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Ethanol enhances endothelial ionic currents and nitric oxide release via intermediate-conductance calcium-activated potassium channel

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ABSTRACT

Aims: Ethanol is known to induce NO release and coronary vasorelaxation. Evidence suggests that K^+ channels, especially a Ca^{2+}-activated K^+ channel (K_{Ca}), may regulate endothelial NO production. We aimed to investigate the ethanol effect on K^+ currents in human coronary artery endothelial cells (HCAECs), identify the K^+ channel type/subtype and signaling pathway involved, and demonstrate the relevance to ethanol-induced NO release.
Main methods: Ionic currents of cultured HCAECs were studied using whole-cell patch clamp technique. NO production were measured using the fluorescent probe, 2,3-diaminonaphthalene.
Key findings: We found that ethanol significantly potentiated HCAEC current (maximal increase to 155.68 ± 18.93%, 20 mM ethanol, +80 mV; mean ± SEM, $n = 9$). Ethanol-induced current was significantly inhibited by blockers of IK_{Ca} or SK_{Ca} (intermediate- or small-conductance K_{Ca}), but not by blocking other K^+ channels. When other known HCAEC channels were inhibited except IK_{Ca}, 20 mM ethanol significantly increased IK_{Ca} current to 198 ± 25.11% ($n = 6$), but it could not enhance SK_{Ca} current that was similarly isolated. Moreover, ethanol-induced NO release was prevented by blocking IK_{Ca} channel, adenosine A_{2A} receptor ($A_{2A}R$), G_s protein, or protein kinase A (PKA).
Significance: This study was the first to demonstrate that acute ethanol exposure could activate endothelial IK_{Ca} channel, via $A_{2A}R$-G_s-PKA signaling, leading to increased whole-cell current and NO release, which could be an important mechanism underlying ethanol-induced NO release and vasodilation.

1. Introduction

Studies in humans and animals showed that ethanol could acutely induce vasodilation or increase blood flow in many vascular beds, including carotid [1], brachial [2], cerebral [3,4], and coronary [5] arteries. In cultured human umbilical vein endothelial cells (HUVECs) and bovine aorta, it was found that acute low dose ethanol application activated endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production [6,7]. Studies in HUVECs further demonstrated that NO release is regulated by endothelial K^+ channels, especially small- and intermediate-conductance calcium-activated K^+ channels (SK_{Ca} and IK_{Ca}) [8]. In another study, acute ethanol application in HUVECs can potentiate large-conductance calcium-activated K^+ channel (BK_{Ca}) activity causing hyperpolarization [9], an initial step mediating agonist-stimulated NO release, which is followed by increased Ca^{2+} entry, increased intracellular Ca^{2+}, activation of NO production, and vasodilation [8,10].

Ethanol may exert its effects on endothelial cells via adenosine receptors. In HUVECs, ethanol increased cell survival via adenosine receptor-mediated protein kinase pathway [11]. Moreover, adenosine agonists were shown to cause hyperpolarization by activating K^+ channels (BK_{Ca} and K_{ATP}, ATP-sensitive K^+ channel) in porcine coronary artery endothelial cells [12]. Protein and mRNA of both adenosine A_{2A} and A_{2B} receptor subtypes have been demonstrated in human and porcine coronary artery endothelial cells [13].

Human coronary artery endothelial cell (HCAEC) ion channels contribute significantly to the regulation of human coronary circulation [14,15]. Major classes of endothelial ion channels are also found in HCAECs, namely, nonselective cation channels (NSC), Cl^- channels, and K^+ channels, including three types of Ca^{2+}-activated K^+ channels (BK_{Ca}, IK_{Ca}, and SK_{Ca}), K_{ATP}, and inwardly rectifying K^+ channels (K_{ir}) [16,17]. However, despite the evidence that ethanol causes coronary vasodilation [5], no study has demonstrated the role of endothelial ion channels in mediating the ethanol effects in the coronary vessels. We

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hypothesized that one of the ways by which ethanol exerts its effect may be to increase ionic currents through one or more K^+ channels in HCAECs leading to NO release. We demonstrated, using whole-cell patch clamp technique, the type of K^+ channel, adenosine receptor subtype, and intracellular pathway involved. Moreover, the role of the ion channel in ethanol-induced NO release was studied using a fluorescent probe of NO and the channel's blocker.

2. Materials and methods

2.1. Cell culture

Cultured HCAEC (3rd passage) was obtained commercially (Lonza, Walkersville, MD, USA), and kept in an endothelium-specific medium as suggested by the manufacturer. The medium was changed at 16–24 h after seeding and every other day thereafter. HCAECs (passages 4–8) were subcultured when they were 70–80% confluent and then plated on 35 mm petri-dishes containing poly-L-lysine-coated coverslips. Cells were kept in a humidified incubator, 37 °C and 5% CO_2, for at least 3–4 h before an electrophysiological experiment. This research was approved by Siriraj Institutional Review Board, Mahidol University, Bangkok, Thailand [078/2558(Exempt)].

2.2. Chemicals

All chemicals were obtained from Sigma-Aldrich, unless otherwise indicated. These are ethanol (Merck, Kenilworth, NJ, USA), apamin, $BaCl_2$, CGS-15943 (9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine), clotrimazole (Calbiochem, San Diego, CA, USA), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), DMSO (dimethyl sulfoxide), glibenclamide, H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), iberiotoxin (Abcam, Cambridge, UK), $LaCl_3$, NF-449 (4,4′,4″,4‴-[carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid octasodium salt; Abcam), and SCH-58261(7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine). Iberiotoxin, apamin, Ba^{2+}, La^{3+}, and NF-449 were dissolved in deionized water. Clotrimazole, glibenclamide, DIDS, CGS-15943, SCH-58261, and H-89 were dissolved in DMSO. The final DMSO volume was < 0.3% v/v, which did not affect HCAEC currents ($n = 4$; data not shown).

2.3. Electrophysiological studies

Membrane currents were recorded in the whole-cell patch clamp configuration, in response to a voltage ramp protocol (a 200-ms pulse, from −110 to +80 mV), using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Molecular Device, CA, USA) and a Digidata 1440A analog-to-digital converter (Axon Instruments). The holding potential was −40 mV. The microelectrode resistance was 2–5 MΩ in the bath solution. The *external solution* contained (in mM): 124 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 5 HEPES, and 58 D-mannitol (320 ± 5 mOsmol/kg, pH 7.3, adjusted with NaOH). D-mannitol concentration was varied to maintain the same external osmolality in 1–50 mM ethanol. The *internal solution* contained (in mM): 140 KCl, 1 $MgCl_2$, 4 $CaCl_2$, 5 HEPES, 7 EGTA, and 3 ATP (290 ± 5 mOsmol/kg, pH 7.2, adjusted with KOH). Calculated free [Ca^{2+}] was 390 nM (Ca-EGTA Calculator 1.3, http://maxchelator.stanford.edu, using constants from Theo Schoenmakers' Chelator). Cells were superperfused with or without tested substance in the external solution. All recordings were made after at least 3-minute equilibration in a new solution. Washout currents were obtained where possible. Whole-cell currents were normalized with cell capacitance and expressed as current density (pA/pF). All experiments were performed at room temperature (22–24 °C).

In six-blocker experiments, currents were recorded in the presence of six blockers which inhibited all known HCAEC channels except for the one being studied. For *IK_{Ca} recording solution*, blockers added to the external solution were 100 nM iberiotoxin, 100 nM apamin, 100 μM Ba^{2+}, 10 μM glibenclamide, 250 μM DIDS, and 10 μM La^{3+}, for blocking BK_{Ca}, SK_{Ca}, K_{ir}, K_{ATP}, Cl^-, and NSC channels, respectively [18–23]. Thus, IK_{Ca} channel was not inhibited. For *SK_{Ca} recording solution*, 100 nM apamin in the IK_{Ca} recording solution was substituted by 10 μM clotrimazole [24], and so SK_{Ca} was not blocked. Similarly, *K_{ATP} recording solution* contained 10 μM clotrimazole instead of 10 μM glibenclamide in the IK_{Ca} recording solution, so mainly K_{ATP} current could be recorded.

2.4. NO production measurement

NO production was measured using a commercial NO assay kit (Abcam), which employed the nitrite-sensitive fluorescent probe, 2,3-diaminonaphthalene (DAN) [25,26]. The culture medium of HCAECs under basal conditions or 30-minute treatment (20 mM ethanol, 10 μM clotrimazole, or both), was collected and centrifuged at 4 °C (11,000 rpm, for 5 min). Then the nitrate in the supernatant was converted to nitrite, using nitrate reductase, before adding the fluorescent probe; thus the resulting changes in fluorescent intensity represented the sum of nitrate and nitrite, i.e., total NO, produced. The excitation and emission wavelengths were 360 and 450 nm, respectively. The fluorescent intensity was detected by SpectraMax M5 spectrofluorometer (Molecular Device) and, after subtracting out the background intensity, was converted to nitrite concentration using a standard curve constructed in each experiment. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad, Munich, Germany), using bovine serum albumin (Sigma-Aldrich) as a protein standard. Values are normalized with protein concentration in the supernatant (pmol/mg protein).

2.5. Data and statistical analyses

Data derived from electrophysiological recordings were initially analyzed with pClamp 10.0, followed by simple calculations using Microsoft® Excel, and expressed as mean ± SEM. Current density-voltage (I-V) curves and bar graphs were plotted using GraphPad PRISM 5 (GraphPad Software, San diego, CA, USA). Data from a cell were discarded if the current amplitude at +80 mV was < 25 pA, the seal resistance was < 800 MΩ, or the maximum voltage error was > 3 mV. Data were tested for normality using Kolmogorov-Smirnov test. Data derived from NO measurement were expressed as mean ± SD of three independent experiments. For all data, %control refers to the percentage of response compared to that before adding a test substance; %difference or %increase refers to the percentage of the difference in response between test and control condition (test – control) compared to control; and %inhibition refers to percentage of response inhibited by a blocker compared to the response before the inhibition. One sample *t*-test or Wilcoxon signed rank test, as appropriate, was used to test the difference of a population's value with a specified mean (e.g. zero or 100%). To compare the mean between two matched groups, paired *t*-test or Wilcoxon signed rank test was used, as appropriate. For comparisons among three groups or more, one-way analysis of variance (ANOVA) with Tukey's multiple comparison or Newman-Keuls multiple comparison test was employed, as appropriate. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Acute ethanol application increases total HCAEC current

Fig. 1A shows an example of a current increase in a representative cell after exposure to 20 mM ethanol for 3–5 min. The average results from nine cells were plotted as current density-voltage (I-V) curves in Fig. 1B. Whole-cell currents in 1, 3, 10, 20, 30, and 50 mM ethanol, expressed in %control at +80 mV [mean ± SEM (n)], were

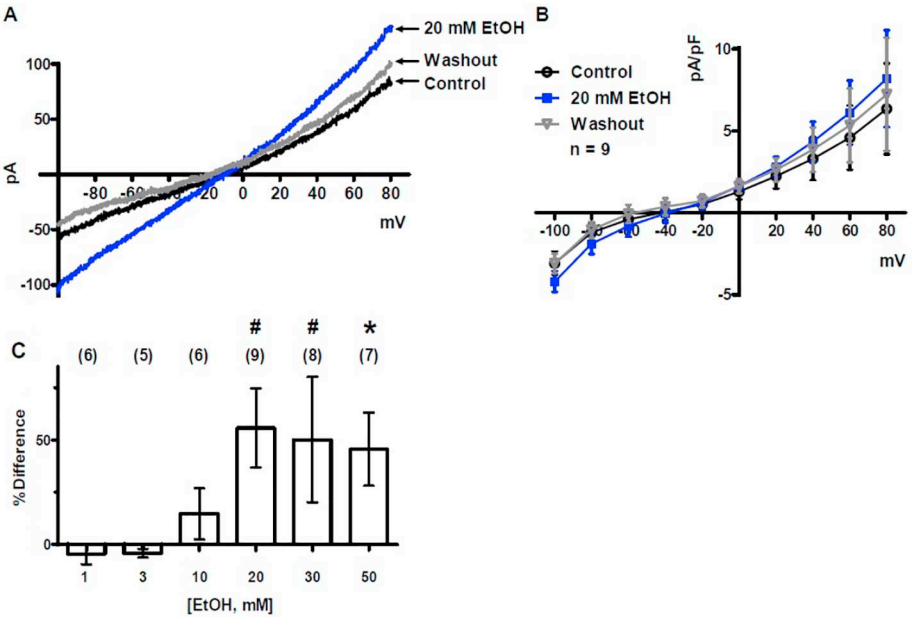

Fig. 1. Acute ethanol exposure increases total HCAEC currents. *A*, Representative traces of total HCAEC currents, before, during, and after 20 mM ethanol application (*Control*, *20 mM EtOH*, and *Washout*, respectively) are shown. *B*, Average I-V curves comparing current density in control, 20 mM ethanol, and washout ($n = 9$). *C*, %Difference of currents after 1–50 mM ethanol compared to before ethanol application (at +80 mV). $^{*}P < 0.05$, one-sample *t*-test or $^{\#}$Wilcoxon signed rank test (data not normally distributed), compared with control. Error bars are SEM; numbers in parentheses indicate sample size.

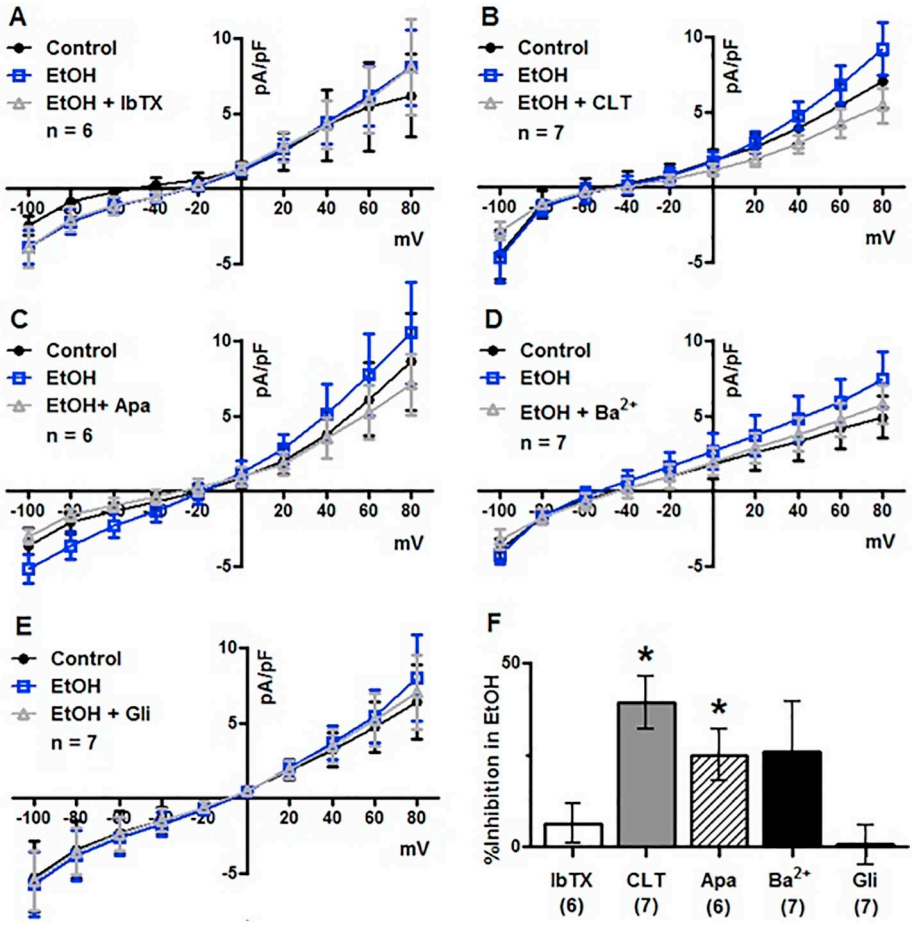

Fig. 2. Ethanol may increase total HCAEC currents via IK_{Ca} and/or SK_{Ca} channel activation: one-blocker experiment. *A–E*, Average I-V curves of current density in control or no ethanol (*Control*, black circles), in ethanol alone (*EtOH*, blue squares), and in ethanol with added blocker (*EtOH* + *blocker*, gray triangles); IbTX, 100 nM iberiotoxin; CLT, 10 μM clotrimazole; Apa, 100 nM apamin; Gli, 10 μM glibenclamide; n, sample size. *F*, %Inhibition of currents in ethanol by each K^+ channel blocker at +80 mV (IbTX, CLT, and Apa) or −100 mV (Gli and 100 μM Ba^{2+}); $^{*}P < 0.05$, one-sample *t*-test compared with EtOH. Error bars are SEM; numbers in parentheses indicate sample size. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

95.42 ± 4.84 (6), 95.75 ± 2.05 (5), 114.60 ± 12.26 (6), 155.68 ± 18.93 (9), 150.08 ± 29.97 (8), and 145.51 ± 17.49% (7), respectively. Significant increases in total HCACE current was found with exposure to 20, 30, and 50 mM ethanol ($P < 0.05$; plotted as % difference in Fig. 1C). Washout currents after 1, 3, 10, 20, 30, and 50 mM ethanol (%control; +80 mV) were 87.28 ± 12.00 (6), 87.80 ± 10.18 (5), 102.92 ± 17.58 (3), 109.33 ± 7.14 (9), 74.11 ± 23.49 (2), and 158.36 ± 88.86% (2), respectively. We noticed that gigaseals were less stable in higher ethanol solutions (30 and 50 mM) and washout current could be obtained from fewer cells. Therefore, in the remaining experiments, 20 mM ethanol was used. We found that application of 20 mM ethanol for up to 60 min did not affect HCAEC viability (see Fig. S1, Supplementary information).

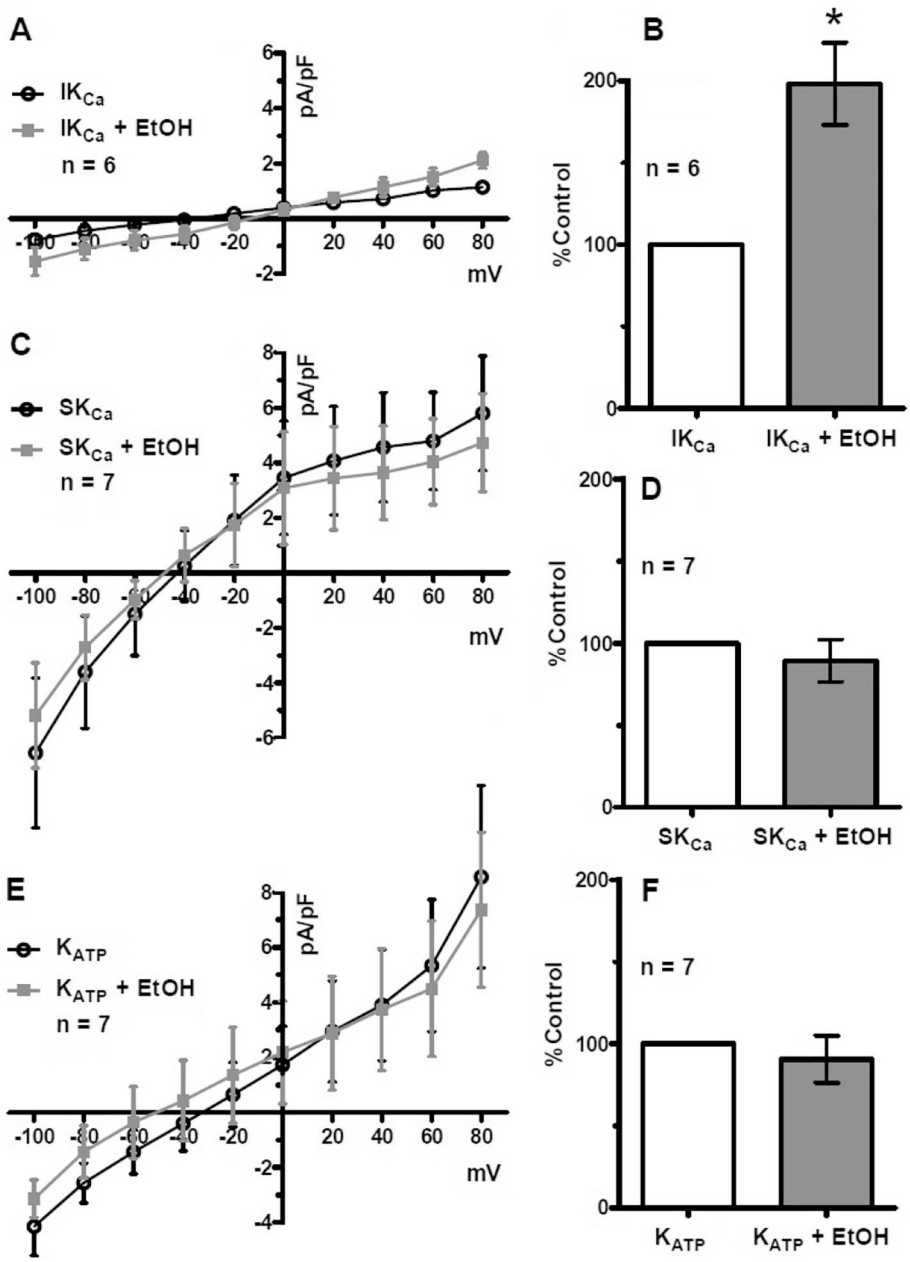

Fig. 3. Ethanol increases total HCAEC currents by enhancing IK_{Ca} current: six-blocker experiment (see text for details). *A*, Average I-V curves and *B*, % control current density at +80 mV, comparing results in IK_{Ca} recording solution without and with ethanol (*IK_{Ca}* and *IK_{Ca} + EtOH*, respectively). *C–D*, Similar plots in SK_{Ca} recording solution (*SK_{Ca}* vs *SK_{Ca} + EtOH*). *E* and *F*, Average I-V curves and % control current density at −100 mV, comparing currents in K_{ATP} recording solution without and with ethanol (*K_{ATP}* and *K_{ATP} + EtOH*, respectively). Only currents in IK_{Ca} recording solution could be increased by ethanol. *$P < 0.05$, one sample *t*-test compared to without ethanol; n, sample size. Error bars are SEM.

3.2. Ethanol increases IK_{Ca} current in HCAECs

3.2.1. One-blocker experiments

To determine the type of K^+ current affected by ethanol, a blocker of each known K^+ channel in HCAEC was tested on ethanol-induced current. These were: 100 nM iberiotoxin, 10 μM clotrimazole, 100 nM apamin, 100 μM Ba^{2+}, or 10 μM glibenclamide, for blocking BK_{Ca}, IK_{Ca}, SK_{Ca}, K_{ir}, or K_{ATP} channel, respectively. Cell current was first recorded in 20 mM ethanol (control), followed by 20 mM ethanol plus a blocker (Fig. 2A-E). Significant inhibition of ethanol-increased current was found with clotrimazole and apamin (Fig. 2F). The %controls (with blocker compared to without, both in ethanol; +80 mV) were: clotrimazole, 60.66 ± 7.17% ($n = 7$, $P = 0.0015$) and apamin, 74.98 ± 7.13% ($n = 6$, $P = 0.0171$). [Washout currents (current in ethanol after removal of a blocker) in %control (+80 mV) were: clotrimazole, 84.16 ± 13.02% ($n = 6$, $P = 0.2780$) and apamin, 89.66 ± 12.27% ($n = 4$, $P = 0.6250$).] Conversely, iberiotoxin, Ba^{2+}, and glibenclamide did not significantly inhibit the ethanol effect: % control: iberiotoxin, 93.70 ± 5.41% (+80 mV, n = 6, $P = 0.2970$); Ba^{2+}, 74.20 ± 13.87% (−100 mV, n = 7, $P = 0.1123$); and glibenclamide, 99.46 ± 5.32% (−100 mV, n = 7, $P = 0.9231$), respectively. [Washout current in %control was: iberiotoxin, 97.11 ± 10.63% ($n = 5$, $P = 0.7997$); Ba^{2+} 104.13 ± 17.43% (n = 6, $P = 0.8221$); and glibenclamide, 110.66 ± 16.40% (n = 6, $P = 0.5445$).] Apparently, ethanol-induced total HCAEC current increase could be mediated via activation of IK_{Ca} and/or SK_{Ca} channels.

3.2.2. Six-blocker experiments

In the previous experiment, it was also possible that blockers may have inhibited already opened channels not activated by ethanol. In the next set of experiments, we tested if ethanol could still exert its effect in the presence of six blockers which inhibited all known HCAEC channels except for the one tested. Superfusing HCAEC with *IK_{Ca} recording solution* (see Materials and methods) reduced the cell current to 43.17 ± 5.05% ($n = 6$); this remaining current should consist mainly of IK_{Ca} current. Addition of 20 mM ethanol significantly increased this remaining current by 98.00 ± 25.11% (vs before ethanol, n = 6, $P = 0.0114$; Fig. 3A–B). [Washout current (on return to six-blocker

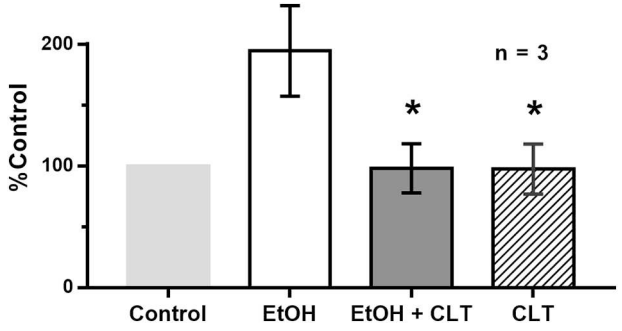

Fig. 4. Blocking IK_{Ca} channel reduces ethanol-induced nitric oxide release. NO levels (in pmol/mg protein) in the supernatant were determined from the relative fluorescent signal of the nitrite-sensitive dye 2,3-diaminonaphthalene, and expressed as %Control (percentage of NO levels in cells exposed to neither ethanol nor clotrimazole, Control bar = 100%). CLT, pre-incubated in 10 μM clotrimazole for 3 min before ethanol exposure; EtOH, incubation in 20 mM ethanol for 30 min. *$P < 0.05$, ANOVA with Tukey's multiple comparison. Error bars are SD.

solution again, after ethanol) was 117.83 ± 22.23% vs before ethanol ($n = 4$, $P = 0.9044$).] Thus, IK_{Ca} channel likely mediated the ethanol-induced HCAEC current.

SK_{Ca} recording solution reduced the control current to 55.84 ± 7.45% ($n = 7$), which presumably contained mainly SK_{Ca} current. Applying 20 mM ethanol, however, did not significantly change the remaining current at +80 mV (current in ethanol was 89.41 ± 12.91% vs before ethanol; $n = 7$, $P = 0.4433$; Fig. 3C–D). [Washout current was 94.03 ± 17.35% vs before ethanol ($n = 6$, $P = 0.7448$).] Thus, SK_{Ca} channel was not involved in ethanol-induced HCAEC current. Finally, we tested the ethanol effect on K_{ATP} current as a negative control. The remaining current in *K_{ATP} recording solution* was 72.30 ± 11.31% of cell current (at −100 mV, n = 7, $P = 0.0498$). Ethanol at 20 mM did not significantly change the current at −100 mV (90.62 ± 14.35% vs before ethanol, n = 7, $P = 0.5376$; Fig. 3E–F). [Washout current was 88.25 ± 21.30% (vs before ethanol, at −100 mV, n = 6, $P = 0.6048$).]

3.3. Blocking IK_{Ca} channel reduces ethanol-induced nitric oxide release

To test the physiological relevance of ethanol-induced IK_{Ca} increase, NO production of HCAEC in ethanol without and with 10 μM clotrimazole were compared. Cells were pretreated with clotrimazole for 3 min before 20 mM ethanol was added to the culture medium for a 30-minute exposure. Results demonstrated that clotrimazole could inhibit ethanol-induced NO increase: %control of NO levels (mean ± SD) in ethanol alone, ethanol + clotrimazole and clotrimazole alone were 194.85 ± 37.36%, 98.13 ± 20.20%, and 97.59 ± 20.60%, respectively ($P < 0.05$; Fig. 4). Thus IK_{Ca} may play an essential role in ethanol-induced NO increase in HCAECs.

3.4. Adenosine receptor subtype 2A, G_s protein, and protein kinase A mediate the ethanol effects

To test whether adenosine receptor was involved in ethanol-induced HCAEC current increase, cells were pretreated (by adding to the culture medium), for at least 10 min, with 10 μM CGS-15943, a nonspecific adenosine receptor blocker [27]. Then currents were compared between before and during ethanol exposure, with 10 μM CGS-15943 present in the external solution throughout the experiment. Results showed that 20 mM ethanol could not increase total HCAEC currents in the presence of CGS-15943 (Fig. 5A–B and J): Ethanol-exposed current was 104.24 ± 6.72% of control (+80 mV; $n = 5$, $P = 0.5627$). Thus, an adenosine receptor mediates the ethanol effect.

To identify the subtype of adenosine receptor involved, cells were pretreated for at least 20 min before an experiment with 100 nM SCH-58261, a specific adenosine A_{2A} receptor ($A_{2A}R$) blocker [28]. In the continued presence of 100 nM SCH-58261, application of 20 mM ethanol could not increase HCAEC current: Ethanol-exposed current was 92.12 ± 11.40% of control (+80 mV; $n = 6$, $P = 0.5204$; Fig. 5C–D and J). Evidently, $A_{2A}R$ mediates the ethanol effect.

A_{2A} receptor has been known to signal via G_s protein and protein kinase A (PKA) [29]. Therefore, we confirmed the role of $A_{2A}R$ by performing similar experiments with blockers of this pathway's components. Similarly, results showed that 20 mM ethanol could not increase currents in HCAEC pretreated for at least 30 min with 10 μM NF-449, a specific G_s blocker [30], or for at least one hour with 10 μM H-89, a specific PKA blocker [31]. In cells pretreated with NF-449, currents in ethanol were 102.69 ± 5.99% of control ($n = 6$, $P = 0.6722$; Fig. 5E–F and J), while in those pretreated with H-89, currents in ethanol were 96.52 ± 7.72% of control ($n = 7$, $P = 0.6678$; Fig. 5G–H and J).

In parallel with the above experiments, we also tested ethanol effects on non-pretreated cells. We found that while ethanol could not significantly change total HCAEC currents in the presence of CGS-15943, SCH-58261, NF-449, or H-89 blockers, it could still increase total currents in non-treated cells (Fig. 5J, first bar graph, "No blocker"). In addition, there was no statistical difference in basal current density between cells pretreated with signaling pathway blockers ($n = 5$–7) and non-pretreated cells ($n = 6$) (see Fig. S2, Supplementary information).

Taken together, the data above indicated that $A_{2A}R$-G_S-PKA signaling pathway mediated ethanol-increased HCAEC currents.

4. Discussion

The effects of ethanol on K^+ currents in human coronary artery endothelial cells (HCAECs), as studied by whole-cell patch clamp technique, were investigated in this study. We found that ethanol could increase total HCAEC currents, with the highest increase to 155.68 ± 18.93% (at 20 mM). By examining all endothelial K^+ channels, and using multiple blockers simultaneously, we demonstrated that, among K^+ channels in HCAECs, only IK_{Ca} channel was principally involved in this ethanol effect. This is relevant to ethanol-induced NO release, because the IK_{Ca} channel blocker clotrimazole could inhibit NO production from cells exposed to ethanol. In addition, the stimulating effects of ethanol could be inhibited by pretreatment with SCH-58261, NF-449, or H-89, demonstrating that acute ethanol exposure acted via $A_{2A}R$, G_s protein, and PKA to enhance IK_{Ca} current in HCAEC. The rapid ethanol action (within 3–5 min) may reflect a non-genomic action similar to those reported previously [32–35].

The millimolar dosage range was consistent with several studies on acute ethanol effects in vascular tissues. For example, ethanol was found to increase BK_{Ca} open probability, eNOS activity, and NO production in HUVECs (10–50 mM) [6,9], enhance basal eNOS activity in bovine aortic endothelial cells (17 and 40 mM) [7,36], and increase porcine coronary vasodilation and guinea pig coronary flow (peaking at ~4 min) (0.17–515 mM) [32]. In contrast, ethanol was reported to cause vasoconstriction in dog epicardial coronary artery [37] and rat hepatic circulation [38]. Furthermore, ethanol could elicit inhibitory effects, such as decreasing BK_{Ca} activity of mouse brain (50–100 mM) [33] and HUVECs (100–150 mM) [9], which may explain the trend in the reduction of mean current increase by 30 and 50 mM ethanol, compared to 20 mM (Fig. 1C, although no statistical difference could be demonstrated).

To the best of our knowledge, this study is the first to demonstrate that endothelial IK_{Ca} channels could be a target of acute ethanol action. Investigations in cells other than HCAECs described ethanol effects on various channel types, but not IK_{Ca}. It has been found that ethanol could activate HUVEC BK_{Ca} channel [9], and also BK_{Ca} and/or SK_{Ca} channels

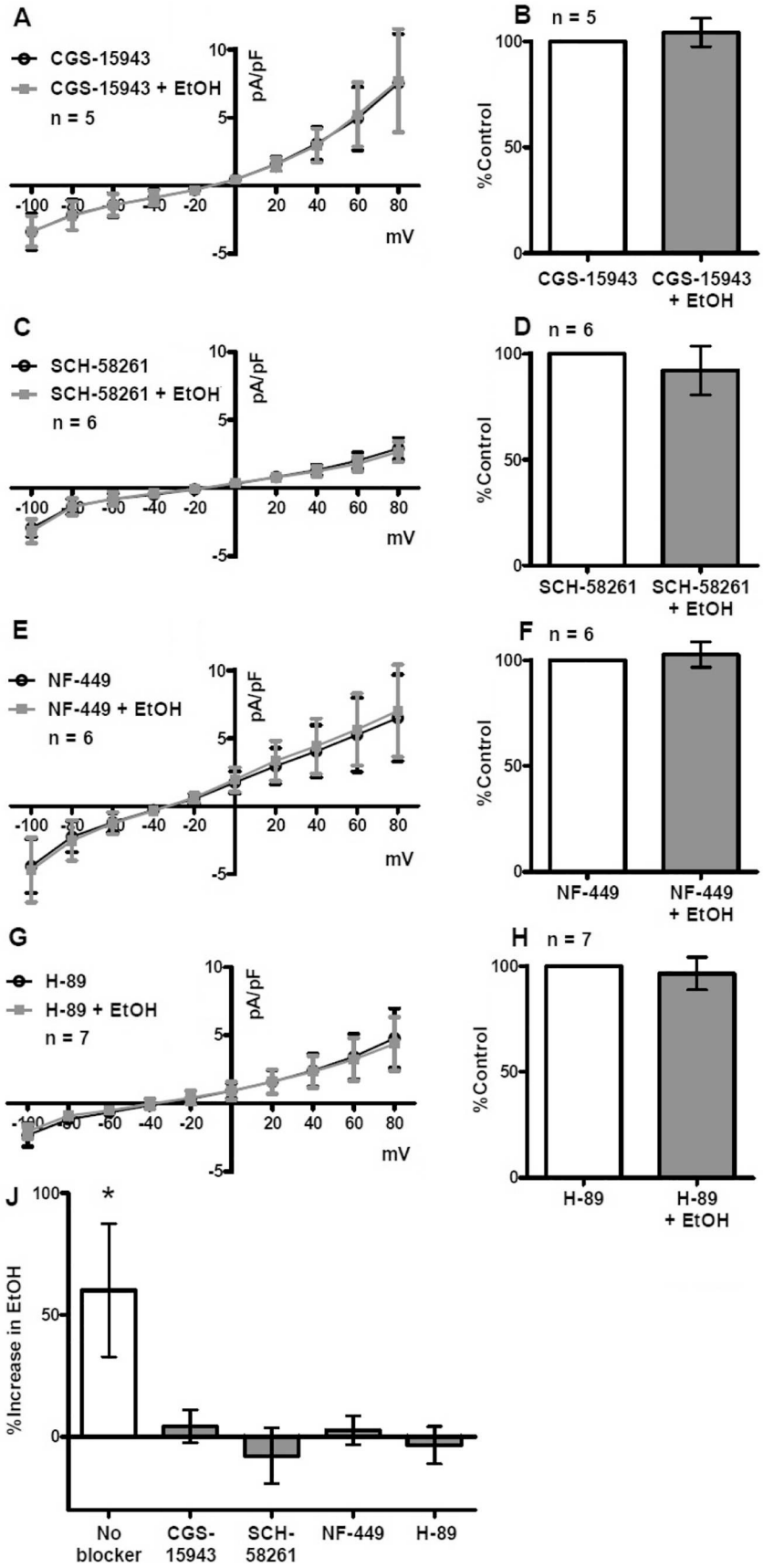

Fig. 5. Ethanol action is mediated by $A_{2A}R$-G_S protein-PKA signal pathway. HCAECs were pre-incubated in CGS-15943 (nonselective adenosine receptor blocker), SCH-58261 [adenosine A_{2A} receptor ($A_{2A}R$) blocker], NF-449 (G_s protein blocker), or H-89 (PKA blocker), and then exposed to 20 mM ethanol. *A*, *C*, *E*, and *G*, average I-V curves comparing current density before vs during ethanol exposure, in the presence of the indicated blocker; *B*, *D*, *F*, and *H*, bar graphs comparing % control current density at +80 mV of the indicated conditions (control refers to before ethanol exposure, i.e. in the presence of the blocker only). *J*, %increase of current density at +80 mV in ethanol (n = 6; $^*P < 0.05$, Wilcoxon signed rank test compared with before ethanol exposure). Ethanol could not increase currents in the presence of adenosine receptor, $A_{2A}R$, G_s protein and PKA blockers. n, sample size; all error bars are SEM.

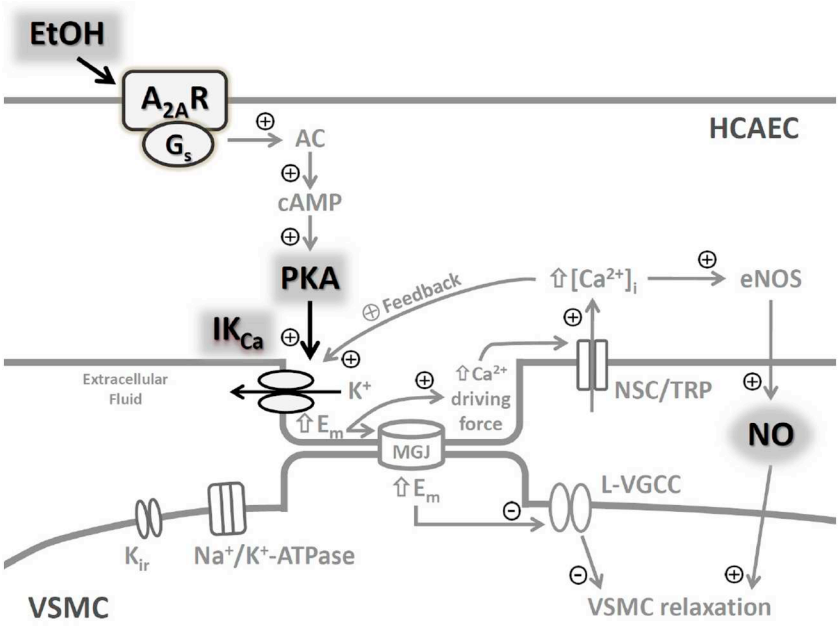

Fig. 6. A possible signaling pathway mediating the acute ethanol effects on whole-cell currents, as derived from our study. (See text for details.) Shadowed labels are those demonstrated by our data to be involved. AC, adenylate cyclase; $A_{2A}R$, adenosine A_{2A} receptor subtype; $\uparrow E_m$, membrane hyperpolarization; eNOS, endothelial nitric oxide synthase; EtOH, ethanol; HCAEC, human coronary artery endothelial cell; IK_{Ca} channel, intermediate-conductance Ca^{2+}-activated K^+ channel; K_{ir}, inwardly rectifying channel; L-VGCC, L-type voltage-gated Ca^{2+} channel; MGJ, myoendothelial gap junction; PKA, protein kinase A; NSC/TRP channel, nonselective cation/transient receptor potential channel; VSMC, vascular smooth muscle cell; ⊕, activation; ⊖, inhibition.

in neurons and vascular smooth muscle [33,39–43]. In addition, acute ethanol has been shown to affect GIRK channel [34] and TRP channels [32,35].

Similar to SK_{Ca}, IK_{Ca} channel protein is abundantly found in coronary artery endothelial cells of human and animals [13]. They may respond to agonists and cause hyperpolarization, $[Ca^{2+}]_i$ increase, and NO generation, leading to vascular smooth muscle cell (VSMC) hyperpolarization and relaxation [8,44]. In contrast to SK_{Ca}, IK_{Ca} channel is located near the myoendothelial gap junctions, the endothelial projections towards the underlying VSMCs [45], while SK_{Ca} is located in the endothelial caveolae. The intracellular segregation of these channels may explain the ethanol preferential activation of IK_{Ca}.

NO release is regulated by endothelial ion channels. For example, Sheng et al. [8] demonstrated in the endothelial cell line EA.hy926 that activation of SK_{Ca} and IK_{Ca} channels, leading to membrane hyperpolarization, was an initial step mediating agonist-stimulated NO release. In HUVECs, Kuhlmann and coworkers [9] showed that acute ethanol application can potentiate BK_{Ca} activity causing hyperpolarization. Moreover, NO synthesis was increased by ethanol, an effect attenuated by blocking K_{Ca} channel [9]. Therefore, in HUVECs ethanol may enhance vasodilation through activation of BK_{Ca} channel which consequently increased NO synthesis. In contrast, our data in HCAECs showed that ethanol mainly activated IK_{Ca}, and blocking IK_{Ca} could abolish NO release by ethanol.

Adenosine receptors have been suggested to mediate ethanol effects in endothelial cells [11]. We demonstrated in this study that the $A_{2A}R$ subtype and its conventional signaling, G_s protein-PKA pathway [46], are involved in HCAEC ethanol-induced current increase. Both $A_{2A}R$ and $A_{2B}R$, but not A_1R, are expressed in human and porcine coronary artery endothelial cells [13]. However, since pretreatment with SCH-58261, one of the most selective $A_{2A}R$ blockers [47], could completely inhibited the ethanol effect, it was less likely that ethanol exerted its action via $A_{2B}R$, and this may suggest a preferential IK_{Ca} activation by $A_{2A}R$. However, further study is needed to prove this supposition. Recently, it has been shown that a selective $A_{2A}R$ agonist could rapidly (30 s) increase NO production in endothelial cells derived from human iliac, porcine coronary and carotid arteries [28,48]. In addition, Olanrewaju et al. [12] demonstrated that $A_{2A}R$ induced porcine coronary artery endothelial hyperpolarization by activating K_{ATP} and BK_{Ca} channels, but IK_{Ca} involvement was not ruled out in the study. On the other hand, ethanol is known to directly affect some channels, especially in neuronal tissues [34,39,49,50]. However, it might be less likely in our system that ethanol could act directly on IK_{Ca} channels, since blocking the second messenger pathway with CGS-15943, SCH-58261, NF-449 or H-89 could virtually abolish the ethanol effect.

Ethanol-enhanced IK_{Ca} current, as well as the $A_{2A}R$-G_s-PKA signaling described in this study, may be involved in ethanol-induced vasodilatation [5]. As mentioned, it is known that IK_{Ca} channel is located within the myoendothelial gap junctions (MGJs) [51]. Opening IK_{Ca} channel leads to K^+ efflux and induces membrane hyperpolarization which can be transmitted to the surrounding VSMCs via the MGJs [45]; VSMC hyperpolarization lowers L-type voltage-gated Ca^{2+} channel activity, causing decreased Ca^{2+} influx and vasorelaxation. Moreover, K^+ accumulation in the intercellular space, a result of opening K^+ channels, can hyperpolarize VSMCs by activating Na^+/K^+-ATPase and K_{ir} channels, resulting in VSMC relaxation [52,53]. In endothelial cells, membrane hyperpolarization can enhance Ca^{2+} driving force, forming a positive-feedback that enhances intracellular Ca^{2+} signaling, leading to endothelial vasoactive substance release, especially NO, causing additional VSMC relaxation [44,54–56]. These may be the sequence of events leading from ethanol activation of endothelial IK_{Ca} channel to ethanol-induced vasodilation (Fig. 6).

4.1. Limitations

Our study employed whole-cell recording and therefore was limited to only membrane-associated components; cytosolic pathways could not be studied. However, the fact that blocking IK_{Ca} with clotrimazole could inhibit NO release from intact cells suggested that the pathway reported here and IK_{Ca} were the major players for the ethanol action. Another limitation was that we could not investigate ethanol effects at concentrations higher than 50 mM, because gigaseals were usually lost or could not be obtained. Previous reports found that higher than 200 mM ethanol could cause a loss of tight seal in inside-out patch experiments [33]. The apparent lower tolerance of our cells may be partly due to the whole-cell configuration. Finally, we could not exclude the effect of ethanol on TRP or Cl^- channels; these channels were blocked in our six-blocker experiments. However, from NO experiments we could say that either TRP or Cl^- channel has little role in ethanol-induced NO release.

5. Conclusions

Our data were the first to demonstrate that acute ethanol could activate IK_{Ca} channel, via $A_{2A}R$-G_s-PKA signaling pathway, leading to increased whole-cell currents and NO release in HCAECs. The results from this study shed lights on the mechanism underlying acute ethanol-induced vasorelaxation, and suggest that IK_{Ca} is critical to the ethanol action. Additionally, this knowledge could be employed for developing novel prevention and treatment strategies for cardiovascular diseases in the future. There have been examples of therapeutic ethanol use in other tissues (apart from topical disinfection), for instance, percutaneous ethanol injection to reduce tumor in hepatocellular carcinoma patients [57]. Thus, it is possible that ethanol injection may be used to temporarily and locally dilate vessels in some tissue or to test vascular function in the future. Alternatively, an activator of IK_{Ca} channel may similarly enhance vasodilatation without the toxic effects of ethanol.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.052.

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