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Nitric Oxide Synthase Mediates Carbon Monoxide-Induced Stimulation of L-type Calcium Currents in Human Jejunal Smooth Muscle Cells

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Exogenous carbon monoxide (0.2%) increases L-type calcium (Ca^{2+}) current in human jejunal circular smooth muscle cells. The stimulatory effect of carbon monoxide (CO) on L-type Ca^{2+} current is inhibited by pre-application of L-NNA, a classical competitive inhibitor of nitric oxide synthase (NOS) with no significant isoform selectivity (Lim, 2003). In the present study, we investigated which isoform of NOS affected CO induced stimulation of L-type Ca^{2+} current in human jejunal circular smooth muscle cells. Cells were voltage clamped by whole-cell mode patch clamp technique, and membrane currents were recorded with 10 mM barium as the charge carrier. Before the addition of CO, cells were pretreated with each inhibitor of three NOS isoforms for 15 minutes. CO-stimulating effect on L-type Ca^{2+} current was partially blocked by N-(3-(Amino-methyl) benzyl) acetamidine · 2HCl (1400W, an iNOS inhibitor). On the other hand, 3-bromo-7-nitroindazole (BNI, a nNOS inhibitor) or N^5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO, an eNOS inhibitor) completely blocked the CO effect. These data suggest that low dose of exogenous CO may stimulate all NOS isoforms to increase L-type Ca^{2+} channel through nitric oxide (NO) pathway in human jejunal circular smooth muscle cells.

Key Words: L-type calcium channels, Carbon monoxide, Intestinal smooth muscle cells, Nitric oxide synthase

INTRODUCTION

Carbon monoxide (CO) and nitric oxide (NO) act as cotransmitters, and both are required for inhibitory neurotransmission in the mouse intestine, because CO mediates inhibitory neurotransmission through NO pathway (Xue et al, 2000). Both activate soluble guanylyl cyclase (sGC) and increase cGMP levels (Farrugia et al, 1998; Zyromski et al, 2001). Most researches on the NO pathway have been performed by applying exogenous NO, by eliminating the messenger with hemoglobin as a scavenger, or by inhibiting its synthesis (Moncada et al, 1991). Arginine derivatives which can inhibit the production of NO by competing with L-arginine or by blocking the NOS (Marletta, 1994) have been widely used. NOS, the mammalian enzyme catalyzing the oxidation of L-arginine to L-citrulline and NO, exists in three isoforms. Because each of the three major isoforms of NOS has distinct physiological functions and they are subject to separate regulatory and disregulatory influences, safe and efficient pharmacological treatment of NOS-mediated pathologies would require isoform-specific inhibitors.

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Neuronal isoform of NOS (nNOS, type 1) is considered as a viable therapeutic target, because over-stimulated nNOS is implicated in ischemia-reperfusion injury following stroke, migraine headache, pain and neurodegenerative disorders (Dawson and Dawson, 1996; Anbar and Gratt, 1997; Lassen et al, 1997). The inducible isoform of NOS (iNOS, type 2) is expressed in response to endotoxin and inflammatory cytokines in macrophages, vascular endothelial cells, smooth muscle cells, and many other cell types (Griffith and Stuehr, 1995; Gross and Wolin, 1995). Because iNOS is often expressed at high levels and essentially unregulated once expressed, locally high cytotoxic concentration of NO can be formed. Animal studies suggest that iNOS inhibitors might be useful in treating septic shock (Thiemermann, 1997), stroke (Zhang et al, 1996), many inflammatory and autoimmune disorders, including rheumatoid arthritis, osteoarthritis (McCartney-Francis et al, 1993), inflammatory bowel disease (Yamada et al, 1993), type 1 diabetes (McDaniel et al, 1996) and multiple sclerosis (Parkinson et al, 1997). Endothelial isoform of NOS (eNOS, type 1) plays a key role in maintaining normal blood pressure, therefore, some pathologies may be associated with hyperactivity of eNOS (Vromen et al, 1996).

Low dose of CO (0.2%) increases L-type calcium (Ca^{2+})

ABBREVIATIONS: CO, carbon monoxide; NOS, nitric oxide synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS

current in human jejunal circular smooth muscle cells. However, N^G-nitro-L-arginine (L-NNA, 100 μM) pretreatment blocks this stimulatory effect of CO on L- type Ca^{2+} currents, suggesting that means CO may activate the current through NO pathway (Lim, 2003). L-NNA is a classical competitive inhibitor of NOS, however, it has no significant isoform selectivity (Babu and Griffity, 1998). 3-bromo-7-nitroindazole (BNI) is a specific nNOS inhibitor that has been reported to be approximately four times more potent than 7-NI (7-nitroindazole), a well-known specific nNOS inhibitor (Rickard et al, 1999). 1400W is the most specific inhibitor of iNOS reported up to date and 200 to 5000-fold more potent against iNOS than eNOS and nNOS (Garvey et al, 1997), whereas L-NIO is approximately 5 times more potent as an inhibitor of eNOS than nNOS (Rees et al, 1990). In order to discern which isoform of NOS that affects CO stimulatory effect on L-type Ca^{2+} channel in the smooth muscle cells, each isoform-specific inhibitors of NOS should individually be tested.

In the present study, we examined the effect of each specific inhibitors of three isoforms of NOS on CO induced stimulation for L-type Ca^{2+} channels in human jejunal circular smooth muscle cells.

METHODS

Preparation of human jejunal circular smooth muscle cell

Human jejunal tissue was obtained from surgical tissue during gastric operations. Tissue specimens were harvested directly into chilled buffer, and dissociation was carried out with the use of a papain-based enzymatic technique (Farrugia et al, 1993). Briefly, the tissue was pinned down with mucosal surface up in a Petri dish filled with normal Krebs solution. The mucosa was removed, and tissue was cut and placed with serosal surface up in normal Krebs solution. The serosa and the longitudinal muscle were removed, and the circular muscle layer and submucosa were left. Strips of circular muscle were pulled off and cut into small pieces. They were placed in enzyme solution containing 15 mg of papain (Sigma, USA) and 3 mg of dithiothreitol (Sigma, USA) in 15 ml of Hanks' solution (Sigma, USA) and magnetically stirred at 37°C. After centrifugation, the tissue was mechanically dissociated to obtain single relaxed circular smooth muscle cells. The fresh isolated cells were used in electrophysiological recording within 6 hours of dissociation.

Patch clamp recordings and data analysis

Glass microelectrodes for whole-cell mode voltage clamp recordings were obtained by using borosilicate glass (WPI, USA), pulled on a electrode puller PP-83 (Narishige, Japan). Electrodes were coated with sylgard and fire polished to a final resistance of 3 to 5 MΩ. Currents were amplified, digitized, and processed using Axopatch 200B amplifier, Digidata 1322, and pCLAMP 9 software (Axon Instruments, USA). Membrane currents were filtered at 2 kHz with an 8 pole Bessel filter, digitized and stored. No leakage subtraction was performed to the original recordings, and all data with visible changes in leakage currents during the course of study were excluded from further analysis. The cell was held at −90 mV, pulsed in 13 steps to voltages ranging from −90 mV to 30 mV and stimulated every 5 sec to record the peak of the current. Each pulse was 500 ms long. The cell was returned to −90 mV between pulses with an interpulse interval of 1 sec to allow complete recovery from inactivation. Current-voltage (I-V) curves were constructed using the current amplitude which measured the maximal negative peak point. All electrophysiological experiments were carried out at room temperature (22~23°C). Data were expressed as means± standard error (S.E.). Differences before and after CO addition in the same cells were evaluated by Students t-test (two-tailed, paired) and the differences between CO effect without any NOS inhibitor and with inhibitor of each NOS isoforms by Students t-test (two-tailed, unpaired). The significant level of difference was determined (*$p<0.05$, **$p<0.01$).

Solutions and chemicals

The pipette solution contained (in mM) $CsMeSO_3$ 125, CsCl 20, EGTA 2, and HEPES 5 (pH adjusted to 7.3 with CsOH). The bath solution contained $BaCl_2$ 10, NaCl 142, KCl 5, HEPES 5, and mannitol to reach an osmolarity of 290 mOsm (pH adjusted to 7.35 with NaOH) for whole cell recordings.

CO solution was freshly prepared just before each experiment. A bulb sealed with a rubber injection port was filled with CO at atmospheric pressure. A gas syringe was used to remove 1 ml of CO which was added to 100 ml of bath solution placed in another gas bulb. One hundred μl of 1 % CO solution was gently added to the bath (500 μl) to 0.2% final concentration. CO solution was applied directly by syringe into bath solution to prevent mechanoactivation of L-type Ca^{2+} channel by continuous flow.

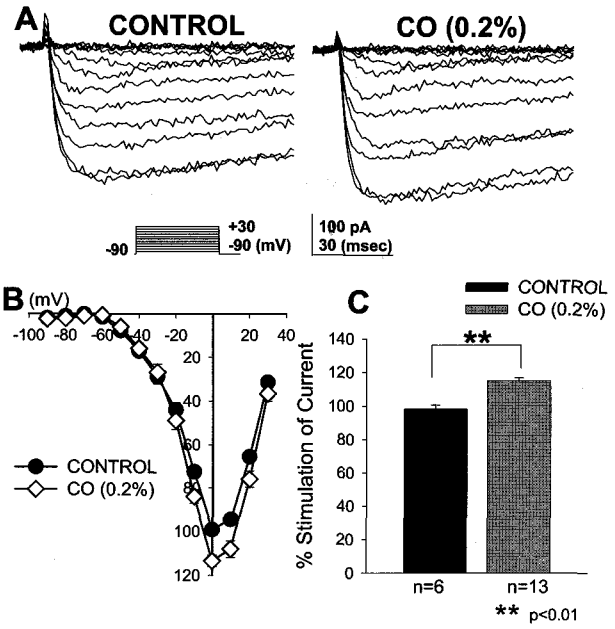

Fig. 1. The effect of exogenous CO on L-type Ca^{2+} currents of human jejunal circular smooth muscle cells.

N-omega-nitro-L-arginine (L-NNA) was purchased from Sigma, 3-bromo-7-nitroindazole (BNI) and N^5-1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO) from Tocris, and N-(3-(Amino-methyl) benzyl) acetamidine · 2HCl (1400W) was from Biomol. BNI was dissolved in dimethylsulfoxide (DMSO), L-NNA, 1400W and L-NIO were dissolved in water.

RESULTS

The human jejunal circular smooth muscle cells were held at −90 mV, and the membrane potential was pulsed in 13 steps from −90 mV to 30 mV for 500 msec. To minimize outward potassium (K^+) current, the intrapipette solution contained 145 mM cesium (Cs^+) to block K^+ channels, and the bath solution contained 10 mM Ba^{2+} as the charge carrier, because Ba^{2+} is more permeable to L-type Ca^{2+} channels than Ca^{2+} itself. The mean amplitude of maximal inward current recorded in control cell was 232±11 pA (n =39), and the inward current was blocked by nifedipine (1 μM), indicating L-type calcium channel (Data are not shown). Peak current of L-type Ca^{2+} current typically increased during the first several minutes of recording before reaching a constant level. After reaching a constant level, CO at 0.2% final concentration, which is within the physiological blood concentration (Vreman et al, 1984), was added to the cells. CO increased significantly L-type Ca^{2+} current by 15.8±1.6% (mean±S.E., n=13, $p<0.01$, Fig. 1) without any pretreatment of NOS inhibitors. No shift in the mean current-voltage (I-V) relationship was noted between before and after CO addition. To verify the increase of the current by direct addition of bath solution on the L-type solution without CO. Direct infusion of the bath solution without CO did not increase the L-type Ca^{2+} current (−1.72 ±2.4% increase, n=6, $p>0.05$).

We tested several isoform-specific inhibitors of NOS to verify which isoform of NOS affected the CO stimulatory effect on the current. Control indicated the L-type Ca^{2+} current in 10 mM Ba^{2+} Ringer solution that pretreated with each NOS isoform-specific inhibitor before CO application. After pre-application of BNI (1 μM, a specific nNOS inhibitor), the stimulatory effect of CO on L-type Ca^{2+} channel was blocked. The representative current of BNI effect is shown in Fig. 2A. There was no significant shift between control and CO stimulated current after BNI in the I-V curve (Fig. 2B). The bar graph in Fig. 2C shows the summary of the CO effect without BNI and with BNI pretreatment for 15 minutes (5.8±1.7%, n=4, $p<0.01$). CO could not increase the L-type Ca^{2+} current after 1400 W (100 nM, a selective iNOS inhibitor) pretreatment (Fig. 3A). No significant I-V shift could be observed between the two groups (Fig. 3B). The bar graph in Fig. 3C shows % stimulation of the current by CO alone and CO with pretreatment of 1400 W (8.6±1.5% increase, n=5, $p<0.01$).

L-NIO, a specific eNOS inhibitor, was also tested to verify whether eNOS is involved in the CO effect. A typical experiment with L-NIO is shown in Fig. 4A. When CO (0.2%) added to the bath solution, L-type Ca^{2+} current in the L-NIO (5 μM) pretreated cells was not increased. Fig. 4B illustrates no significant change in the typical I-V relationship between the control and CO-added group after L-NIO pre-treatment. The bar graph in Fig. 4C shows % stimulation of the current by CO after L-NIO pretreatment (3.5±1.6% increase, n=5, $p<0.01$).

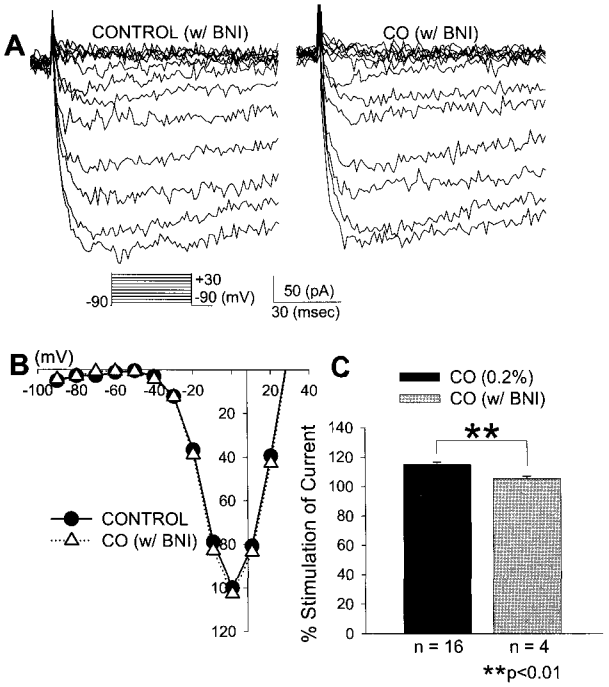

Fig. 2. 3-bromo-7-nitroindazole (BNI) pretreatment blocked CO stimulatory effect on L-type Ca^{2+} currents.

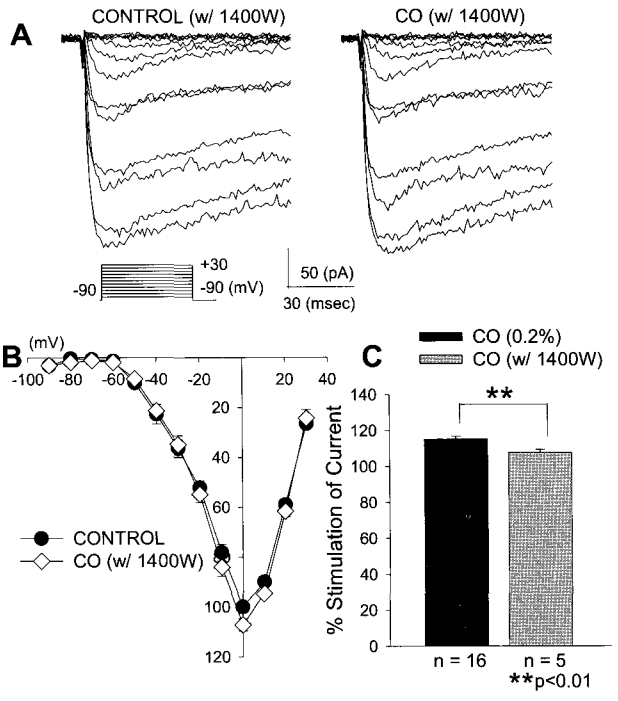

Fig. 3. 1400W pretreatment blocked CO stimulating effect on L-type Ca^{2+} current.

Ca^{2+} channel, we conducted similar experiment with bath

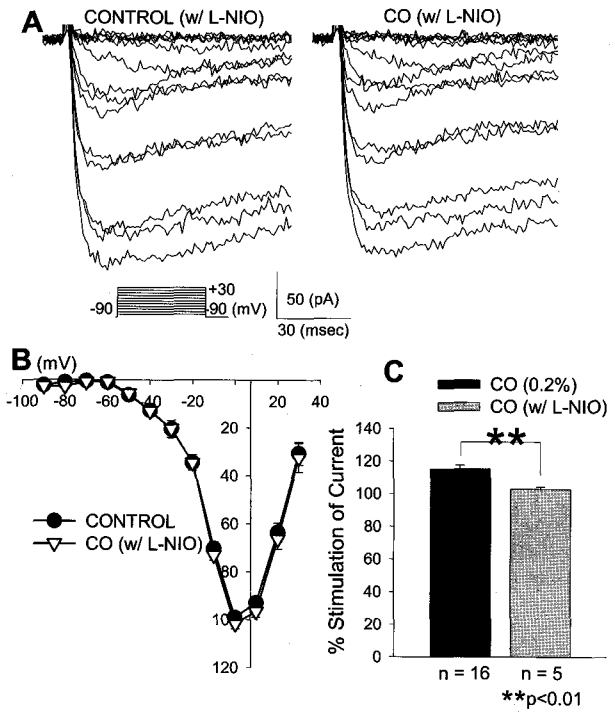

Fig. 4. L-NIO blocked the CO effect on L-type Ca^{2+} channel currents.

DISCUSSION

CO and NO are low molecular weight oxide gases, and they are endogeneously produced under physiological conditions. They collaborate as co-transmitters to mediate intestinal neurotransmission (Xue et al, 2000), therefore, they are known as smooth muscle relaxant through soluble guanylyl cyclase activation, but CO exhibits a poor vasorelaxant activity than NO (Villamor et al, 2000). CO can activate guanylyl cyclase to produce cGMP (Verma et al, 1993), which modulates NO release (Hallen et al, 2001). There are some reports to indicate cross-talk between CO and NO. NO increases heme oxygenase-1 (HO-1) expression by induction of mRNA transcription (Hartsfield et al, 1997), and HO coexists with NOS in gastrointestinal tracts, as revealed by immunoreactivity for NOS (Ny et al, 1997). CO regulates NOS activity in a concentration-dependent manner; with high CO levels inhibit NOS activity and low CO levels raise NO levels. Also, CO induces NO release that could be attributed to either stimulation of eNOS or NO displacement from cellular storage pool (Thorup et al, 1999). In addition, L-NNA blocks the effect of CO on L-type Ca^{2+} channel in human jejunal circular smooth muscle cells (Lim, 2003), suggesting that CO not only induces release of preformed NO, but also stimulates NO production.

Neuronal NOS (nNOS), as with eNOS, is constitutive and tightly regulated by Ca^{2+} and calmodulin (Ca^{2+}/CaM). It has been identified in vascular smooth muscle cells (Buchwalow et al, 2002), central and peripheral neuronal cells where found to be co-localized with HO-2 (Vincent et al, 1994; Ny et al, 1996; 1997). The inducible NOS (iNOS) expressed in response to endotoxin and inflammatory cytokines in macrophages, vascular endothelial and smooth muscle cells (Griffith and Stuehr, 1995). The endothelial NOS (eNOS) plays a key role in maintaining normal blood pressure (Hlatky et al, 2003).

To investigate the nature of NOS isoforms involved, we examined the influence of specific NOS inhibitors of the NOS isoform. Our experiment revealed that inhibitors of all isoforms, BNI (a nNOS inhibitor), 1400W (an iNOS inhibitor), and L-NIO (an eNOS inhibitor) attenuated the CO stimulation effect on the L-type Ca^{2+} current.

We did not attempt to determine the presence of NOS isoforms in jejunal smooth muscle cells. However, several reports that all three NOS isoforms are present in smooth muscle cells; eNOS and nNOS are detected in jejunal smooth muscle cell of human, rabbit (Teng et al, 1998) and rat (Chen et al, 2002), and in the gastric fundus of human and rat (Fischer et al, 1999), whereas iNOS is possibly induced by the procedure to prepare the smooth muscle cells of gastric fundus of guinea pig (Dick et al, 2000) and rat (Zheng et al, 1999). By novel supersensitive immunohistochemical technique of signal amplification with tyramide and electron microscopic immunogold labelling complemented with Western blotting, all three NOS isoforms were found to be expressed in vacular smooth labelling muscle cells (Buchwalow et al, 2002).

In the present study, the inhibitors of eNOS and nNOS blocked CO-stimulation effect more sensitive than iNOS. Both eNOS and nNOS are constitutively expressed enzymes, whose activities are stimulated by intracellular calcium increase. We used barium ion as the charge carrier to increase the amplitude of L-type calcium current. Calcium and barium ions stimulate L-citrulline formation in the presence of calmodulin in identical fractions, indicating a possible action of Ba^{2+} on NOS through the calmodulin-dependent pathway (Yamazaki et al, 1996). According to this report, barium ions may not decrease eNOS and nNOS effect on L-type calcium channel. For the reason why iNOS effect is weaker than eNOS and nNOS, we can offer at least two possible mechanisms; the amount of iNOS produced by cell isolation procedure was not sufficient enough to make the CO stimulatory effect, and iNOS was too weak to stimulate L-type Ca^{2+} channel because of its low calcium-dependency. However, based on our results, we were unable to decide which is more reasonable mechanism. To elucidate the exact mechanism, we need further studies to examine the expression patterns and levels of each NOS isoform after CO treatment. In summary, our results suggest that exogenous CO may stimulate all NOS isoforms to increase L-type Ca^{2+} currents through NO pathway in human jejunal circular smooth muscle cells.

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