0026-895X/05/6705-1723–1731$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 67:1723–1731, 2005

Vol. 67, No. 5
8953/1201289
Printed in U.S.A.

The Effect of Hydroxylamine on K_{ATP} Channels in Vascular Smooth Muscle and Underlying Mechanisms

Guanghua Tang, Lingyun Wu, and Rui Wang

Departments of Physiology (G.T., R.W.) and Pharmacology (L.W.), College of Medicine, University of Saskatchewan, Saskatoon, Canada

Received November 5, 2004; accepted February 16, 2005

ABSTRACT

Hydroxylamine (HA) is a putative intermediate in the conversion of L-arginine to nitric oxide (NO). HA was reported to cause the relaxation of precontracted aorta strips; however, the ionic mechanisms of HA-induced vasorelaxation were not yet known. In the present study, the whole-cell patch-clamp technique was used to examine the effects of HA on ATP-sensitive K^+ (K_{ATP}) currents and membrane potentials in vascular smooth muscle cells from rat mesenteric arteries and underlying mechanisms. It was found that bath-applied HA reversibly enhanced K_{ATP} currents in a concentration-dependent fashion with an EC_{50} of 54 ± 3.4 μM and hyperpolarized the cell membrane from −48 ± 5.2 to −65 ± 7.5 mV ($n = 6$, $p < 0.01$). The increase in K_{ATP} currents induced by HA was suppressed by superoxide dismutase (−380 ± 45 to −160 ± 20 pA, $n = 4$, $p < 0.01$) and *N*-acetyl-L-cysteine (−385 ± 55 to −150 ± 16 pA, $n = 5$, $p < 0.01$), indicating the involvement of different free radicals, including superoxide anion. Hypoxanthine/xanthine oxidase increased not only basal K_{ATP} currents, but also HA-enhanced K_{ATP} currents (from −355 ± 40 to −480 ± 62 pA, $n = 6$, $p < 0.05$). Sodium nitroprusside, a spontaneous NO donor, and a membrane-permeable cGMP analog (8-bromo-cGMP) were without effects on HA-enhanced K_{ATP} currents or basal K_{ATP} currents. Our results indicate that HA augmented K_{ATP} channel activity and hyperpolarized cell membrane, possibly via increased free radical generation.

Hydroxylamine (HA) is a natural product of cellular metabolism. The vasodilatory properties of HA have been documented in rabbit and rat aortic strips or rings (Rapoport and Murad, 1984; DeMaster et al., 1989; Thomas and Ramwell, 1989; Feelisch et al., 1994; Huang, 1998), rat kidney vessels (Moore et al., 1989), and rodent pulmonary vasculature (Santoian et al., 1993). HA is also a putative intermediate in the oxidative conversion of L-arginine to NO (DeMaster et al., 1989). This process requires a catalase-dependent reaction and involves the hydrolysis of oxime arginine to L-citrulline and *N*-hydroxylamine. *N*-Hydroxylamine is in turn converted by catalase to NO and superoxide anion (O_2^-) in the presence of hydrogen peroxide (H_2O_2) (Ohta et al., 1997; Klink et al., 2001); therefore, HA-induced vasorelaxation may be associated with the generation of HA-derived NO and O_2^- (DeMaster et al., 1989; Taira et al., 1997; Huang, 1998). NO acts on ion channels in vascular tissues either directly or indirectly by stimulating the soluble guanylyl cyclase (sGC)-cGMP pathway. For example, NO activates Ca^{2+}-activated K^+ (K_{Ca}) channels (Robertson et al., 1993; Bolotina et al., 1994), voltage-dependent K^+ (K_V) channels (Hermann and Erxleben, 2001), or K_{ATP} channels (Kubo et al., 1994; Murphy and Brayden, 1995) directly via a nitrosylation mechanism (Stamler, 1994) or indirectly via the NO-sGC-cGMP pathway (DeMaster et al., 1989; Thomas and Ramwell, 1989; Robertson et al., 1993). Nevertheless, whether the vasorelaxant effects of HA involve the activation of ion channels and hyperpolarization of the cell membrane has never been defined.

In the present study, it was hypothesized that the reactive oxygen species generated from the metabolism of HA to NO may activate K_{ATP} channels and lead to the hyperpolarization of cell membrane. The whole-cell patch-clamp recording technique was used to examine the effects of HA on K_{ATP} channel currents in freshly isolated single vascular smooth muscle cells (VSMC) from rat mesenteric artery. Whether

This work was supported by an operating grant from the Canadian Institutes of Health Research (CIHR) (to R.W.). R.W. and L.W. were supported by the Investigator Awards of CIHR. G.T. was supported by a Doctoral Research Award from the Heart and Stroke Foundation of Canada.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.008953.

ABBREVIATIONS: HA, hydroxylamine; sGC, soluble guanylyl cyclase; VSMC, vascular smooth muscle cell(s); PSS, physiological salt solution; 8-Br-cGMP, 8-bromo-cGMP; NAC, *N*-acetyl-L-cysteine; SOD, superoxide dismutase; SNP, sodium nitroprusside; Glib, glibenclamide; IbTX, iberiotoxin; HX, hypoxanthine; XO, xanthine oxidase; LY83583, 6-anilino-5,8-quinolinedione; X, xanthine; HP, holding potential; TP, testing potential; MP, maximum potential.

HA activated K_{ATP} channels via enhanced production of free radicals was further investigated.

Materials and Methods

Single VSMC Preparation. Single mesenteric artery VSMC were isolated according to our previously published method with modifications. In brief, male Sprague-Dawley rats (120–150 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg b.wt.). Small mesenteric arteries below the second branch off the main mesenteric artery were dissected and kept in ice-cold physiological salt solution (PSS) that contained 137 mM NaCl, 5.6 mM KCl, 0.44 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose (pH adjusted to 7.4 with NaOH). Connective tissues were gently removed under a dissecting microscope with surgical tweezers. The freshly isolated tissues were cut into 5-mm pieces and then incubated for 40 min at 37°C in Ca^{2+}-free PSS containing 1 mg/ml albumin, 0.5 mg/ml papain, and 1 mg/ml dithiothreitol and for another 30 min in the nominally Ca^{2+}-free PSS containing 1 mg/ml albumin, 0.8 mg/ml collagenase, and 0.8 mg/ml hyaluronidase. Single cells released by gentle triturating through a Pasteur pipette exhibited long and spindle shapes under a microscope. Cells were stored in Ca^{2+}-free PSS at 4°C and used within the same day of isolation.

Electrophysiological Recording of Membrane Potential and K_{ATP} Channel Currents. The whole-cell patch-clamp technique was used to record K_{ATP} channel currents. In brief, two or three drops of cell suspension were added to the recording chamber inside a Petri dish that was mounted on the stage of an Olympus IX70 inverted phase-contrast microscope (Olympus, Tokyo, Japan). Cells were left to stick to the glass coverslip in the recording chamber for 5 to 10 min before an experiment was started. Pipettes were pulled from soft microhematocrit capillary tubes (Fisher Scientific Co., Nepean, ON) with a tip resistance of 2 to 4 MΩ when filled with the pipette solution. Currents were recorded with an Axopatch 200-B amplifier (Axon Instruments Inc., Union City, CA) and controlled by a Digidata 1200 interface and pCLAMP software (version 6.0; Axon Instruments Inc.). Membrane currents were filtered at 1 kHz with a four-pole Bessel filter, digitized, and stored. At the beginning of each experiment, junction potential between pipette and bath solutions was electronically adjusted to zero.

In the current-clamp mode, the membrane potential of single VSMC was measured using the nystatin-perforated patch-recording technique as the current was held at 0 pA. A stable recording of membrane potential was achieved at least 2 min after the penetration of cell membrane. The bath solution contained 140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES, 2 mM EGTA, and 5 mM glucose (pH adjusted to 7.4 with NaOH). The pipette solution used in the nystatin-perforated whole-cell recording contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM glucose, and 250 μg/ml nystatin. Because nystatin may destabilize the cell, the appearance of nystatin at the tip of the electrode was avoided by dipping the pipette tip into a nystatin-free solution and backfilling the remainder of the pipette with a nystatin-containing solution.

In the voltage-clamp mode, K_{ATP} channel currents of single VSMC were mostly recorded at a membrane potential of −60 mV with symmetrical 140 mM K^+. In some experiments, test pulses were made with a 10-mV increment from −80 to +70 mV at a holding potential of −60 mV with extracellular 5.4 mM K^+ or from −150 to +50 mV at a holding potential of −20 mV with extracellular 40 mM K^+. A 600-ms test pulse to different membrane potentials was applied every 10 s. In other experiments, the voltage ramps ranging from −150 to +100 mV were applied with a holding potential of −60 mV. A 600-ms ramp pulse was used every 10 s. The bath solution for recording the whole-cell K_{ATP} currents contained 140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, and 5 mM glucose (pH adjusted to 7.4 with NaOH). The pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM glucose, 0.3 mM Na_2ATP, and 0.5 mM MgGDP (pH adjusted to 7.2 with KOH). The K^+ concentration of bath solutions was increased, in some experiments, to 40 or 140 mM by the removal of equimolar NaCl. The cells were superfused continuously with the bath solution at a rate of approximately 2 ml/min. A complete solution change in the recording chamber was accomplished within 30 s. The absence of Ca^{2+} in the bath and pipette solutions, the presence of EGTA in the pipette solution, and the recording made at a negative membrane potential (−60 mV) would minimize K_{Ca} and K_V currents. All electrophysiological experiments were conducted at room temperature (20–22°C).

Chemicals and Data Analysis. Pinacidil, nystatin, GDP, ATP, 8-bromo-cGMP (8-Br-cGMP), *N*-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), HA, sodium nitroprusside (SNP), and adenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer, were purchased from Sigma-Aldrich (St. Louis, MO); glibenclamide (Glib) was purchased from Sigma/RBI (Natick, MA); and iberiotoxin was purchased from Alomone Labs (Jerusalem, Israel). Stock solutions of pinacidil and glibenclamide were made in dimethyl sulfoxide and diluted to the desired concentrations immediately before use. Dimethyl sulfoxide alone was without effect at the concentration used (up to 0.3%). Na_2ATP, MgGDP, and nystatin were directly dissolved in the pipette solution to achieve the desired concentrations on the day of experiments.

All data were expressed as means ± S.E.M. and analyzed using Student's *t* test or analysis of variance in conjunction with Newman-Keuls test, where applicable. Group differences were considered statistically significant at $p < 0.05$.

Results

Basal K_{ATP} Currents in Rat Mesenteric Artery VSMC. K_{ATP} channels in VSMC are activated by GDP, and a low concentration of ATP facilitates channel opening (Zhang and Bolton, 1995). Cell capacitance of the isolated rat mesenteric artery VSMC was 11.2 ± 0.7 pF ($n = 54$). The current densities of K_{ATP} currents were significantly higher with the inclusion of 0.3 mM Na_2ATP and 0.5 mM MgGDP in the pipette solution than that without the inclusion of ATP and GDP (at +40 mV; $n = 8$ for each group) (Fig. 1A). With ATP and GDP in the pipette solution, basal K_{ATP} currents in VSMC were increased from −11 ± 6 to −156 ± 19 pA by elevating KCl concentrations of the bath solution from 5 to 140 mM (at −60 mV; $n = 4$, $p < 0.01$) (Fig. 1, B and C). High-K^+-enhanced K_{ATP} currents were not sensitive to externally applied Ba^{2+} at 10 μM (−156 ± 19 versus −142 ± 15 pA at −60 mV; $n = 4$, $p > 0.05$). Pinacidil, a K_{ATP} channel opener, further increased K_{ATP} channel current to −286 ± 37 pA at 10 μM ($n = 4$, $p < 0.01$), and glibenclamide inhibited K_{ATP} channel currents to −76 ± 15 pA at 10 μM ($n = 4$, $p < 0.01$) (Fig. 1, B and C). The current-voltage relationship curves showed that the reversal potentials were shifted from −78 ± 2.1 mV ($n = 4$) in 5.4 mM $[K^+]_o$ to −28 ± 1.2 mV ($n = 5$) in 40 mM $[K^+]_o$, quite close to the calculated K^+ electrochemical equilibrium potentials (E_K) of −80.1 and −32.6 mV, respectively (Fig. 1D), indicating that the recorded current is K^+-selective. With 40 mM $[K^+]_o$, inward K_{ATP} currents were enhanced by increased K^+ driving force. The inward currents were also stimulated and suppressed by pinacidil and glibenclamide (Fig. 1D), respectively. All of these results demonstrated that the recorded membrane currents under our recording conditions were mainly conducted by K_{ATP} channels.

HA Stimulated K_{ATP} Currents and Hyperpolarized Cell Membrane in VSMC. Bath-applied HA at 0.5 mM with symmetrical 140 mM K^+ increased K_{ATP} currents from -180 ± 32 to -380 ± 70 pA ($n = 8$, $p < 0.01$), which were inhibited by glibenclamide to -110 ± 13 pA ($n = 8$, $p < 0.01$) (Fig. 2, A and B). HA activated K_{ATP} currents in a concentration-dependent fashion with an EC_{50} of 54 ± 3.4 μM (Fig. 2C). Bath-applied HA at 0.5 mM hyperpolarized cell membrane from -48 ± 5.2 to -65 ± 7.5 mV ($n = 6$, $p < 0.01$), which was inhibited by glibenclamide to -34 ± 3 mV ($n = 6$, $p < 0.01$). With extracellular physiological K^+ concentration ($[K^+]_o = 5.4$ mM), the whole-cell K_{ATP} currents were increased by including 0.5 mM HA in the pipette solution in a time-dependent fashion (Fig. 3A). The inward currents (at -120 mV) were increased by 98 ± 5.4, 135 ± 6.2, and $160 \pm 8.6\%$ at 10, 15, and 20 min after HA dialysis, respectively (Fig. 3B). Outward K_{ATP} currents became noisier with the increase of depolarizing stimuli (Fig. 3A). To exclude the possibility of K_{Ca} channel contamination, 200 nM iberiotoxin (IbTX), a selective K_{Ca} channel blocker, was used, and it failed to prevent the HA-induced K_{ATP} current increase under conditions of Ca^{2+}-free recording solutions (-195 ± 21 to -255 ± 30 pA at -120 mV; $n = 5$, $p < 0.05$) (Fig. 3B). The contamination of our results by Kv channels is unlikely, because at this negative membrane potential, the activation of Kv channels is impossible. After the elevation of $[K^+]_o$ to 40 mM, K_{ATP} currents were profoundly increased by HA with the testing potentials of -150 to $+50$ mV, especially the

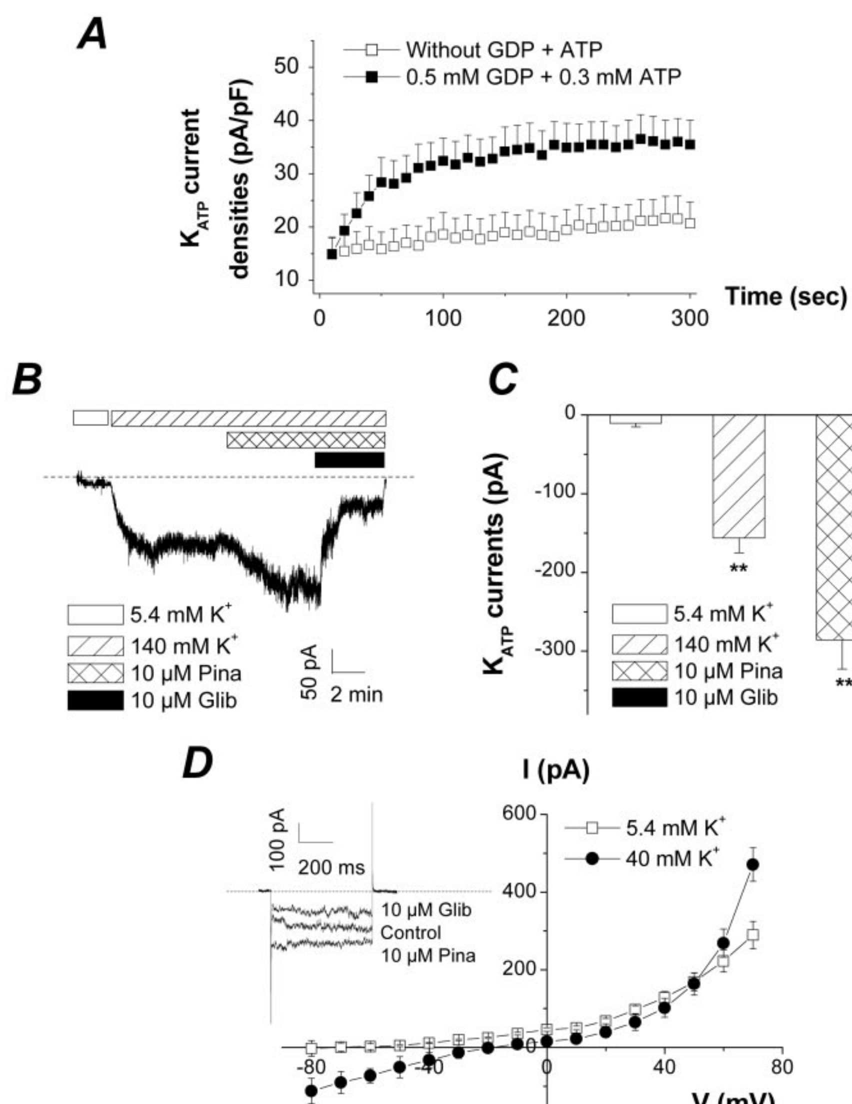

Fig. 1. The pharmacological properties and reversal potentials of basal K_{ATP} currents in VSMC dialyzed with 0.3 mM ATP and 0.5 mM GDP. A, summary of time-dependent increase of basal K_{ATP} current densities by the dialysis of 0.3 mM ATP and 0.5 mM GDP compared with the lack of dialysis of ATP and GDP. Holding potential (HP), -60 mV; testing potential (TP), $+40$ mV ($n = 8$ for each group). B, representative original recording of basal K_{ATP} currents activated by 10 μM pinacidil (Pina) and then inhibited by 10 μM Glib with symmetrical 140 mM K^+. Membrane potential (MP), -60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5 to 1 min. The dashed line indicates zero current. C, summary of basal K_{ATP} currents activated by Pina and inhibited by Glib ($n = 5$ for each group). **, $p < 0.01$ (140 mM K^+ versus 5.4 mM K^+; 10 μM Pina versus 140 mM K^+; 10 μM Glib versus 10 μM Pina). D, average current-voltage relationship curves of basal K_{ATP} currents with 5.4 or 40 mM $[K^+]_o$, showing that the reversal potentials were shifted rightward with a rise in $[K^+]_o$ ($n = 5$). Inset, representative original traces of inward part of basal K_{ATP} currents activated by Pina and then inhibited by Glib with 40 mM $[K^+]_o$. The dashed line indicates zero current. TP, -80 mV; HP, -60 mV.

inward current component (Fig. 4A). The inward K_{ATP} currents were increased in a time-dependent fashion after HA dialysis (Fig. 4, B and C). However, HA-increased currents were not significantly inhibited by extracellularly applied Ba^{2+} at 10 μM (-657 ± 45 versus -624 ± 52 pA at -150 mV; $n = 5$) but were blocked by a high 0.5 mM concentration of Ba^{2+} from -624 ± 52 to -334 ± 22 pA at -150 mV ($n = 5$, $p < 0.01$).

Effects of Free Radical Generating System and Scavengers on K_{ATP} Channels in VSMC. To determine the involvement of free radicals in HA-induced effects, a free radical generation system, hypoxanthine (HX)/xanthine oxidase (XO), was applied to VSMC. With 5.4 mM K^+ in the bath solution, basal K_{ATP} currents recorded by a ramp pulse were increased by HX/XO (100 μM/20 mU/ml) by 118% (at -120 mV), which was blocked by SOD by 60% (Fig. 5, A and B), although HX alone at 100 μM had no effect on K_{ATP} currents. With symmetrical 140 mM K^+ solutions, the combined application of HX at 100 μM and XO at 20 mU/ml enhanced HA-elicited K_{ATP} currents at -60 mV from -355 ± 40 to -480 ± 62 pA ($n = 6$, $p < 0.05$), which were blocked by 500 U/ml SOD to -150 ± 20 pA ($n = 6$, $p < 0.01$) (Fig. 5, C and D). On the other hand, the bath-applied HA at 0.5 mM enhanced K_{ATP} currents with symmetrical 140 mM K^+ solutions from -250 ± 26 to -380 ± 45 pA ($n = 4$, $p < 0.05$), which were inhibited by SOD to -160 ± 20 pA ($n = 4$, $p < 0.01$) and further inhibited by glibenclamide to -45 ± 3 pA ($n = 4$, $p < 0.01$) (Fig. 6, A and B). To confirm the inhibition of HA-enhanced K_{ATP} currents by SOD, another free radical scavenger, NAC, was applied. K_{ATP} currents enhanced by the bath-applied HA were inhibited reversibly by 300 and 600 μM NAC by $48 \pm 5\%$ ($n = 5$, $p < 0.01$) and $61 \pm 9\%$ ($n = 5$, $p < 0.01$), respectively, and also inhibited by SOD by $43 \pm 6\%$ ($n = 5$, $p < 0.05$) (Fig. 6, C and D).

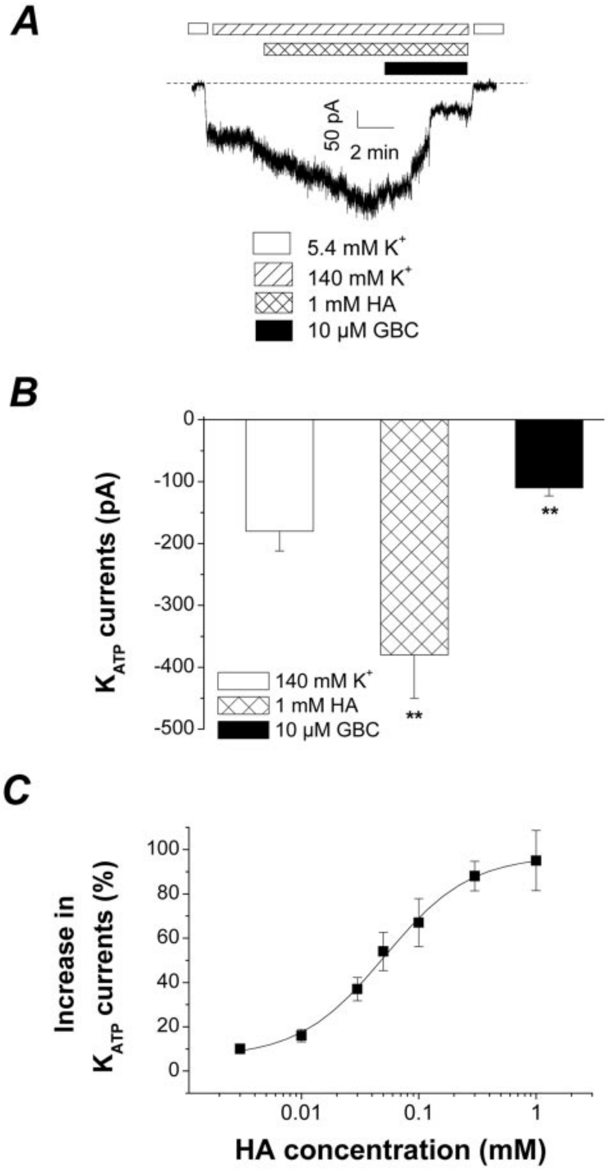

Fig. 2. Effects of HA on K_{ATP} currents and membrane potentials in VSMC. A, representative original current recording showing that bath-applied HA enhanced K_{ATP} currents, and these currents were inhibited by Glib with symmetrical 140 mM K^+. MP, -60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5~1 min. The dashed line indicates zero current. B, summary of the effects of HA and Glib on K_{ATP} currents. MP, -60 mV. **, $p < 0.01$ (0.5 mM HA versus control; 10 μM Glib versus 0.5 mM HA) ($n = 8$ for each group). C, concentration-dependent effect of HA on K_{ATP} channel currents with symmetrical 140 mM K^+. MP, -60 mV ($n = 5$–7 for each group).

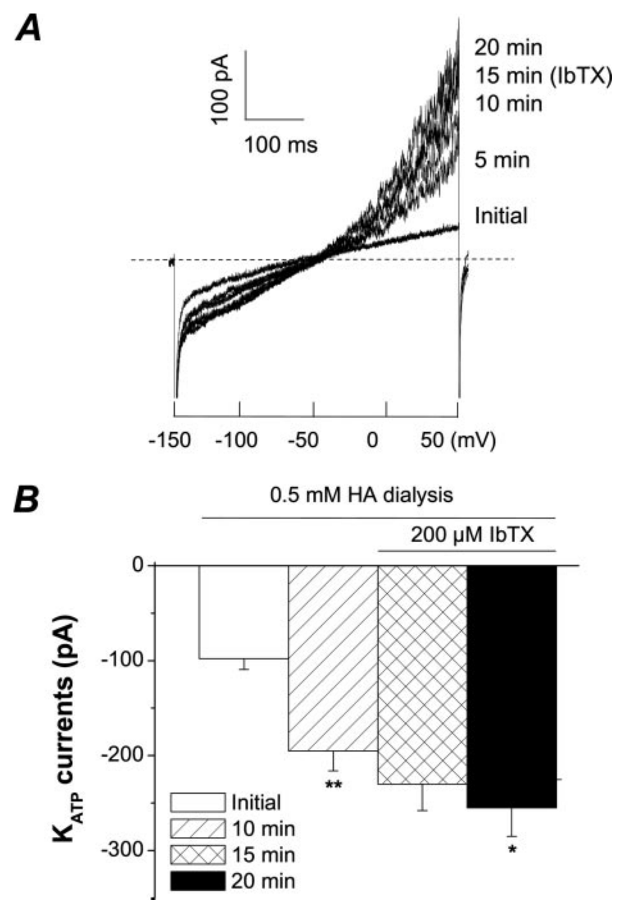

Fig. 3. Hydroxylamine enhanced K_{ATP} currents in VSMC with extracellular 5.4 mM K^+. A, original current recording showing intracellularly applied 0.5 mM HA enhanced IbTX-insensitive K_{ATP} currents. The ramp pulse was set from -150 to $+50$ mV with HP of -20 mV. The dashed line indicates zero current. B, effect of bath-applied IbTX (at 200 μM) on K_{ATP} currents at different time points after the dialysis of cells with 0.5 mM HA. The testing potential was set at -120 mV with HP of -20 mV ($n = 5$ for each group). *, $p < 0.05$ (20 min versus initial); **, $p < 0.01$ (10 min versus initial).

Effects of NO Donor and cGMP Analog on K_{ATP} Currents in VSMC. To examine whether the NO-sGC-cGMP signaling pathway mediated HA effects, NO donor and cGMP analog were used to test K_{ATP} currents. The NO donor SNP had no effect on HA-stimulated K_{ATP} currents with symmetrical 140 mM K^+ at 0.5 mM (−293 ± 46 versus −284 ± 32 pA; $n = 5$, $p > 0.05$) (Fig. 7, A and B). With the same recording conditions, the membrane-permeable cGMP analog 8-Br-cGMP failed to affect HA-increased K_{ATP} currents (−232 ± 30 versus −248 ± 34 pA; $n = 5$, $p > 0.05$) (Fig. 7, C and D). Basal K_{ATP} currents were not affected by SNP (−182 ± 23 versus −200 ± 30 pA; $n = 5$, $p > 0.05$) or 8-Br-cGMP (−142 ± 21 versus −165 ± 23 pA; $n = 5$, $p > 0.05$). However, HA-increased K_{ATP} currents were inhibited completely by glibenclamide at 10 μM, indicating that HA-activated currents are K_{ATP} currents. Furthermore, when 100 μM Rp-cAMP was included in the pipette solution to inhibit the cAMP-dependent protein kinase pathway (Wellman et al., 1998), the stimulatory effect of bath-applied HA on K_{ATP} currents was significantly reduced from −175 ± 21 to −204 ± 26 pA (at the testing potential of −60 mV; $n = 5$, $p > 0.05$). This result suggests that the phosphorylation of K_{ATP} channels by the cAMP-dependent protein kinase pathway alter the sensitivity of K_{ATP} channels to HA modulation (Quayle et al., 1994).

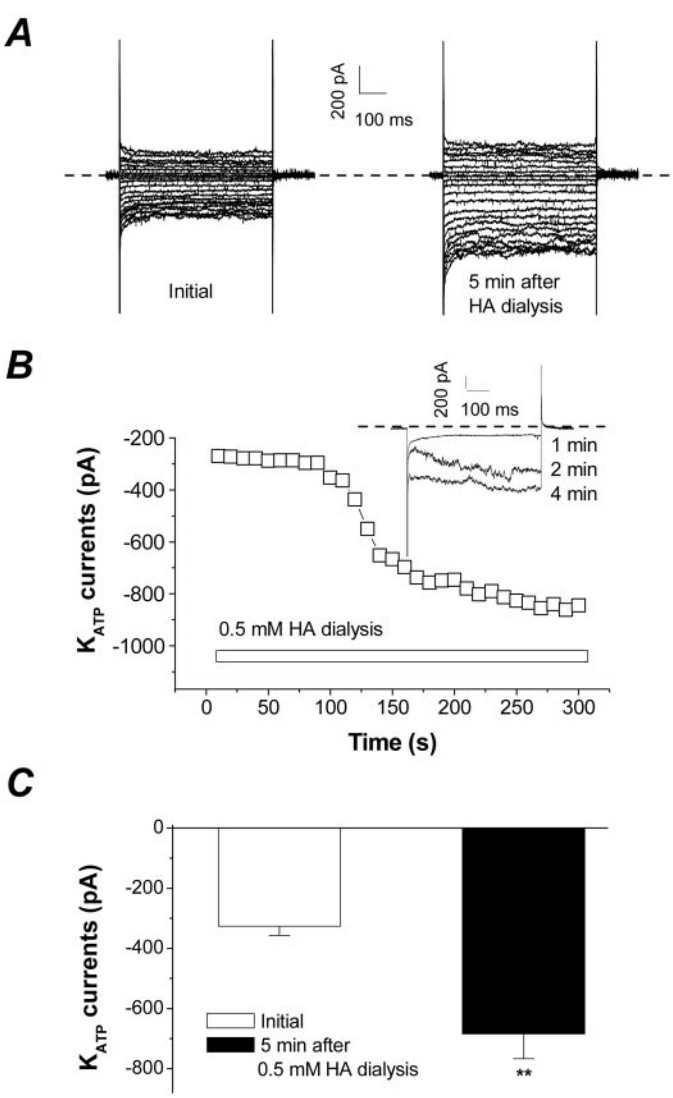

Fig. 4. HA enhanced K_{ATP} currents in VSMC with extracellular 40 mM K^+. A, original current recordings on the effect of HA dialysis on K_{ATP} currents. The dashed line indicates zero current. HP, −20 mV; TP, −150 to +50 mV. B, representative time-dependent effect of HA dialysis on K_{ATP} currents with HP of −20 mV and TP of −150 mV. Inset, original current traces of HA-increased K_{ATP} currents 1, 2, and 4 min after HA dialysis. The dashed line indicates zero current. C, summary of HA-increased K_{ATP} currents at initial and 5 min after the dialysis of 0.5 mM HA. HP, −20 mV; TP, −150 mV. **, $p < 0.01$ ($n = 6$ for each group).

Discussion

The novel findings of this study are summarized as follows. 1) Bath-applied HA reversibly enhanced K_{ATP} currents in a concentration-dependent fashion with an EC_{50} of 54 ± 3.4 μM and hyperpolarized cell membrane of rat mesenteric artery VSMC. 2) HA activated K_{ATP} channels with different $[K^+]_o$. The HA-stimulated inward currents increased with the elevation of $[K^+]_o$ (140 > 40 >5.4 mM). 3) HA-induced K_{ATP} channel activation and hyperpolarization were reduced by free radical scavengers (SOD and NAC). 4) The free radical generating system (HX/XO) mimicked and augmented the effect of HA on K_{ATP} currents, indicating the activation of K_{ATP} channels by $O_2^{\cdot-}$. 5) SNP and 8-Br-cGMP had no effect on basal and HA-stimulated K_{ATP} currents. Thus, the activation of K_{ATP} channels by HA is probably caused by increased free radical generation.

HA Targeted on K_{ATP} Channels in VSMC from Rat Mesenteric Artery. Although inward rectifier K^+ currents are known to be expressed in rat mesenteric artery VSMC (Bradley et al., 1999), the recorded K^+ currents in our recording condition are conducted through K_{ATP} channels. The following lines of evidence support this notion. 1) The recorded K^+ current was enhanced by the dialysis with GDP and ATP. The NDP-induced activation is a hallmark of vascular K_{ATP} channels in VSMC (Zhang and Bolton, 1995). 2) The recorded K^+ current was activated by K_{ATP} channel openers, such as pinacidil, and inhibited by glibenclamide. Glibenclamide suppressed not only high K^+-enhanced currents, but also GDP-activated basal currents. 3) The recorded K^+ current exhibited a weak inward rectification without the voltage dependence, whereas the classic inward rectifier current was activated by hyperpolarization with strong inward rectification (Quayle et al., 1993, 1994). 4) It has been reported that glibenclamide inhibited or blocked K_{ATP} channels at 10 μM but had no effect on Kir channels (Quayle et al., 1993). In our study, both basal K^+ currents and HA-enhanced K^+ currents were inhibited by 10 μM glibenclamide, supporting a K_{ATP} channel entity under the current investigation. 5) In cells dialyzed with 0.5 mM GDP and 0.3 mM ATP, high K^+-increased currents and HA-enhanced currents were not reduced by 10 μM Ba^{2+}, indicating that no Kir channel is activated in this recording condition (Bradley et al., 1999). Quayle et al. (1993) showed that Ba^{2+} at 10 μM blocked Kir currents but not K_{ATP} current; thus, Ba^{2+} was used to identify or separate Kir from K_{ATP} currents.

HA Evoked K_{ATP} Channel Activation and Membrane Hyperpolarization in VSMC. The HA-induced vasodilation of different vascular tissues has been reported (Rapoport and Murad, 1984; DeMaster et al., 1989; Thomas and Ramwell, 1989; Feelisch et al., 1994; Huang, 1998); however, the exact cellular mechanisms underlying the vasorelaxant effect of HA has been largely unclear. It was reported that HA increased the rate of ^{86}Rb outflow from perfused pancreatic islets, which was counteracted by glibenclamide, indicating that K_{ATP} channels were involved in HA-inhibited insulin release (Antoine et al., 1996). HA was also reported to activate voltage-dependent K^+ channels in crustacean skeletal muscle (Hermann and Erxleben, 2001). But a high 10 mM concentration of HA blocked the inactivating K^+ channels (*Shaker-B*) expressed in *Xenopus laevis* oocytes by an unknown mechanism (Yool, 1994) and depolarized cell membrane by inhibiting K^+ channels (Mongin et al., 1998). Our results demonstrated for the first time that HA enhanced K_{ATP} currents in VSMC and hyperpolarized the cell membrane. HA-induced hyperpolarization by K_{ATP} channel activation may close voltage-dependent L-type Ca^{2+} channels and then decrease intracellular free $[Ca^{2+}]_i$, leading to vasorelaxation.

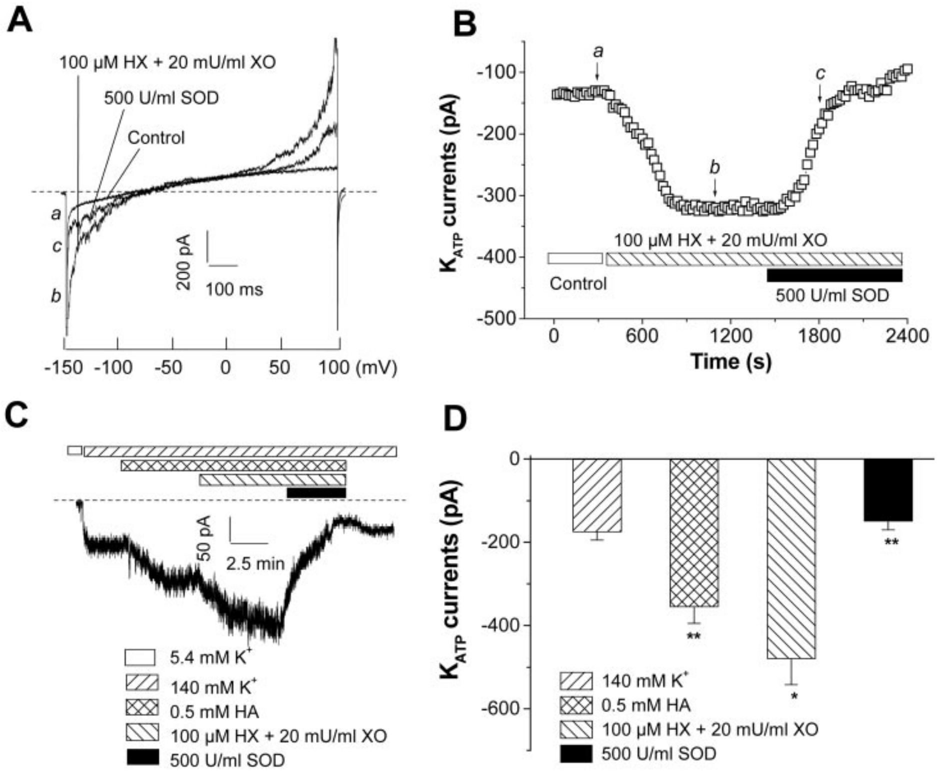

Fig. 5. Effects of HX and XO on K_{ATP} currents in VSMC. A, original current recordings of the effects of HX/XO (*b*) and SOD (*c*) on basal K_{ATP} currents (*a*) with extracellular 5.4 mM K^+. The ramp pulse was set from −150 to +100 mV with HP of −20 mV. The dashed line indicates zero current. B, time-dependent effects of HX/XO (*b*) and SOD (*c*) on basal K_{ATP} currents (*a*). HP, −20 mV; TP, −120 mV. C, original current traces showing the effects of 100 μM HX with 20 mU/ml XO and 500 U/ml SOD on HA-enhanced K_{ATP} currents with symmetrical 140 mM K^+. MP was held at −60 mV. The current amplitude with slow activation was measured at the maximal value of different treatments for 0.5 to 1 min. The dashed line indicates zero current. D, summary of the augmentation and suppression of HA-enhanced K_{ATP} currents by HX/XO and SOD, respectively. MP, −60 mV (n = 5–6 for each group). *, $p < 0.05$ (HX + XO versus 0.5 mM HA); **, $p < 0.01$ (0.5 mM HA versus 140 mM K^+; 500 U/ml SOD versus HX + XO).

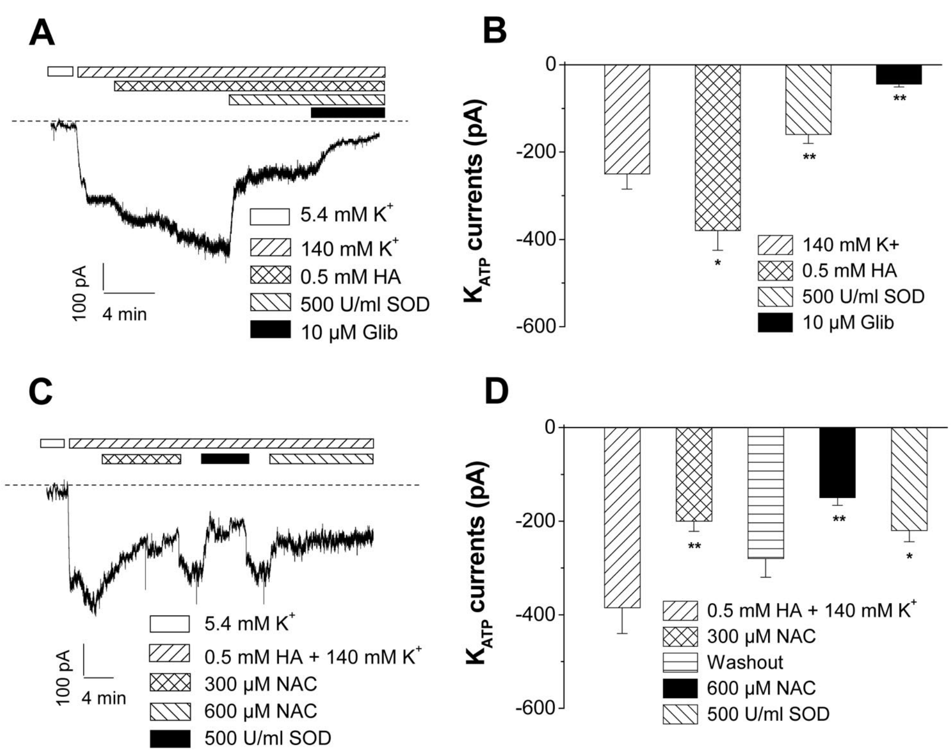

Fig. 6. Effects of free radical scavengers on HA-enhanced K_{ATP} currents with symmetric 140 mM K^+. A, original current recording of the inhibitory effects of 500 U/ml SOD and 10 μM Glib on HA-enhanced K_{ATP} currents. MP was held at −60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5 to 1 min. The dashed line indicates zero current. B, summary of the inhibition of HA-enhanced K_{ATP} currents by SOD and Glib (n = 4 for each group). *, $p < 0.05$ (0.5 mM HA versus 140 mM K^+); **, $p < 0.01$ (500 U/ml SOD versus 0.5 mM HA; 10 μM Glib versus 500 U/ml SOD). C, original current recording of the reversible inhibition of HA-enhanced K_{ATP} currents by NAC and SOD. MP, −60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5 to 1 min. The dashed line indicates zero current. D, summary of the inhibition of HA-enhanced K_{ATP} currents by NAC and SOD. *, $p < 0.05$ (500 U/ml SOD versus washout); **, $p < 0.01$ (300 μM NAC versus 0.5 mM HA; 600 μM NAC versus washout) (n = 5 for each group).

NO-sGC-cGMP Signaling Pathway Did Not Mediate the Effect of HA on K_{ATP} Channels. Among the known endogenous K_{ATP} channel modulators is endogenous NO, which activated K_{ATP} channels in cell-attached patches via the activation of sGC in cultured VSMC from porcine coronary artery (Kubo et al., 1994). Bath-applied atrial natriuretic factor and isosorbide dinitrate, which are activators of particulate and soluble guanylyl cyclase, respectively, activated unitary K_{ATP} channel currents. These effects were abolished by methylene blue (an sGC inhibitor) but potentiated by 8-Br-cGMP, suggesting that the cGMP pathway mediated the effects of atrial natriuretic factor and isosorbide dinitrate (Kubo et al., 1994). At the tissue level, SNP elicited the dilation of pial arterioles from anesthetized piglets, which was blocked by cGMP-dependent protein kinase inhibitor (8-Br-cGMP, *R*p-isomer) and sGC inhibitor (LY83583), indicating that NO primarily elicited its effects via cGMP production (Armstead, 1996). Furthermore, SNP- and 8-Br-cGMP-elicited dilation of newborn pig pial artery was blunted by the K_{ATP} channel antagonist glibenclamide, indicating that NO and cGMP might interact with K_{ATP} channels (Armstead, 1999). However, SNP- and HA-induced vasorelaxation of rat aortic rings was not affected by glibenclamide, disproving the involvement of K_{ATP} channels in NO-induced vasorelaxation (Huang, 1998). SNP did not increase whole-cell K_{ATP} currents with symmetrical 140 mM K^+, indicating that the activation of the NO-sGC-cGMP pathway did not lead to K_{ATP} channel activation (Quayle et al., 1994; Wellman et al., 1998). Therefore, NO effects on K_{ATP} channels in different vascular beds are controversial without clear mechanisms.

Some studies have shown hyperpolarization of smooth muscle by NO via activation of K_{ATP} channels. SNP activated cGMP-dependent protein kinase and produced glibenclamide-sensitive membrane hyperpolarization in rabbit mesenteric arteries (Murphy and Brayden, 1995); however, other studies in rabbit cerebral and canine coronary arteries failed to demonstrate hyperpolarization induced by exogenous NO (8–30 μM) (Tare et al., 1990). SNP-induced hyperpolarization may result from the cross-activation of protein kinase A by cGMP (Quayle et al., 1994). Only a large amount of NO could produce a hyperpolarizing effect in VSMC from rat mesenteric artery (Zhao et al., 2000). *S*-Nitroso-*N*-acetyl-penicillamine at a high 400 μM concentration caused membrane hyperpolarization that was reversed by glibenclamide and completely blocked by treatment with Tiron, a scavenger of $O_2^{\cdot}$, suggesting that peroxynitrite ($OONO^-$) other than NO exerts the hyperpolarizing effect via the activation of K_{ATP} channels (Zhao et al., 2000).

Our results present evidence that HA directly activated whole-cell K_{ATP} channels and hyperpolarized cell membrane, whereas both SNP and 8-Br-cGMP had no effect on basal K_{ATP} currents and HA-stimulated K_{ATP} currents. These observations suggested that the activation of the NO-sGC-cGMP signaling pathway did not mediate K_{ATP} channel activity in rat mesenteric artery VSMC. It is tempting to speculate that HA activated K_{ATP} channels via another mechanism. The yield of free radicals, including $O_2^{\cdot}$ by HA could be one such mechanism (Santoian et al., 1993; Market et al., 1994; Vetrovsky et al., 1996).

Free Radical Generation Mainly Underlies the Effect of HA on K_{ATP} Channels. The modulation of K^+ channel activity by cellular oxidative stress has been recognized as a significant determinant of vascular tone. Under certain conditions, many extracellular ligands generated and/or required free radicals to transmit biological signals to intracellular milieu as second messengers. Different kinds of free radicals can modify various types of K^+ channels in vascular tissues. At the tissue level, $O_2^{\cdot}$, H_2O_2, and $OONO^-$ dilated

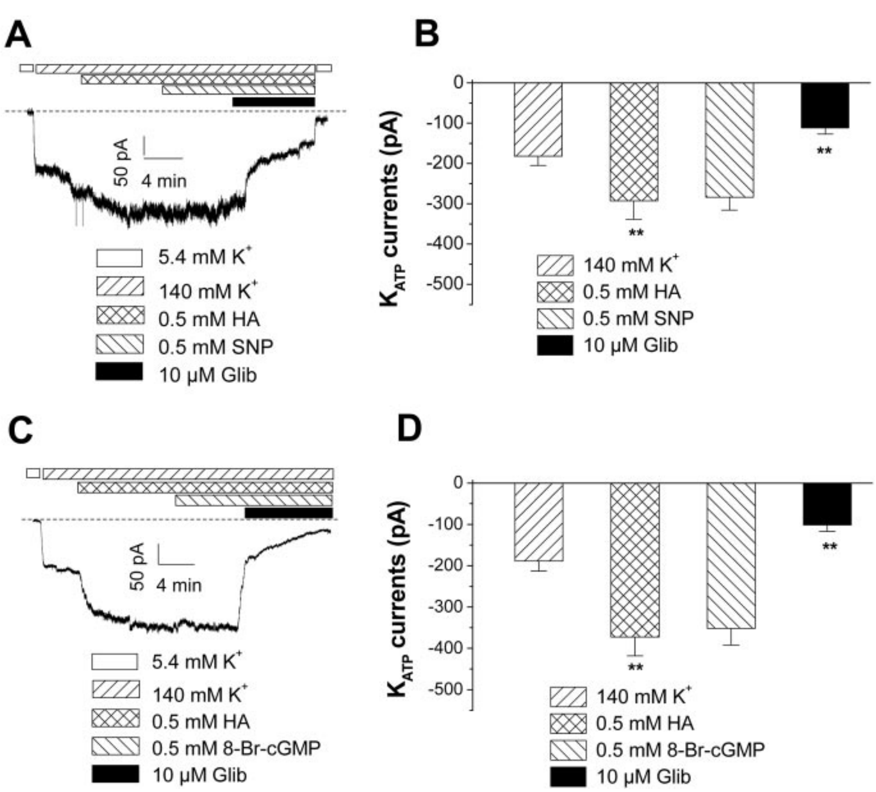

Fig. 7. Effects of SNP and 8-Br-cGMP on HA-enhanced K_{ATP} currents with symmetrical 140 mM K^+. A, original current recording of the effect of SNP and Glib on HA-enhanced K_{ATP} currents. MP was held at −60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5 to 1 min. The dashed line indicates zero current. B, summary of the effects of SNP and Glib on HA-enhanced K_{ATP} currents. **, $p < 0.01$ (0.5 mM HA versus 140 mM K^+; 10 μM Glib versus 0.5 mM SNP) ($n = 5$ for each group). C, original current traces of the effect of 8-Br-cGMP and Glib on HA-enhanced K_{ATP} currents. MP, −60 mV. The current amplitude with slow activation is measured at the maximal value of different treatments for 0.5 to 1 min. The dashed line indicates zero current. D, summary of the effect of 8-Br-cGMP and Glib on HA-enhanced K_{ATP} currents. **, $p < 0.01$ (0.5 mM HA versus 140 mM K^+; 10 μM Glib versus 0.5 mM 8-Br-cGMP) ($n = 5$ for each group).

cerebral vasculature, which was not mediated by sGC activation (Wei et al., 1996). Both H_2O_2 and $OONO^-$ elicited dilation via activating K_{ATP} channels, whereas O_2^- dilated cerebral arterioles by opening K_{Ca} channels (Wei et al., 1996). H_2O_2 induced a glibenclamide-sensitive dose-dependent dilation in cat cerebral arterioles and rat gracilis skeletal muscle arterioles (Wei et al., 1996; Cseko et al., 2004). $OONO^-$ elicited vasodilation in several vascular beds, including coronary (Liu et al., 1994), renal, mesenteric (Benkusky et al., 1998), and cerebral arteries (Wei et al., 1996, 1998; Liu et al., 2002). Dilation of cerebral and coronary arteries to $OONO^-$ is blocked by glibenclamide, suggesting a role of K_{ATP} channels (Wei et al., 1996, 1998; Liu et al., 2002).

At the cellular level, knowledge about the modulation of K^+ channels by free radicals in single VSMC is still limited. O_2^- produced by xanthine (X)/XO or high glucose reduced the whole-cell Kv current density in freshly isolated rat coronary VSMC, which was reversed partially by SOD (Liu et al., 2001). However, O_2^- generated by X/XO did not significantly alter the open-state probability of K_{Ca} channels (Liu et al., 2002). H_2O_2 activated macroscopic and unitary BK_{Ca} channel currents in porcine coronary arteries via a phospholipase A_2-arachidonic acid signaling cascade (Barlow et al., 2000). In isolated coronary arteriole VSMC, the IbTX-sensitive whole-cell K^+ current density was reduced by $OONO^-$ generated from the mixture of SNP with X/XO. $OONO^-$ greatly decreased the open-state probability of K_{Ca} channels in inside-out excision, contributing to the inhibition of K_{Ca} channel activity (Liu et al., 2002); however, electrophysiological evidence for the effects of free radicals on K_{ATP} channel activity is largely lacking in VSMC. Our study provides for the first time the electrophysiological evidence that HA activated K_{ATP} channels in single VSMC from rat mesenteric artery, which was mimicked or augmented by the free radical generating system HX/XO and reduced by free radical scavengers such as SOD and NAC. It should be noted that HA in the cytosol is converted into NO and O_2^-, which are likely to form $OONO^-$ (Liu et al., 1994; Pryor and Squadrito, 1995). Whether HA-induced K_{ATP} channel activation and vasodilation are linked to $OONO^-$ generation remains to be investigated.

Although HX/XO is widely used as the free radical generating system, direct effects of HX/XO on K^+ channels in single VSMC are rarely reported. When HX is oxidized by XO in the presence of O_2, an electron from the reaction of HX with XO is transferred to O_2 to form O_2^-. The dismutation of O_2^- generated H_2O_2 via cytosolic or mitochondrial SOD. Further oxidation of H_2O_2 leads to highly potent OH^- via the catalysis of transient metal such as ferrous iron (Graf, 1984). Thus, HX/XO may generate various reactive species such as O_2^-, H_2O_2, and OH^-, which determine different effects of HX/XO, along with species- and tissue-specific differences in various vascular beds. Application of HX/XO together with $FeCl_3$ to pial artery in vivo resulted in attenuated vasodilatation induced by K_{ATP} channel agonists (cromakalim and calcitonin gene-related peptide), NO donors (SNP and *S*-nitroso-*N*-acetyl-penicillamine), and 8-Br-cGMP (Armstead, 1999). From these results, however, one cannot conclude that O_2^- inhibits K_{ATP} channel in VSMC. Changes in the diameter of pial artery in vivo are influenced by many vasoactive substances with multiple mechanisms. Blocking a common downstream cellular event by HX/XO would not only inhibit the vasodilatory effect of K_{ATP} channel agonists, but also that of many other vasodilators that may not interact with K_{ATP} channels at all. The direct effect of HX/XO on the basal diameter of pial artery was not examined. Electrophysiological evidence for the effect of HX/XO on K_{ATP} channels in VSMC of pial artery was also unavailable. In our present study, direct electrophysiological recording of K_{ATP} channel currents was carried out on isolated VSMC from rat mesenteric artery. Both electrophysiological and pharmacological results in our study demonstrated that HX/XO reaction in fact activated K_{ATP} channels in single VSMC. This effect is probably mediated by O_2^- because HX/XO-activated K_{ATP} currents were reduced by SOD.

In summary, HA-induced K_{ATP} channel activation and resultant hyperpolarization in VSMC may underlie HA-induced vasorelaxation via enhanced production of free radicals. These observations will lead to a better understanding of the physiological functions of HA and the underlying cellular and molecular mechanisms. Novel therapeutic approaches in dealing with K_{ATP} channel abnormality-related disorders may also be yielded from the observations of this study.

References

Antoine MH, Ouedraogo R, Sergooris J, Hermann M, Herchuelz A, and Lebrun P (1996) Hydroxylamine, a nitric oxide donor, inhibits insulin release and activates K_{ATP} channels. *Eur J Pharmacol* **313:**229–235.

Armstead WM (1996) Role of ATP-sensitive K^+ channels in cGMP-mediated pial artery vasodilation. *Am J Physiol* **270:**H423–H426.

Armstead WM (1999) Superoxide generation links protein kinase C activation to impaired ATP-sensitive K^+ channel function after brain injury. *Stroke* **30:**153–159.

Barlow RS, El-Mowafy AM, and White RE (2000) H_2O_2 opens BK_{Ca} channels via the PLA_2-arachidonic acid signaling cascade in coronary artery smooth muscle. *Am J Physiol* **279:**H475–H483.

Benkusky NA, Lewis SJ, and Kooy NW (1998) Attenuation of vascular relaxation after development of tachyphylaxis to peroxynitrite in vivo. *Am J Physiol* **275:**H501–H508.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, and Cohen RA (1994) Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature (Lond)* **368:**850–853.

Bradley KK, Jaggar JH, Bonev AD, Heppner TJ, Flynn ER, Nelson MT, and Horowitz B (1999) Kir2.1 encodes the inward rectifier potassium channel in rat arterial smooth muscle cells. *J Physiol* **515:**639–651.

Cseko C, Bagi Z, and Koller A (2004) Biphasic effect of hydrogen peroxide on skeletal muscle arteriolar tone via activation of endothelial and smooth muscle signaling pathways. *J Appl Physiol* **93:**1130–1137.

DeMaster EG, Raij L, Archer SL, and Weir EK (1989) Hydroxylamine is a vasorelaxant and a possible intermediate in the oxidative conversion of L-arginine to nitric oxide. *Biochem Biophys Res Commun* **163:**527–533.

Feelisch M, te Poel M, Zamora R, Deussen A, and Moncada S (1994) Understanding the controversy over the identity of EDRF. *Nature (Lond)* **368:**62–65.

Graf E, Mahoney JR, Bryant RG, and Eaton JW (1984) Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *J Biol Chem* **259:**3620–3624.

Hermann A and Erxleben C (2001) Nitric oxide activates voltage-dependent potassium currents of crustacean skeletal muscle. *Nitric Oxide* **5:**361–369.

Huang Y (1998) Hydroxylamine-induced relaxation inhibited by K^+ channel blockers in rat aortic rings. *Eur J Pharmacol* **349:**53–60.

Klink M, Swierzko A, and Sulowska Z (2001) Nitric oxide generation from hydroxylamine in the presence of neutrophils and in the cell-free system. *APMIS* **109:**493–499.

Kubo M, Nakaya Y, Matsuoka S, Saito K, and Kuroda Y (1994) Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K^+ channels in cultured vascular smooth muscle cells. *Circ Res* **74:**471–476.

Liu S, Beckman JS, and Ku DD (1994) Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. *J Pharmacol Exp Ther* **268:**1114–1121.

Liu Y, Terata K, Chai Q, Li H, Kleinman LH, and Gutterman DD (2002) Peroxynitrite inhibits Ca^{2+}-activated K^+ channel activity in smooth muscle of human coronary arterioles. *Circ Res* **91:**1070–1076.

Liu Y, Terata K, Rusch NJ, and Gutterman DD (2001) High glucose impairs voltage-gated K^+ channel current in rat small coronary arteries. *Circ Res* **89:**146–152.

Market M, Carnal B, and Mauel J (1994) Nitric oxide production by activated human neutrophils exposed to sodium azide and hydroxylamine: the role of oxygen radical. *Biochem Biophys Res Commun* **199:**1245–1249.

Mongin AA, Nedvetsky PI, and Fedorovich SV (1998) Depolarization of isolated brain nerve endings by nitric oxide donors: membrane mechanisms. *Biochemistry (Mosc)* **63:**662–670.

Moore PK, Burrows L, and Bhardwaj R (1989) Hydroxylamine dilates resistance

blood vessels of the perfused rat kidney and mesentery. *J Pharm Pharmacol* **41:**426–429.
Murphy M and Brayden JE (1995) Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J Physiol.* **186:**47–58.
Ohta K, Rosner G, and Graf R (1997) Nitric oxide generation from sodium nitroprusside and hydroxylamine in brain. *Neuroreport* **8:**2229–2235.
Pryor WA and Squadrito GL (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* **268:**L699–L722.
Quayle JM, Bonev AD, Brayden JE, and Nelson MT (1994) Calcitonin gene-related peptide activated ATP-sensitive K^+ currents in rabbit arterial smooth muscle via protein kinase *Am J Physiol* **475:**9–13.
Quayle JM, McCarron JG, Brayden JE, and Nelson MT (1993) Inward rectifier K^+ currents in smooth muscle cells from rat resistance-sized cerebral arteries. *Am J Physiol* **265:**C1363–C1370.
Rapoport RM and Murad F (1984) Effect of cyanide on nitrovasodilator-induced relaxation, cyclic GMP accumulation and guanylate cyclase activation in rat aorta. *Eur J Pharmacol* **104:**61–70.
Robertson BE, Schubert R, Hescheler J, and Nelson MT (1993) cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am J Physiol* **265:**C299–C303.
Santoian EC, Thomas G, Augerio AD, Kot PA, and Ramwell PW (1993) Vasodilator effects of hydroxylamine in the isolated rodent lung. *Angiology* **44:**897–901.
Stamler JS (1994) Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* **78:**931–936.
Taira J, Misik V, and Riesz P (1997) Nitric oxide formation from hydroxylamine by myoglobin and peroxide. *Biochem Biophys Acta* **1336:**502–508.
Tare M, Parkington HC, Coleman HA, Neild TO, and Dusting GJ (1990) Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature (Lond)* **346:**69–71.
Thomas G and Ramwell PW (1989) Vascular relaxation mediated by hydroxylamines and oximes: their conversion to nitrites and mechanism of endothelium dependent vascular relaxation. *Biochem Biophys Res Commun* **164:**889–893.
Vetrovsky P, Stoclet JC, and Entlicher G (1996) Possible mechanism of nitric oxide production from N^G-hydroxy-L-arginine or hydroxylamine by superoxide ion. *Int J Biochem Cell Biol* **28:**1311–1318.
Wei EP, Kontos HA, and Beckman JS (1996) Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide and peroxynitrite. *Am J Physiol* **271:**H1262–H1266.
Wei EP, Kontos HA, and Beckman JS (1998) Antioxidants inhibit ATP-sensitive potassium channels in cerebral arterioles. *Stroke* **29:**817–822.
Wellman GC, Quayle JM, and Standen NB (1998) ATP-sensitive K^+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. *J Physiol* **507:**117–129.
Yool AJ (1994) Block of the inactivating potassium channel by clofilium and hydroxylamine depends on the sequence of the pore region. *Mol Pharmacol* **46:**970–976.
Zhang H and Bolton TB (1995) Activation by intracellular GDP, metabolic inhibition and pinacidil of a glibenclamide-sensitive K-channel in smooth muscle cells of rat mesenteric artery. *Br J Pharmacol* **114:**662–672.
Zhao KS, Liu J, Yang GY, Jin C, Huang Q, and Huang X (2000) Peroxynitrite leads to arteriolar smooth muscle cell membrane hyperpolarization and low vasoreactivity in severe shock. *Clin Hemorheol Microcirc* **23:**259–267.

Address correspondence to: Dr. Rui Wang, FAHA, Department of Physiology, College of Medicine, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada. E-mail: rui.wang@lakeheadu.ca