endothelial cells between passages 5 to 8 were used for patch-clamp experiments and they were placed on a 5x5mm cover glass which was transferred to a chamber (1 ml) mounted on the stage of a Nikon inverted microscope for the experiments.

Patch-Clamp Experiments

We used PP81 Narishege puller to draw patch electrodes and the electrode resistance was 3-5M Ω when filled with 138 mM KCl. Because BK channel activity in the cultured endothelial cells is low, we used the whole-cell patch-clamp recording to study the single BK channel activity in HUVEC. This increased the possibility to find the BK channels. Briefly, when the high resistance seal was formed, the cell membrane under the patch pipette was ruptured to form the whole cell

patch configuration. The seal resistance was measured before forming whole cell patch recording or in the presence of Ba^{2+} after forming the whole cell recording. We have also performed the experiments with perforated patches in each set of experiments. Because no difference between two approaches was observed, we pooled data. The duration of patch clamp experiments was 10 min and no channel run-down was observed within 10 min. If the seal resistance was not changed after forming whole-cell patch mode, we clamped the cell membrane potential from -120mV to +120mV in one second (ramp) to determine whether BK channel activity could be detected. If BK channel activity was detected, we clamped the cell membrane potential at +50 mV (depolarization) which activates BK channels and studied the channel activity at the same holding potential.

An Axopatch200A amplifier (Axon Instruments, CA) was used for recording the BK channel activity. The signal was filtered at 1 kHz and digitized by an interface, digitada1200 (Axon Ins.). Data were collected at sample rate of 5 kHz and were analyzed with software, Axon patch 7.0. Channel activity was defined as NP_o, that was calculated from data samples of 120 seconds duration in the steady state as follows:

$$\text{NP}_o = \Sigma(t_1 + 2t_2 + \dots + t_i)$$

where t_1 is the fractional open time spent at each of the observed current levels. The BK channel conductance was measured at several holding potentials.

Measurement of superoxide and H_2O_2

We have used 2',7'-dichlorofluorescein (DCF) (Invetrogen, Carlsbad, CA) to detect the superoxide and H_2O_2 . HUVEC cells were loaded with 5 μM DCF for 30 min in the dark. After loading, HUVEC cells were washed three times with HEPES-buffered Ringer solution and fluorescence intensity correlated with superoxide and H_2O_2 in HUVEC was detected with an Olympics confocal microscope (excitation at 488 nm).

Experimental Solution, Chemicals and Statistics

A high K bath solution was composed of (in mM) 138 KCl, 0.5 MgCl_2 , 1.5 CaCl_2 , 10 HEPES, (pH 7.4). We also added 0.1mM niflumic acid to the bath to block possible Cl^- channels. Niflumic acid at concentrations used in the present study did not affect BK channel activity. The pipette solution was composed of (in mM) 138 KCl, 4 MgCl_2 , 5 EGTA, 10 HEPES (pH 7.2). The reason for using Ca^{2+} -free intracellular solution was to investigate whether H_2O_2 could stimulate BK channels by a Ca^{2+} -independent way. The stock solution of 10 mM H_2O_2 was freshly prepared before experiments by diluting concentrated H_2O_2 with 138 mM KCl bath solution. The control solution was prepared by adding the same amount of water as H_2O_2 to the saline. The final change in solution osmolality was less than 1 mosm/l which had no effect on channel activity.

Peroxyntirite and degraded product of peroxyntirite were purchased from Upstate (Temecula, CA) and KT5823 was obtained from Biomol (USA). All other chemicals used in the present study were purchased from Sigma-Aldrich (St Louis, MO). Data are presented as means $\pm$ SEM and significance was determined using Student's t-test or Anova one way test. $P < 0.05$ was considered significant.

Fig.1. (A) An I/V curve of BK channels in symmetrical 138 mM KCl solution in the presence (open circle) or absence of iberitoxin (close circle). (B) Single channel recordings demonstrate the effect of H_2O_2 (100 μM) on BK channel activity in HUVEC. The top trace shows the time course of the experiments and two parts of the recording are extended to demonstrate the channel activity at fast time resolution. Arrow indicates the addition of H_2O_2 or H_2O to the bath. The control solution (H_2O) was prepared by adding the same amount of water as H_2O_2 to the saline. The final change in solution osmolarity was less than 1 mosm/l. Fig.1C is a bar graph summarizing the effect of H_2O_2 on BK channels in the absence and presence of 10 μM ebselen. Asterisks indicate the significant difference between control and the experimental group.

Results

Fig. 1A is an I/V curve confirming the previous finding that a 201 pS BK channel was expressed in the cultured human endothelial cells and the channel was inhibited by 100 nM iberitoxin [1]. Fig. 1B is a recording showing the channel activity in a whole cell-patch clamp mode when the cell membrane potential was held at +50 mV. We next examined the effect of H_2O_2 (100 μM) (dissolved in 138 mM KCl bath solution) on BK channels in an endothelial cell. From inspection of Fig. 1B, it is apparent that addition of H_2O_2 increased BK channel activity defined by NP_0 to $210 \pm 20\%$ ($n=11$) of the control value within 4–6 min (The control solution was prepared by adding the same amount of water as H_2O_2 to the saline). In contrast, the channel activity was not significantly altered in the same time period when the control solution was added into the bath solution. Data summarized in Figure 1C show that H_2O_2 increased the BK channel activity in a dose-dependent manner from 50 μM to 200 μM ($300 \pm 70\%$) ($n=10$). Moreover, adding 10 μM ebselen, a scavenger of ROS, in the pipette solution (intracellular solution) completely abolished the stimulatory effect of H_2O_2 (100 μM) on BK channels.

After demonstrating that adding exogenous H_2O_2 stimulates the BK channels, we examined whether stimulation of endogenous superoxide production could also mimic the effect of H_2O_2 and stimulate the BK channels in endothelial cells. Thus, we used GO to generate H_2O_2 in the presence of glucose. Fig. 2A is a

confocal image showing that addition of 1 U/ml GO to the bath increased H_2O_2 in HUVEC cells loaded with DCF by $120 \pm 30\%$ ($N=4$). Fig. 2B is a recording showing the effect of GO (1 unit/ml) on the BK channels. GO was directly added to the bath solution in the presence of 5 mM glucose. Similar to H_2O_2 , stimulation of endogenous H_2O_2 generation significantly increased BK channel activity and increased NP_0 to $192 \pm 30\%$ ($n=8$) of the control value (with solvent in the bath) (Fig. 2C). Also, adding 10 μM ebselen or catalase (1000 U/ml) completely abolished the stimulation of BK channels by GO. Thus, results indicate that BK channels in cultured endothelial cells are stimulated by H_2O_2 .

It has been reported that H_2O_2 increases nitric oxide (NO) production in endothelial cells in a previous study [12, 13]. Because NO has been shown to stimulate BK activity in the cultured human endothelial cells in previous study [1], we examined whether the stimulatory effect of H_2O_2 on BK channels was mediated by a NO-dependent mechanism. Thus, we treated endothelial cells with L-NAME to block the NOS and examined the effect of 100 μM H_2O_2 on BK channels in the presence of L-NAME. Inhibition of NOS had no significant effect on BK channels (data not shown), suggesting that NOS was not actively involved in determining the basal activity of BK channels under control conditions. However, inhibition of NOS with L-NAME (100 μM) completely abolished the stimulatory effect of H_2O_2 on BK channels (Fig. 3A). Data summarized in Fig 3B demonstrate that H_2O_2 did not increase the BK channel activity ($98 \pm 10\%$ of the

Fig. 2. (A) Confocal image showing the fluorescence intensity correlated with H_2O_2 level in HUVEC cells measured with DCF. (B) Single channel recordings show the effect of 1 unit/ml glucose oxidase on BK channels in the absence or presence of $10\mu\text{M}$ ebselen. The top trace shows the time course of the experiments and two parts of the recording are extended to demonstrate the channel activity at fast time resolution. Arrow indicates the addition of H_2O_2 to the bath. (C) A bar graph summarizes the effect of GO on BK channels in the absence and presence of $10\mu\text{M}$ ebselen or catalase (1000 U/ml). Asterisk indicates that H_2O_2 treatment significantly increased channel activity in comparison to any other groups ($P<0.001$). Ctr: control; GO: glucose oxidase; Cat: catalase.

Fig. 3. (A) A channel recording shows the effect of H_2O_2 ($100\mu\text{M}$) on BK channel activity in the cells pretreated with L-NAME ($100\mu\text{M}$). The top trace demonstrates the time course of the experiments and two parts of the recording are extended to show channel activity at fast time resolution. B, A bar graph shows the effect of L-NAME on H_2O_2 -induced increase of BK activity. Asterisk indicates the significant difference between control and the experimental group. “##” indicates that difference between H_2O_2 and L-NAME+ H_2O_2 is significant. ** $P<0.01$; ## $P<0.01$. Ctr: control.

control value) in the presence of L-NAME ($n=10$). This suggests that the effect of H_2O_2 on BK channels in cultured endothelial cells was the result of stimulating endogenous NOS. Also, L-name treatment abolished the effect of GO on BK channels (data not shown).

Because superoxide has been shown to interact with NO to form peroxynitrite, we examined whether other ROS, such as peroxynitrite, could mimic the effect of H_2O_2 and stimulate BK channels in the endothelial cells. Thus, we tested the effect of peroxynitrite on BK activity. Figure 4 summarizes the effect of peroxynitrite on BK channels in the cultured endothelial cells. We also used degraded components of peroxynitrite as a negative control. Application of 5, 10 and $100\mu\text{M}$ peroxynitrite had no significant effect on BK channels in comparison to that with the degraded products of peroxynitrite ($n=8$). Further increasing peroxynitrite to $500\mu\text{M}$ induced an unspecific cell injury and was not possible for the patch-clamp experiments.

Fig. 4. A bar graph shows the effect of peroxynitrite on BK channels at different concentrations. DP: degraded product of peroxynitrite.

Fig. 5. A channel recording showing the effect of 100 μ M 8-bromo-cGMP on the BK channels (A) and the effect of H₂O₂ on BK channels in the presence of 100 μ M 8-bromo-cGMP (B). (C) A bar graph summarizes the effect of H₂O₂ on BK channels in the absence and presence of 8-bromo-cGMP.

After showing that H₂O₂ stimulates BK channels by a NO-dependent mechanism, we explored the possibility that the effect of H₂O₂ on BK channels was mediated by NO-cGMP pathway. Fig. 5 summarizes the effect of 8-bromo-cGMP on BK channels in the cultured endothelial cells. Application of 8-bromo-cGMP (100 μ M) increased BK channel activity by $250 \pm 30\%$ ($N=4$). Moreover, application of H₂O₂ did not further increase BK channel activity in the presence of 100 μ M 8-bromo-cGMP ($n=3$). Thus, the effect of H₂O₂ and cGMP was not additive. The notion that the stimulatory effect of H₂O₂ on BK channels was the result of increasing cGMP is also supported by experiments in which the effect of H₂O₂ on BK channels was examined in the presence of 1 μ M ODQ, an inhibitor of sGC. Although ODQ did not alter the basal level of BK channel activity, inhibition of sGC completely abolished the stimulatory effect of H₂O₂ on BK channels ($n=9$) (Fig. 6). This suggests that the effect of H₂O₂ on BK channels is the result of stimulating sGC and increasing cGMP. Because cGMP could stimulate protein kinase G (PKG) or protein kinase A (PKA) by inhibiting cGMP-sensitive phosphodiesterase [14], we next examined whether PKG or PKA was involved in mediating the effect of H₂O₂ on BK channels in the endothelial cells. Data summarized in Fig. 6 show that inhibiting PKG with 1 μ M KT5823 ($n=8$), but not inhibiting PKA with 0.5 μ M KT5720 ($n=7$), abolished the effect of H₂O₂ on BK channels. Therefore, the

Fig. 6. A bar graph shows the effect of H₂O₂ (100 μ M) on BK channels in the presence of 1 μ M ODQ, 1 μ M KT5823 or 0.5 μ M KT5720. Asterisk indicates the significant difference between control and experimental groups.

stimulatory effect of H₂O₂ on BK channels is mediated by NO-cGMP-PKG pathway.

Discussion

Several studies have reported that H₂O₂ stimulates NO production and cGMP production in endothelial cells [15, 16]. Moreover, H₂O₂ has been shown to cause vasorelaxation by direct oxidation of cGMP-dependent protein kinase G [17]. Also, it is known that H₂O₂ activates BK channels in endothelial cells [11]. Thus, the main contribution of the present study is to demonstrate that H₂O₂ stimulates BK channels in the cultured endothelial cells by a NO-dependent mechanism. Although eNOS activity was expected to be low under Ca²⁺-free pipette solution (equivalent intracellular solution) before adding H₂O₂, inhibition of NOS abolished the effect of H₂O₂ on BK channels. This suggests H₂O₂ may stimulate NO release by a Ca²⁺-independent mechanism. In this regard, it has been reported that stimulation of G-protein could activate eNOS and increase NO production in lung microvascular endothelial cells by a Ca²⁺-independent mechanism [18]. BK channels are located in both smooth muscle cells and endothelial cells [3, 19] and play an important role in the regulation of vasoactivity. The regulation of BK channels in smooth muscle cells is intensively studied and has been demonstrated to be

stimulated by NO, CO and epoxyeicosatrienoic acid (EET) and to be inhibited by 20-hydroxyeicosatetraenoic acid (20-HETE) [19-23]. In contrast, the regulatory mechanism of BK channels in endothelial cells is poorly understood. We have previously demonstrated that BK channels in the cultured endothelial cells were stimulated by NO and CO. Now we have demonstrated that H_2O_2 is also involved in stimulating BK channels in the endothelial cells.

H_2O_2 has been suggested to be a candidate of endothelial derived hyperpolarization factor (EDHF) which causes vasodilation [6, 24, 25]. The production of H_2O_2 is increased by stimulation of eNOS activity induced by acetylcholine [6]. Moreover, NO may have a positive feed back effect on the generation of H_2O_2 because activation of NOS increases dilatory H_2O_2 production [26]. The notion that increasing eNOS activity stimulates the H_2O_2 generation was further supported by the observation that deletion of eNOS diminished the H_2O_2 generation induced by acetylcholine. In rat femoral artery, acetylcholine stimulates H_2O_2 generation in endothelial cells and H_2O_2 may be one of mediators for the acetylcholine-induced vasodilation [8]. Although H_2O_2 has been suggested to be involved in mediating the effect of acetylcholine on vasoactivity, the mechanism by which H_2O_2 causes vasodilation is not completely understood. Several studies indicate that the vasodilation induced by H_2O_2 is the result of stimulating Ca^{2+} -activated K channels in endothelial cells [11], Ca^{2+} -activated BK channels [1, 10, 27] and ATP-sensitive K channels in vascular smooth muscle [28].

The present study has suggested that H_2O_2 -induced stimulation of BK channels in endothelial cells may be partially responsible for H_2O_2 -mediated vasodilation. However, it is unlikely that H_2O_2 -induced activation of BK channels is the only factor which mediates the vasodilation in response to H_2O_2 because K channels other than BK channels have also been shown to play a role in determining the membrane potential in endothelial cells [3]. H_2O_2 has been shown to stimulate BK channels in smooth muscle cells. But, the mechanism by which H_2O_2 stimulates BK channels in smooth muscle cells may be different from that in endothelial cells. It has been shown that exogenous H_2O_2 could still cause vasodilation in the endothelium-stripped artery [6]. This suggests that a factor other than NO mediates the effect of H_2O_2 on K channel activity. In this regard, it has been reported that H_2O_2 -induced vasodilation is induced by stimulating cyclooxygenase and increasing PGE_2 production which activates BK channels in smooth

muscle cells [10, 27]. In contrast, three lines of evidence strongly suggested that H_2O_2 -induced stimulation of BK channels in endothelial cells is mediated by a NO-cGMP-dependent pathway: 1) Inhibition of either NOS or sGC completely abolished the effect of H_2O_2 on BK channels; 2) The stimulatory effect of H_2O_2 and cGMP on BK channels was not additive; 3) Inhibition of PKG abolished the effect of H_2O_2 on BK channels. Although our data support the role of NO-cGMP in mediating the effect of H_2O_2 on BK channels, the conclusion was largely based on experiments in which pharmacological approaches were used. Thus, it is desirable in the future study to directly measure cGMP level or protein kinase G activity in response to H_2O_2 treatment to strengthen the conclusion that H_2O_2 stimulates BK channels by NO-cGMP pathway in HUVEC. In addition to stimulation of PKG, cGMP has been demonstrated to inactivate phosphodiesterase and increase cAMP production which stimulates PKA [14]. However, the observation that inhibition of PKA did not abolish the stimulatory effect of H_2O_2 on BK channels excluded the possibility that PKA is responsible for mediating the effect of H_2O_2 on BK channels.

Not only causing vasodilation, H_2O_2 has also been shown to be an endothelium-dependent contracting factor in rat renal artery [29] and acts as a vasoconstrictor in resistance vessels and contributes to the regulation of blood pressure [30]. Moreover, several studies reported that H_2O_2 inhibited BK channels in smooth muscle cells [31] and endothelial cells [32]. It has been shown that superoxide anions inhibit α -subunit of BK channels by altering cysteine-mediated Ca^{2+} -sensing [10]. Also, it has been demonstrated that H_2O_2 inhibited α subunit of BK channels expressed in HEK293 cells by oxidation of cysteine residue 911 [33]. However, we have now shown that H_2O_2 stimulates BK channels in HUVEC. The discrepancy may be caused by two different experimental conditions. First, those experiments were performed in HEK cells transfected with BK channel α -subunit and in excised patches. Thus, it is unlikely that H_2O_2 could stimulate NOS under such experimental conditions. Second, a high concentration of H_2O_2 (>1 mM) was used in those experiments while a low concentration of H_2O_2 (0.1 mM) was employed in the present experiment. As a matter of fact, application of 250 μ M H_2O_2 had no effect on BK channel activity expressed in HEK293 cells in that study [33]. Considering the low permeability of endothelial cells to H_2O_2 , the actual intracellular

concentration of H_2O_2 would be even lower than 0.1 mM. We speculate that ROS-induced inhibition of BK channels by modulating cysteine residue may be overridden in intact endothelial cells because H_2O_2 stimulates NO-cGMP signaling. Another possible interpretation for the controversial observation about H_2O_2 effect on vasoactivity may be that the oxidation status of the tissue could affect the final effect of H_2O_2 on BK channels. It is well established that NO could interact with superoxide to form peroxynitrite [34, 35] which has been shown to inhibit Ca^{2+} -activated BK channels in smooth muscle cells in rat cerebral artery and human coronary arterioles [36, 37]. If tissue has a high superoxide level, increased NO release induced by H_2O_2 could interact with superoxide to form peroxynitrite which inhibits K channels in vascular tissue and causes vasoconstriction. Indeed, peroxynitrite (100 μM) has been shown to inhibit BK channels expressed in HEK293 cells [33]. However, the observation that peroxynitrite (up to 100 μM) had no significant effect on BK channels in the cultured endothelial cells suggests that BK channels in endothelial cells may not be sensitive to the interaction between superoxide and NO. Alternatively, different beta-subunit of BK channels expressed in smooth muscle cells and endothelial cells may modify the effect of peroxynitrite on BK channel activity.

The effect of H_2O_2 on the BK channels in the endothelial cells should contribute to the H_2O_2 -induced vasodilation. H_2O_2 activates BK channels and hyperpolarizes the membrane of endothelial cells. As consequence of increasing driving force for Ca^{2+} entry, H_2O_2 should increase the intracellular Ca^{2+} concentration which stimulates NO production [38, 39]. Related to this speculation is the report that H_2O_2 has been shown to stimulate NOS and increase NO production in vessels [12, 13]. Since NO is known to stimulate BK channels in endothelial cells and smooth muscle cells [1, 23, 40], the synergized effect of H_2O_2 on BK channels in both smooth muscle cells and endothelial cells should lead to vasodilation. We conclude that H_2O_2 stimulates BK channels in the cultured endothelial cells and that the effect of H_2O_2 on BK channels is mediated by PKG.

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