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Different mechanisms underlying the stimulation of K_{Ca} channels by nitric oxide and carbon monoxide

Lingyun Wu,¹ Kun Cao,² Yanjie Lu,² and Rui Wang²

¹Department of Anatomy and Cell Biology, and

²Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

The molecular mechanisms underlying the effects of nitric oxide (NO) and carbon monoxide (CO), individually and collectively, on large-conductance calcium-activated K^+ (K_{Ca}) channels were investigated in rat vascular smooth muscle cells (SMCs). Both NO and CO increased the activity of native K_{Ca} channels. Dehydrosoyasaponin-I, a specific agonist for β subunit of K_{Ca} channels, increased the open probability of native K_{Ca} channels only when it was delivered to the cytoplasmic surface of membrane. CO, but not NO, further increased the activity of native K_{Ca} channels that had been maximally stimulated by dehydrosoyasaponin-I. After treatment of SMCs with anti- $K_{Ca,\beta}$ subunit antisense oligodeoxynucleotides, the stimulatory effect of NO, but not of CO, on K_{Ca} channels was nullified. CO, but not NO, enhanced the K_{Ca} current densities of heterologously expressed cloned $K_{Ca,\alpha}$ subunit, showing that the presence of $K_{Ca,\beta}$ subunit is not a necessity for the effect of CO but essential for that of NO. Finally, pretreatment of SMCs with NO abolished the effects of subsequently applied CO or diethyl pyrocarbonate on K_{Ca} channels. In summary, the stimulatory effects of CO and NO on K_{Ca} channels rely on the specific interactions of these gases with $K_{Ca,\alpha}$ and $K_{Ca,\beta}$ subunits.

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Introduction

Nitric oxide (NO) is an endogenous vasorelaxant gas that is synthesized from the terminal guanidino nitrogen atoms of L-arginine by NO synthase (1). Carbon monoxide (CO) is another endogenously generated biological gas. The major route for the endogenous generation of CO involves heme oxygenase (HO), which works in concert with NADPH-cytochrome P450 reductase to cleave the heme ring in hemoproteins into biliverdin, CO, and iron (2). Results from our laboratory (3) and others (4–7) have demonstrated that endogenously generated CO can effectively relax vascular tissues. The physiological role of endogenous CO is thus indicated.

NO and CO are not redundant messenger molecules. For instance, NO potently dilated hepatic artery, but only slightly dilated the portal venous vascular bed. In contrast, CO had no effect on the hepatic artery, but relaxed the portal venous vascular bed (8). In some

cases, NO and CO may even have opposite effects. CO inhibited, but NO increased, the release of IL-1 β from rat hypothalamus stimulated by high K^+ concentrations (9). NO and CO do have at least two common targets in vascular smooth muscle cells (SMCs), i.e., soluble guanylyl cyclase (sGC) and large-conductance calcium-activated K^+ (K_{Ca}) channels (10–14). Although the debate has not been settled on whether NO and CO directly act on K_{Ca} channels or indirectly by altering intracellular second messengers, many single channel studies on the cell-free membrane patches have been supportive for a direct interaction of NO and CO with K_{Ca} channel proteins. Even for these single channel studies different mechanisms for the effects of NO and CO on K_{Ca} channels have been presented. The NO effect on K_{Ca} channels in rabbit aortic SMCs was related to the changes in sulfhydryl groups (10). Our previous study showed that CO acted on the histidine residue of K_{Ca} channel proteins in rat tail artery SMCs (13).

Large-conductance K_{Ca} channels are stimulated by increased intracellular calcium concentration and by membrane depolarization. These K_{Ca} channels are composed of two noncovalently linked subunits: the pore-forming α subunit and the accessory β subunit, which affects the electrophysiological and pharmacological properties of K_{Ca} channel complexes (15). The interactions of NO and CO with α and β subunits of K_{Ca} channels may be the determining factor for the selective modulation of K_{Ca} channels, which has not been addressed.

The interaction between NO and CO on the regulation of vascular contractility is another important but still largely uncharted issue. The activation of large-conductance K_{Ca} channels in SMCs accounts for a significant part of the relaxation induced by NO (16) or

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Address correspondence to: Rui Wang, Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E5. Phone: (306) 966-6592; Fax: (306) 966-6532; E-mail: wangrui@duke.usask.ca.

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Nonstandard abbreviations used: heme oxygenase (HO); smooth muscle cells (SMCs); soluble guanylyl cyclase (sGC); calcium-activated K^+ (K_{Ca}); sodium nitroprusside (SNP); open probability (P_o); number of multiple channels (N); intracellular free Ca^{2+} ($[Ca^{2+}]_i$); oligodeoxynucleotide (ODN); S-nitroso-N-acetyl-penicillamine (SNAP); diethyl pyrocarbonate (DEPC); N-ethylmaleimide (NEM); chromium mesoporphyrin (Cr-MP); heme-L-lysinate (HLL); iberiotoxin (IbTX); dehydrosoyasaponin I (DHS); half-activation potential ($V_{1/2}$); green fluorescent protein (GFP); picoamperes/picofarads (pA/pF); superoxide dismutase (SOD).

CO (20–50%) (3). The K_{Ca} channel-mediated vasorelaxing effect of CO was significantly decreased approximately 40% in the presence of sodium nitroprusside (SNP) (17). These results indicated that NO might desensitize K_{Ca} channels toward CO. Here is an intriguing situation. On one hand, we accept the argument that the vascular effects of NO and CO have different profiles. On the other hand, most studies, if not all, have been focused only on the individual vascular effects of these gases. Although physiologically more relevant, the integrated vascular effect of these gases and the underlying mechanisms have been unknown.

The present study was aimed at investigating the functional interaction of NO/CO with $K_{Ca,\alpha}$ and $K_{Ca,\beta}$ subunits and the collective effect of NO and CO on large-conductance K_{Ca} channels in vascular SMCs. To accomplish these goals we used the patch-clamp technique to characterize the effects of NO and CO, individually and collectively, on K_{Ca} channel currents in vascular SMCs. The function of $K_{Ca,\beta}$ subunit was pharmacologically modulated, and the expression of $K_{Ca,\beta}$ subunit protein was suppressed by antisense oligonucleotide treatment. We also examined the effects of CO and NO on the heterologously expressed cloned $K_{Ca,\alpha}$ subunit. Our results indicate that the stimulatory effects of CO and NO on K_{Ca} channels rely on the specific interactions of these gases with $K_{Ca,\alpha}$ and $K_{Ca,\beta}$ subunits. The collective acts of NO and CO constitute a feedback mechanism controlling the functions of large-conductance K_{Ca} channels in vascular SMCs.

Methods

Cell preparation. Male Sprague-Dawley adult rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). Single SMCs were dispersed from tail artery enzymatically following our established procedure (12, 13). The enzymatically dispersed cells were plated onto 35-mm Petri dishes in DMEM (Life Technologies Inc., Burlington, Ontario, Canada) containing penicillin (100 U/ml; Sigma-Aldrich, St. Louis, Missouri, USA) and streptomycin (0.1 mg/ml; Sigma-Aldrich) and maintained at 4°C for at least 4 hours. These freshly isolated cells were used in electrophysiological recording within 8–24 hours of isolation.

Animal use protocols were approved by the University Committee on Animal Care and Supply of the University of Saskatchewan.

Single channel and whole-cell recordings. The inside-out and outside-out configurations of the patch-clamp technique were used to record single K_{Ca} channel currents as described previously (12, 13). Pipettes with a resistance of 6–8 M Ω were used, and the seal resistance was usually greater than 10 G Ω . Membrane patches with no more than three channels were used for experiments. Single channel currents were filtered at 2 kHz (8-pole Bessel, –3 dB) and recorded with a 5- μ s sampling interval in a gap-free mode. The channel open probability (P_o) was calculated from the ratio between

the open time and the total time. The presence of multiple channels (N) in one membrane patch was distinguished by simultaneous channel openings when exposing the inner surface of the membrane to 100 μ M Ca^{2+} and depolarizing the patch to 50 mV. NP_o values represented the P_o of N channels. The unit amplitude of K_{Ca} channels and the channel open duration were determined from all point histograms using a Fetchan program (Axon Instruments Inc., Foster City, California, USA). A current level higher than 50% of the unit channel current was considered to reflect a channel opening. For each concentration of tested agent at least 60 seconds of channel activity was recorded directly on the hard disk of a computer. NP_o of K_{Ca} channels was averaged over 2–5 minutes of recording to describe the changes in channel activity following different treatments. Membrane patches with unstable NP_o over time were excluded from further analysis. The outer surface of membrane patches was bathed in a solution containing 145 mM KCl, 10 mM HEPES, and 10 mM glucose. The inner surface of membrane patches was exposed to a solution containing 145 mM KCl, 10 mM HEPES, 1.2 mM $MgCl_2$, 10 mM glucose, 1 mM EGTA, and different amounts of $CaCl_2$ to reach the desired final intracellular free Ca^{2+} concentrations, $[Ca^{2+}]_i$. Unless otherwise specified, $[Ca^{2+}]_i$ was 0.5 μ M, calculated using a computer program (EQCAL; Biosoft, Ferguson, Missouri, USA).

The whole-cell K_{Ca} currents were recorded as described previously with an Axopatch 1-D amplifier (Axon Instruments Inc.), controlled by a Digidata 1200 interface and a pClamp software program (version 6.02; Axon Instruments Inc.) (12). Membrane currents were filtered at 1 kHz with a 4-pole Bessel filter, digitized, and stored. At the beginning of each experiment, junctional potential between pipette solution and bath solution was electronically adjusted to zero. No leakage subtraction was performed to the original recordings, and all cells with visible changes in leakage currents during the course of study were excluded from further analysis. Test pulses were made at 10-mV increments from –80 to +60 mV. The holding potential was set at –80 mV. I-V curves were constructed using the current amplitude measured between the 300 to 600 ms of the test pulses. The bath solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose (pH adjusted to 7.3 with NaOH). The pipette solution was composed of 145 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 5 mM glucose, 2 mM Na_2 -ATP (pH adjusted to 7.3 with KOH). $[Ca^{2+}]_i$ in the pipette solution was adjusted to 50 nM by adding adequate amount of $CaCl_2$. Cells were continuously superfused with the bath solution containing tested chemicals. Osmolalities and pH of all solutions were adjusted to 290 mOsm/l and 7.4, respectively. All electrophysiological experiments were carried out at room temperature.

Transient transfection of COS-1 cells with $K_{Ca,\alpha}$ subunit. The *mSlo* (BK $_{Ca,\alpha}$ subunit) cDNA was kindly provided by B. Ganetzky (University of Wisconsin, Madison, Wisconsin,

USA) (GenBank accession no. U09383). Most of the 5' noncoding region has been removed to improve expression. The cDNA was cleaved with the PmeI endonuclease from pcDNA3.1-*mSlo* construct and subcloned into a pIRES2-EGFP expression vector (CLONTECH Laboratories Inc., Palo Alto, California, USA) pretreated with SmaI endonuclease. After being transformed into competent *Escherichia coli* cells, colonies were screened for the clones containing the construct of right orientation using appropriate endonucleases.

COS-1 cells were purchased from American Type Culture Collection (Rockville, Maryland, USA). These cells were cultured at 37°C in a humidified incubator with 95% air and 5% CO₂ in DMEM with 4 mM L-glutamine containing 4.5 g/l of glucose and 1.5 g/l of sodium bicarbonate and supplemented with 10% FBS and penicillin/streptomycin. The cells in 35-mm cell culture dishes were 70–80% confluent prior to the transfection experiment. The pIRES2-EGFP-*mSlo* construct was mixed with a FuGENE 6 transfection reagent (Roche Diagnostics, Laval, Quebec, Canada) in a ratio of 1 µg to 3 µl in 100 µl of FBS-free DMEM. After incubating for 45 minutes at room temperature, the mixture was added to COS-1 cells in 2 ml FBS-free DMEM (cell density: 8 × 10⁴/35-mm dish). Mock transfection (vector only transfection) and nontransfected native COS-1 cells were included as negative controls for the experiment. The cells were allowed to express the transfected *mSlo* cDNA for 48–72 hours prior to electrophysiological experiments. The emitted green fluorescence served as the marker for selecting the transfected cells.

Antisense oligodeoxynucleotide treatment of cells. The oligodeoxynucleotide (ODN) was designed to target the transcript segment encompassing the translation-initiation codon of rat K_{Ca}β subunit (GenBank accession no. AF020712). The antisense ODN (5'-TCTTC-CCCATGGCCACAGG-3') and the scrambled ODN (5'-CTAGACATGCTGCCT CCGC-3') were synthesized by Bio/Can Scientific (Mississauga, Ontario, Canada). The underlined nucleotides were the phosphorothioate backbone of ODN. The scrambled ODN contained the same base composition as the antisense sequence, but no complementary sequence to the scrambled ODN could be found in the entire GenBank database (using the basic local alignment search tool, or BLAST).

Freshly isolated rat tail artery SMCs were cultured at 37°C in an atmosphere of 5% CO₂ in DMEM containing 10% FCS for 18–24 hours before ODN treatments. To increase the cellular uptake, antisense or scrambled ODN diluted in serum-free and antibiotic-free DMEM were first mixed with a cationic liposome solution (Lipofectin; Life Technologies Inc.) at room temperature for 10–20 minutes. After washing cultured cells twice with serum-free and antibiotic-free DMEM, the antisense or scrambled ODN-Lipofectin (final concentration 5 µM and 6 µM, respectively) complexes were added to 35-mm Petri dish in which SMCs were incubated with antibiotic-free DMEM containing 5% FCS. The ODN-Lipofectin-containing medium was re-

placed after an incubation period of 8–12 hours at 37°C in a CO₂ incubator by normal culture medium (DMEM with 10% FCS). The cells were then continuously cultured at 37°C in a CO₂ incubator in the absence of ODN-Lipofectin for 12–24 hours before the patch-clamp studies were conducted on these cells.

Chemicals and data analysis. Sodium nitroprusside (SNP), S-nitroso-N-acetyl-penicillamine (SNAP), diethyl pyrocarbonate (DEPC), N-ethylmaleimide (NEM), L-lysine, and deferoxamine were from Sigma-Aldrich. Chromium mesoporphyrin (Cr-MP) was purchased from Porphyrin Products (Logan, Utah, USA). Heme-L-lysinate (HLL) (38 mM) was prepared as previously described by Tenhunen et al. (18), stored at -20°C, and protected from light until use. Iberiotoxin (IbTX) was from Alomone Labs (Jerusalem, Israel). Dehydrosoyasaponin I (DHS) was a gift from Merck Research Laboratories (Rahway, New Jersey, USA). Saturated CO solution was prepared fresh before each experiment and then diluted immediately to the desired concentrations with bath solution (3). DEPC was freshly diluted with anhydrous ethanol prior to each experiment and directly added to the bath or pipette solution at pH 7.0 in different experiments. NEM was dissolved in 1 M KOH as a 500-mM stock solution, dissolved in the bath solution immediately prior to the experiment, and the pH was readjusted to 7.4 (19). DHS stock solution (250 µM) was prepared in distilled water, dispensed into aliquots, and stored at -20°C.

Data were expressed as means plus or minus SE. Differences between treatments in the same cells were evaluated by paired Student *t* test or in conjunction with Newman-Keuls test where the same patches were treated by multiple agents. The significant level of difference was determined when *P* values were less than 0.05.

Results

Our previous studies have shown that exogenous CO increased the *P*_o of K_{Ca} channels, but not the single channel conductance, in a concentration-dependent manner in rat tail artery SMCs (12, 13). The interaction of endogenous CO with K_{Ca} channels in this cell preparation was, to our knowledge, first examined in the present study. In the presence of Cr-MP, an inhibitor of HO, whole-cell K_{Ca} channel currents were significantly reduced (Figure 1a). To increase endogenous level of CO, we applied HLL, a substrate of HO (20), to tail artery SMCs. K_{Ca} channel currents were significantly increased 10 minutes after the application of HLL (Figure 1b). Whether the effect of HLL could be caused by free iron and biliverdin, another two end products of heme metabolism in addition to CO, was further investigated. Biliverdin failed to affect K_{Ca} currents, and HLL still significantly increased K_{Ca} currents in the presence of free iron scavenger deferoxamine (Figure 1c). In addition, L-lysinate, the vehicle of HLL, had no effect on K_{Ca} currents (Figure 1c). These results demonstrate that the K_{Ca} channels in rat tail artery SMCs are modulated by endogenous CO.

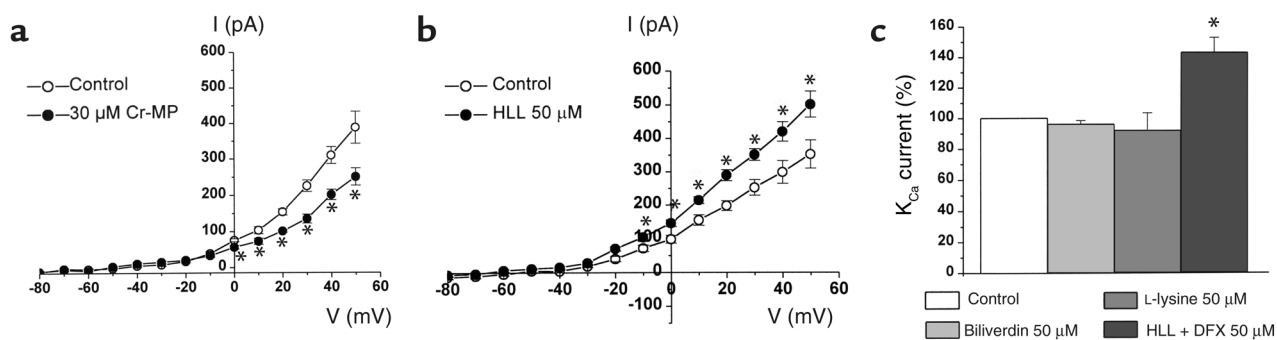


Figure 1

The effect of endogenous CO on whole-cell K_{Ca} currents in rat tail artery SMCs. (a) K_{Ca} currents were suppressed by Cr-MP (30 μ M). The I-V relationships of the K_{Ca} channels were constructed 10 minutes before and after the application of Cr-MP. $n = 7$. (b) K_{Ca} currents were increased by HLL (50 μ M). The I-V relationships of the K_{Ca} channels were constructed 10 minutes before and after the application of HLL. $n = 6$. (c) K_{Ca} currents were not affected by treatment with biliverdin or L-lysine for 10 minutes. $n = 6$ in each group. In the presence of deferoxamine (DFX), HLL still increased K_{Ca} channel currents. Holding potential, -80 mV; test potential, $+40$ mV. $*P < 0.05$ vs. control. Data of each group in this figure were pooled from at least two batches of cells. I, current; V, membrane potential.

The effect of NO on K_{Ca} channels in rat tail artery SMCs was also examined in the present study. SNP increased the NP_o of single K_{Ca} channels in both outside-out (Figure 2) and inside-out membrane patches (not shown). The increase in the NP_o became significant with 1 μ M SNP and reached the plateau with a concentration of SNP around 100 μ M. The excitatory effect of SNP was sustained for the period of SNP application (5–20 minutes). The SNP-stimulated single channel activities were blocked by IbTX (100 nM) (Figure 2), corroborating the nature of large-conductance K_{Ca} channels under investigation. SNP (1–100 μ M) had no effect, however, on single channel conductance (232 ± 11 pS and 241 ± 9 pS before and after the application of SNP; $n = 6$, $P > 0.05$).

In order to identify different mechanisms underlying the effects of NO and CO on K_{Ca} channels in vas-

cular SMCs, sulfhydryl groups of K_{Ca} channel complex were first modified with NEM. Treatment of inside-out patches with NEM (5 mM) led to a significant decrease in the open frequency of single K_{Ca} channels by $72\% \pm 3\%$ ($n = 5$, $P < 0.01$), whereas the mean open time for each individual opening was not affected (not shown). Consequently, NP_o of single K_{Ca} channels was significantly reduced (Figure 3). The stimulatory effect of SNP (10 μ M) on K_{Ca} channels was diminished in the presence of NEM ($n = 5$, not shown). NEM treatment did not, however, alter the stimulatory effect of CO on the P_o of single K_{Ca} channels (Figure 3).

Both α and β subunits contribute to the functionality of native K_{Ca} channels. To identify which subunit of K_{Ca} channels was the potential target of CO or NO, we examined the effect of DHS on single K_{Ca} channel cur-

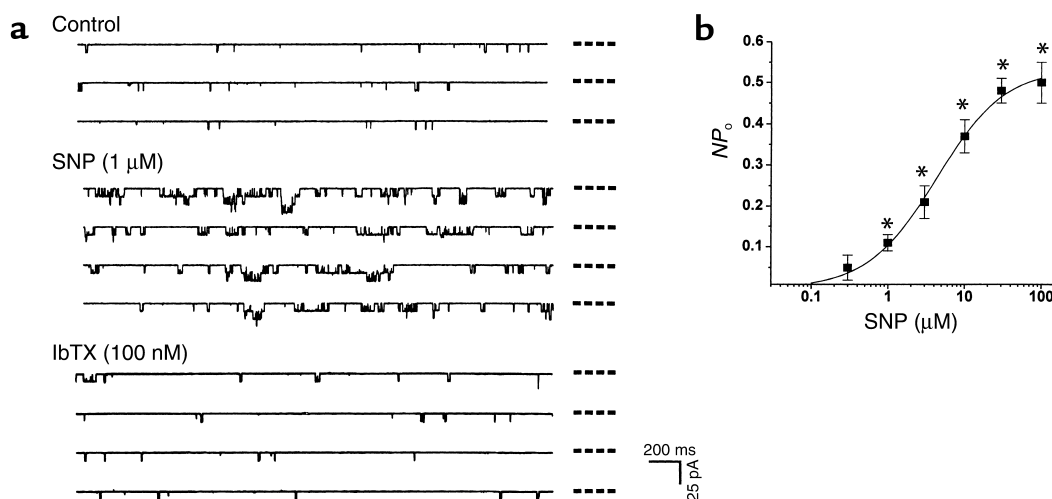


Figure 2

Effects of SNP on K_{Ca} currents in outside-out membrane patches of vascular SMCs. (a) SNP increased, but the subsequently applied IbTX blocked the single channels in the same membrane patch. The dashed lines beside each trace indicate the closed channel level. (b) The concentration-dependent excitatory effect of SNP on the NP_o of K_{Ca} channels. $n = 6$ for each data point. A total of three batches of cells were used in this study. Membrane potential, -30 mV. $*P < 0.05$ vs. control. ms, millisecond; pA, picoampere.

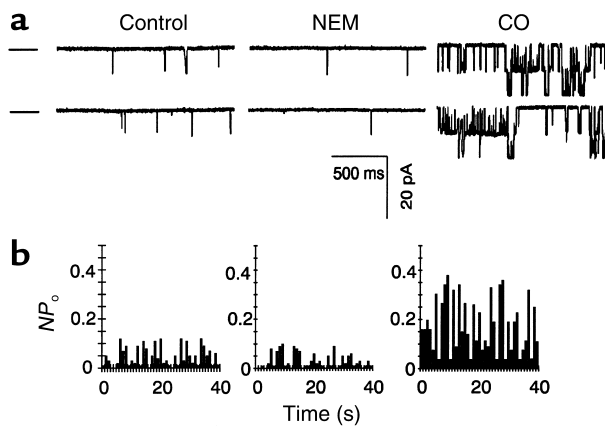


Figure 3
Effects of NEM (5 mM) and CO (30 μ M) on single K_{Ca} channels recorded from inside-out membrane patches held at a membrane potential of -30 mV. (a) Original K_{Ca} current traces recorded in the absence and then the presence of NEM and CO, consecutively, in the same patch. The dashed lines beside the current traces denote the closed state of the channels. (b) NEM decreased the P_o of single K_{Ca} channels. However, the presence of NEM did not block CO-induced modification of single K_{Ca} channels. $n = 6$ from two batches of cell preparations.

rents. DHS is a non-membrane permeable-specific probe for the functionality of β subunit of K_{Ca} channels. DHS (500 nM) did not significantly change the NP_o of K_{Ca} channels in outside-out membrane patches ($n = 5$, not shown). However, DHS concentration dependently increased the NP_o when applied to the inner membrane surface (Figure 4). After the maximum excitatory effect of DHS at 300 nM reached a stable level, the same membrane patches were exposed to either SNP or CO in different sets of experiments. The preconditioning with DHS abolished the excitatory effect of SNP on K_{Ca} channels, but not that of CO. These results summarized in Figure 5 indicated that the effects of DHS and SNP on native K_{Ca} channels might be mediated by the same β subunit-related mechanism, but CO might act on K_{Ca} channels in a β subunit-independent manner.

To abridge the functional coupling of β subunit with α subunit in K_{Ca} channel complexes, SMCs were treated with anti- $K_{Ca,\beta}$ antisense ODN. Single channel conductance of K_{Ca} channels was not altered by the antisense ODN or scrambled ODN treatments (not shown). The decreased voltage dependence and calcium sensitivity after anti- $K_{Ca,\beta}$ antisense ODN treatment were indicative of K_{Ca} channels that existed as the product of one gene that encodes α subunit. Anti- $K_{Ca,\beta}$ antisense ODN treatment decreased the half-activation potential ($V_{1/2}$) of K_{Ca} channels from -62.9 ± 5.7 mV to 20.4 ± 3.7 mV ($n = 5$ in each group of cells, $P < 0.01$) (21). The slopes of the voltage-dependence curves were 18.8 ± 5.8 mV and 19.3 ± 4.7 mV with or without anti- $K_{Ca,\beta}$ antisense ODN treatment, respectively ($P < 0.05$). The presence of β subunit is known to give rise to different degrees of the significant shift in $V_{1/2}$ of K_{Ca} channels, depending on the free calcium concentrations (21, 22).

After anti- $K_{Ca,\beta}$ antisense ODN treatment, the calcium concentration needed to activate 50% of K_{Ca} channels ($Ca_{1/2}$) at $+50$ mV was increased from 5.7 ± 0.5 μ M to 14.5 ± 1.1 μ M ($n = 5$ in each group, $P < 0.01$). Similar values of $Ca_{1/2}$ have been reported for the K_{Ca} channels composed of both α and β subunits or α subunit alone, respectively (21, 22). It is apparent from these results that the participation of $K_{Ca,\beta}$ subunit in the formation of K_{Ca} channel complexes had been effectively reduced by anti- $K_{Ca,\beta}$ antisense ODN treatment.

Should the effects on K_{Ca} channels of both DHS and NO rely on the presence of β subunit, the uncoupling of α and β subunits by anti- $K_{Ca,\beta}$ antisense ODN treatment would render the insensitivity of K_{Ca} channels to DHS and NO. This scenario is portrayed in Figure 6, showing that the stimulatory effects of SNP and DHS on single K_{Ca} channels in inside-out patches were nullified by anti- $K_{Ca,\beta}$ antisense ODN treatment. To further confirm the involvement of NO in the effect of SNP, another NO donor (SNAP) was used. SNAP increased the activity of K_{Ca} channels in the cells treated with scrambled ODN (control) but had no effect on K_{Ca} channels after anti- $K_{Ca,\beta}$ antisense ODN treatment. In contrast, the stimu-

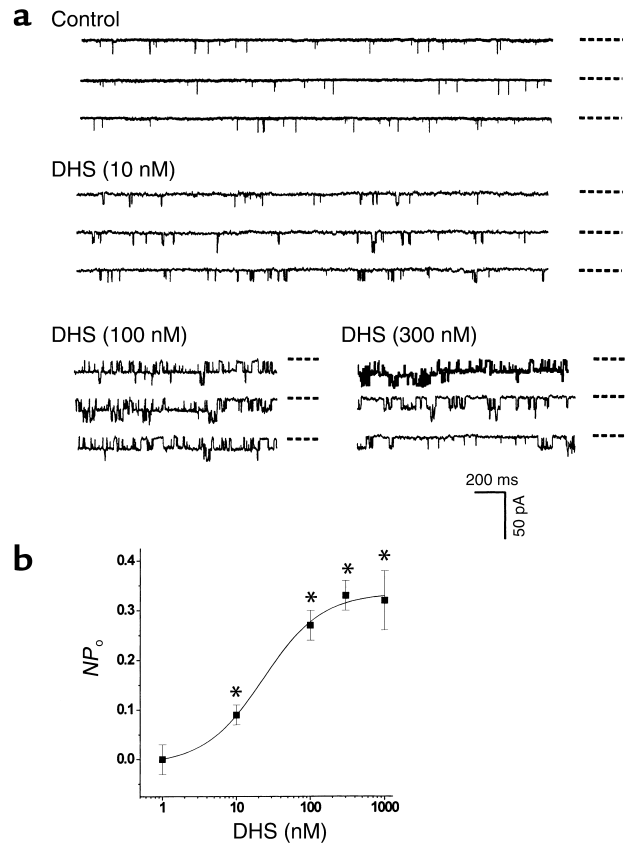


Figure 4
The concentration-dependent effect of DHS on the NP_o of K_{Ca} channels. (a) Actual single channel current traces recorded in inside-out membrane patches. The dashed lines beside each trace indicate the closed state of the channels. (b) Summary of the effects of DHS. $n = 6$ (from three batches of cells) for each data point. Membrane potential, -30 mV. * $P < 0.05$ vs. control.

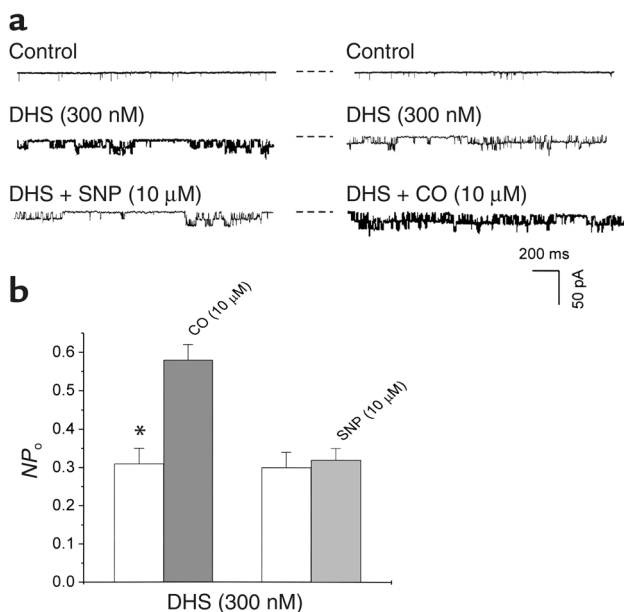


Figure 5
The activation of K_{Ca} channels in vascular SMCs. (a) Representative single channel current recordings showing two sets of experiments in which K_{Ca} channels were preconditioned with DHS, and either SNP (left panel) or CO (right panel) was subsequently applied. (b) The preconditioning with DHS abolished the effect of SNP on the NP_o , but not that of CO. $n = 6$ (from two batches of cells) for each group. $*P < 0.05$. Inside-out patches. Membrane potential, -30 mV. The dashed lines beside each trace indicate the closed channel current level.

latory effect of CO on K_{Ca} channels was not affected by anti- $K_{Ca\beta}$ antisense ODN treatment (Figure 6).

COS-1 cells transfected with cDNAs encoding green fluorescent protein (GFP) alone did not yield K_{Ca} currents in the presence of 50 nM cytoplasmic free Ca^{2+} (Figure 7a) ($n = 11$). In contrast, the cells cotransfected with GFP and *mSlo* cDNAs showed a big current that appeared noisy under the same recording conditions (Figure 7b). SNP (10 μ M) did not affect the current density of the expressed K_{Ca} channels (60.8 ± 4.5 and 64.7 ± 12.2 picoamperes/picofarads [pA/pF] before and after the application of SNP; $+50$ mV, $n = 8$) (Figure 7c). CO (150 μ M), on the other hand, significantly increased K_{Ca} current density from 65.4 ± 12.6 to 100.1 ± 17.7 pA/pF ($+50$ mV; $n = 5$). This effect became obvious at membrane potentials positive to $+20$ mV and was reversible by the washout procedure (Figure 7d).

The collective effect of NO and CO on native K_{Ca} channels in SMCs was not a simple algebraic addition of their individual effects. After the cell membranes were pretreated with SNP for 10 minutes, subsequent application of CO failed to further increase the NP_o of K_{Ca} channels in outside-out patches. However, pretreatment of the cell membranes with CO for 10 minutes did not prevent SNP from further increasing the NP_o (Figure 8). The possibility was thus raised that the CO-sensitive α subunit of K_{Ca} channels might be modified by NO so that the α subunit became desensitized

to CO. In contrast, the NO-sensitive β subunit of K_{Ca} channels was likely resistant to CO, and the effects of CO and the subsequently applied NO on β subunit would become additive.

We demonstrated previously that CO increased K_{Ca} channel activities by activating a histidine residue of K_{Ca} channel proteins located on the outer surface of the cell membrane (13). To explore the molecular basis of the interaction of NO and CO, we set out to examine whether NO modified the histidine-related reactivity of K_{Ca} channels. Because the application of DEPC to the inner membrane surface did not affect K_{Ca} channel activity or the CO effect on K_{Ca} channels (13), DEPC in the present study was always applied to the outer membrane surface. After the SNP-induced increase in the NP_o became stable, the outside-out membrane patches were superfused with the bath solution containing 0.5 mM DEPC for at least 5 minutes. During this period of time, no reduction in the SNP-enhanced K_{Ca} channel activity was spotted (Figure 9a). The NP_o were 0.22 ± 0.04 and 0.19 ± 0.07 before and after the application of DEPC ($n = 6$ from two batches of cell preparations, $P > 0.05$). Since DHS affected K_{Ca} channels only when it was given intracellularly (Figure 4), in the next set of outside-out single-channel recording experiments we included DHS (300 nM) in the pipette solution. The exposure of the same patches to DEPC (0.5 mM) led to a significant decrease in the mean NP_o of K_{Ca} channels (0.32 ± 0.07 vs. 0.15 ± 0.04 ; $n = 5$, $P < 0.05$) (Figure 9b). These results suggested that in addition to the stimulation of $K_{Ca\beta}$ subunit, NO might also somehow act on the histidine of α subunit so that DEPC could not chemically modify this residue. After the outer surface-located histidine residue of K_{Ca} channels was

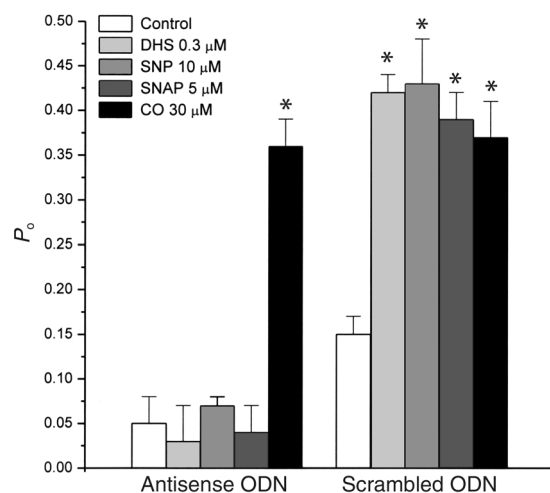


Figure 6
The effects of DHS, SNP, SNAP, and CO on the normalized P_o of K_{Ca} channels in inside-out patches of vascular SMCs after anti- $K_{Ca\beta}$ antisense ODN treatment. Membrane potential, $+30$ mV. $[Ca^{2+}]_i$, 1 μ M. $n = 7$ (from at least two batches of cells) for each group. $*P < 0.01$ vs. control.

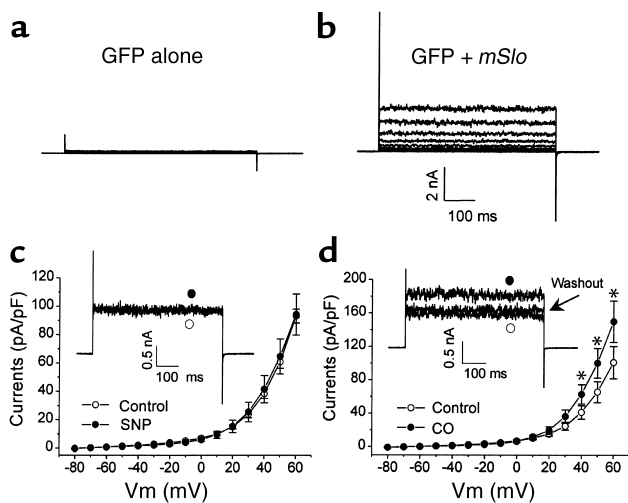


Figure 7 Effects of SNP and CO on the whole-cell K_{Ca} currents generated by heterologous expression of $K_{Ca,\alpha}$ subunit in COS-1 cells. Membrane potential was stepped with 600-ms duration from -80 to $+60$ mV in 10-mV increments from a holding potential of -80 mV at 0.1 Hz. (a) The cells transfected with GFP cDNA alone did not yield K_{Ca} currents. (b) K_{Ca} currents were recorded in COS-1 cells transiently cotransfected with GFP and *mSlo* cDNAs. (c) SNP had no effect on the *mSlo*-encoded K_{Ca} currents ($n = 8$ from three batches of cell preparations). The inset shows the original current traces at $+50$ mV. (d) CO significantly increased the *mSlo*-encoded K_{Ca} currents ($n = 5$ from three batches of cell preparations). The inset shows the original current traces at $+50$ mV. * $P < 0.05$ vs. control. nA, nanoampere.

modified by DEPC, the subsequent bath-applied SNP ($n = 6$ from two batches of cells) or DHS ($n = 5$ from two batches of cells) still significantly increased K_{Ca} channel activities in inside-out membrane patches (Figure 9, c and d). Finally, in the presence of superoxide dismutase (SOD), SNP still inhibited the excitatory effect of CO on single K_{Ca} channels ($n = 6$ from two batches of cells) (Figure 9e).

Discussion

Large-conductance K_{Ca} channels in vascular SMCs are one of the important targets of NO and CO actions. Our present study addressed the molecular mechanisms for the individual as well as integrated effects of NO and CO on K_{Ca} channels. Our novel discoveries are summarized below. First, not only exogenous CO but also endogenous CO increased K_{Ca} channel currents in rat tail artery SMCs. Second, NO increased the NP_o of large-conductance K_{Ca} channels in rat tail artery SMCs by a mechanism related to the modification of the sulfhydryl group of the K_{Ca} channel complex. Third, the excitatory effects of CO and NO on K_{Ca} channels were likely mediated, respectively, by α and β subunits of K_{Ca} channel complex. Fourth, the interaction of CO and histidine residue of K_{Ca} channels did not interfere with the excitatory effect of NO on K_{Ca} channels. Fifth, although NO had no effect on $K_{Ca,\alpha}$ subunit in the absence of $K_{Ca,\beta}$ subunit, the interaction of K_{Ca} chan-

nel complex with NO significantly inhibited the excitatory effect of CO. And sixth, the inhibition of CO effect on K_{Ca} channels by NO likely resulted from a repulsive effect of NO on, instead of oxidation of, histidine residue. Taken together, our results provided molecular basis for understanding the acute interaction of NO and CO, two endogenous vasoactive gases, on the regulation of vascular contractility.

A higher concentration of CO was needed to act on the K_{Ca} channel complex to achieve the similar excitation as induced by a lower concentration of NO, consistent with different vasorelaxant potencies of these two gases. This could be partially explained by the difference in the chemical interactions of CO and NO with $K_{Ca,\alpha}$ and $K_{Ca,\beta}$ subunits, respectively. A relatively weak reaction between CO and the imidazole group of histidine occurs by the formation of a hydrogen bond, which could be largely responsible for the effect of CO on K_{Ca} channels (13). On the other hand, the modification of sulfhydryl groups by NO, named s-nitrosylation, is likely related to the formation of a disulfide bond between NO and the affected protein, including K_{Ca} channels (23). A much weaker hydrogen bond, as compared with the disulfide bond, may result

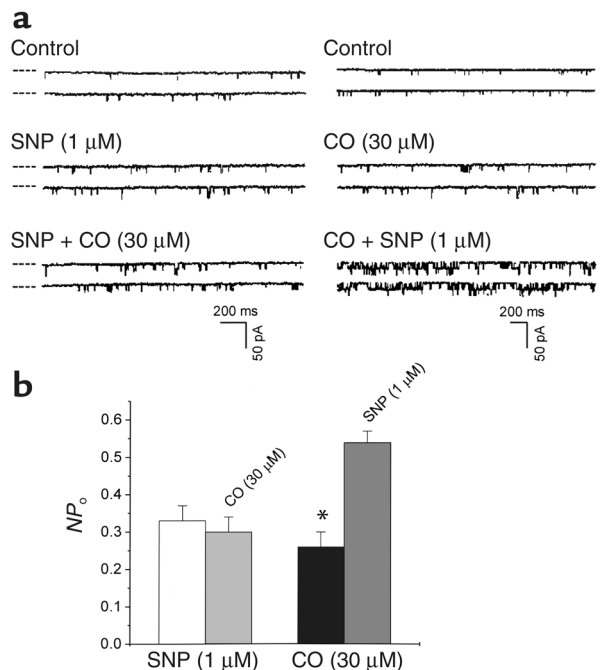


Figure 8 The interaction of NO and CO on K_{Ca} channels in vascular SMCs. (a) Representative recordings showing the modulation of K_{Ca} currents by the sequential application of SNP and CO (left panel) or CO and then SNP (right panel) to different outside-out membrane patches. The dashed lines beside each trace indicate the current level of closed channels. (b) Pretreatment of cell membrane with SNP for 10 minutes abolished the effect of subsequently applied CO on K_{Ca} currents. However, pretreatment of cell membrane with CO for 10 minutes did not eliminate the excitatory effect of SNP on K_{Ca} currents. Membrane potential, -30 mV. $n = 5$ (from at least two batches of cells) for each group. * $P < 0.05$.

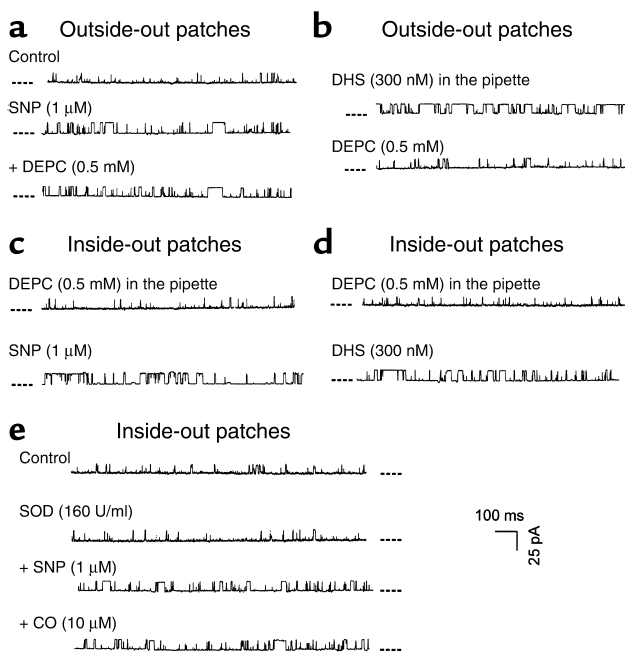


Figure 9
The mechanisms underlying the inhibition of CO effect on K_{Ca} channels by NO. The dashed lines beside each trace indicate the closed channel current level. Membrane potential, +30 mV. $[Ca^{2+}]_i$ in these experiments was 1 μ M. (a) Pretreatment of the outside-out membrane patch with SNP rendered the K_{Ca} channels insensitive to DEPC modification. (b) Inclusion of DHS in the pipette solution increased the NP_o of the outside-out patches but did not prevent K_{Ca} channels from inhibition by the subsequent bath-applied DEPC. (c) Inclusion of DEPC in the pipette solution did not interfere with the excitatory effect of the bath-applied SNP on the NP_o in the inside-out patches. (d) Inclusion of DEPC in the pipette solution did not interfere with the excitatory effect of the bath-applied DHS on the NP_o in the inside-out patches. (e) The presence of SOD in the bath solution did not remove the inhibition of CO effect-by SNP.

in a greater dissociation rate between CO and its target than between NO and K_{Ca} channels. This speculation merits further investigation.

Physiologically, vascular tone may be under the tonic influence of CO (ascribed to the long life span and low vasorelaxant potency of the gas) and regulated by the phasic effect of NO that has a short half life and greater vasorelaxant potency. It can be perceived, based on our results, that the persistent presence of CO in the surroundings of vascular SMCs would not affect the sensitivity of K_{Ca} channels to NO so that the vascular contractility could be acutely and precisely regulated by NO surge. In case a prolonged NO exposure at normal or higher concentrations is encountered, as in ischemia-reperfusion damage, septicemia, or other pathophysiological situations, the tonic relaxant influence of CO on vascular tone would be reduced due to its decreased effect on K_{Ca} channels in vascular SMCs. Thus, the interaction between NO and CO would provide a feedback mechanism to help regain the control of the disturbed vascular contractility. Elucidating the interaction of NO and CO on the function of K_{Ca} chan-

nels in vascular SMCs was the first endeavor and provided important progress leading to a better understanding of the integrated fine-tuning of cardiovascular functions by gaseous vasoactive factors.

Since our results are largely derived from cell-free patch-clamp studies in which the involvement of second messengers has been minimized, a direct action of CO and NO on K_{Ca} channel complex in rat tail artery SMCs under our experimental conditions is envisioned. It is worth noting that our conclusion by no means excludes the possibility that NO and CO may still affect K_{Ca} channels indirectly in other types of vascular SMCs. Mounting evidence shows that NO (10, 16, 24) and CO (11, 12) can directly act on K_{Ca} channel proteins to alter the channel gating. Other studies show that the activation of K_{Ca} channels by NO is mediated by cGMP-dependent mechanisms (25, 26). No study has reported a cGMP-dependent effect of CO on K_{Ca} channels, to our knowledge. The inconsistency in the cGMP dependency of the effect of NO on K_{Ca} channels cannot yet be readily explained. One putative explanation is the difference in cell types from different vascular beds, which have been used under different experimental conditions (26).

Antisense ODN treatment introduces DNA complementary in sequence to a portion of the target mRNA to block its translation. The use of antisense ODN in combination with electrophysiology measurement offers a useful approach for evaluating the contribution of different genes to the native ionic currents. The present study used a specific phosphorothioate antisense ODN to hybridize the translation initiation codon of $K_{Ca,\beta}$ subunit mRNA to eliminate the participation of β subunit in the assembly of native K_{Ca} channel complex in rat tail artery SMCs. Due to the unavailability of commercial Ab against $K_{Ca,\beta}$ subunit, we were not able to confirm the diminished expression of $K_{Ca,\beta}$ subunit at protein levels after anti- $K_{Ca,\beta}$ antisense ODN treatment. However, the decreased calcium sensitivity and voltage dependence of K_{Ca} channels as well as the lack of effect of DHS on K_{Ca} channels after anti- $K_{Ca,\beta}$ antisense ODN treatment speak strongly for the reduction of β subunit as part of the K_{Ca} channel complex (27). Since the effect of NO on K_{Ca} channels was abolished after anti- $K_{Ca,\beta}$ antisense ODN treatment, it is reasoned that the effect of NO may rely on the presence of β subunit. The specificity of anti- $K_{Ca,\beta}$ antisense ODN was further demonstrated by the scrambled ODN that did not alter the sensitivities of K_{Ca} channels whatsoever to NO, CO, and DHS. On the other hand, that CO still increased K_{Ca} channel activity after anti- $K_{Ca,\beta}$ antisense ODN treatment claims $K_{Ca,\alpha}$ subunit as the primary target of this gas. The cell membranes were not treated with anti- $K_{Ca,\alpha}$ antisense ODN in the present study since no K_{Ca} currents could be conducted if the translation of the pore-forming element $K_{Ca,\alpha}$ subunit were blocked. Importantly, the conclusion derived from the antisense experiments was supported by our study of the heterologous expression

of $K_{Ca}\alpha$ subunit. CO, but not NO, significantly increased the K_{Ca} currents generated by $K_{Ca}\alpha$ subunits in COS-1 cells. Although the possibility that NO also reacts with $K_{Ca}\alpha$ subunit still remains, our results leave little doubt that the $K_{Ca}\beta$ subunit is irreplaceable for NO to affect K_{Ca} channel activities.

A pharmacological approach in our study to investigate the subunit-dependent effects of NO and CO on K_{Ca} channels was to examine the interaction of DHS with NO/CO. DHS is a glycosylated triterpene that acts on $K_{Ca}\beta$ subunit in smooth muscles (21, 22). The inhibition of IbTX binding by DHS has also been used as the evidence for the presence of $K_{Ca}\beta$ subunit in rat uterus smooth muscle and in HEK293 cells expressing $K_{Ca}\alpha$ and $K_{Ca}\beta$ subunits (28). By the same token, the absence of $K_{Ca}\beta$ subunit in rat brain and in HEK293 cells expressing only $K_{Ca}\alpha$ subunit was concluded based on the failure of DHS to inhibit the IbTX binding to plasma membranes. In our study, after the saturated excitatory effect of DHS on K_{Ca} channels was achieved, the stimulatory effect of NO on K_{Ca} channels was nullified, but CO was still capable of increasing the NP_o of K_{Ca} channels. Obviously, the NO effect was related to the functional status of $K_{Ca}\beta$ subunit and CO effect was not.

Multiple subtypes or isoforms of large-conductance K_{Ca} channels with large variation in calcium sensitivity have been observed (29). One of the explanations for this diversity is the different coupling affinity or ratio between $K_{Ca}\alpha$ and $K_{Ca}\beta$ subunits (30). There are several lines of evidence corroborating this notion. First, the calcium sensitivity of K_{Ca} channels is greatly affected by the presence of β subunit. Second, although the α/β complex form of K_{Ca} channels was predominant, K_{Ca} channels comprising α subunit alone existed (21). Third, tissue distribution patterns of α subunit and β subunit did not always match, and in certain types of tissues they might not even couple (31). Combining the importance of α/β subunit coupling to the functionality of K_{Ca} channels with our present results may help to elucidate the mechanisms for the different effects of NO or CO on K_{Ca} channels in different types of vascular SMCs. For instance, with the presence of $K_{Ca}\beta$ subunit as the functional necessity for the NO effect, NO might not have a significant effect on K_{Ca} channels in certain types of vascular SMCs if the coupling of $K_{Ca}\beta$ subunit to $K_{Ca}\alpha$ subunit is lacking or weakened.

As shown in our previous study, DEPC induced a relatively irreversible covalent modification of histidine residue and decreased K_{Ca} channel activity. After the chemical modification with DEPC, K_{Ca} channels lost their responses to CO stimulation (13). Our present study showed that pretreatment of K_{Ca} channels with SNP significantly inhibited the effect of the subsequently applied CO on K_{Ca} channels. It was speculated that SNP might act on the CO-sensitive histidine to desensitize K_{Ca} channels. If this were the case, not only CO but also DEPC would have no effect on K_{Ca} channels after the NO treatment. Our results shown in Fig-

ure 9a support this theory. Moreover, the saturated intracellular DHS effect on K_{Ca} channels did not prevent CO from increasing (Figure 5), or DEPC from inhibiting (Figure 9b), K_{Ca} channels though it abolished the effect of NO. It seems that NO has two distinct acting sites. By acting on the inner surface-located DHS-sensitive site (likely on β subunit), NO increased K_{Ca} channel openings. By acting on the outer surface-located DEPC-sensitive histidine residue, NO reduced K_{Ca} channel sensitivity to the agents that targeted on histidine. Interestingly, after the outer membrane surface was first treated with DEPC, the subsequently applied NO or DHS (inner surface) still increased the NP_o of K_{Ca} channels (Figure 9, c and d). These results suggested that the DEPC-sensitive histidine is likely located on $K_{Ca}\alpha$ subunit and its structural change would not affect the $K_{Ca}\beta$ subunit-dependent excitatory effects of NO or DHS.

The nature of the interaction between NO and CO on K_{Ca} channels is unknown. In reaction with superoxide anions, NO would transform to peroxynitrite, which leads to the oxidation of many proteins. However, this mechanism could not explain the observed NO effect since the inclusion of SOD did not alter the effect of NO on K_{Ca} channels, nor change the inhibitory effect of NO on the CO-induced increase in NP_o . Alternatively, the action of NO on $K_{Ca}\beta$ subunit may exert an allosteric effect on the neighboring $K_{Ca}\alpha$ subunit. The noncovalent association of α and β subunits of K_{Ca} channels is tight, with a distance in between of less than 12 Å (32). The conformational changes of $K_{Ca}\beta$ subunit would prevent the formation of hydrogen bonds between CO and the imidazole group of histidine of $K_{Ca}\alpha$ subunit (13). This putative "repulsive" mechanism is reminiscent of the effect of NO on the activation of sGC. Upon binding to sGC, NO induces the displacement of the iron atom, and this repulsive *trans* effect prevents the access to and binding of the proximal histidine of sGC to the heme iron (33).

Acknowledgments

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