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Opening of small and intermediate calcium-activated potassium channels induce relaxation mainly mediated by NO release in large arteries and EDHF in small arteries from rat

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Running Title: NS309 vasorelaxation is mediated by NO and EDHF

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Non-standard abbreviation list: ADMA, N^G, N^G-asymmetric dimethyl-L-arginine; BK_{Ca} channel; large conductance calcium-activated potassium channel; ChTX, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factor; IbTX, iberiotoxin; IK_{Ca} channel, intermediate conductance calcium-activated potassium channel; NO, nitric oxide; NOS, nitric oxide synthase; NS309, 6,7-dichloro-1*H*-indole-2,3-dione 3-oxime; PSS, physiological saline solution; SK_{Ca} channel, small conductance calcium-activated potassium channel; TRAM 34, 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole; U46619, 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$.

A recommended section assignment: Cardiovascular pharmacology

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Abstract

This study was designed to investigate whether calcium-activated potassium channels of small (SK_{Ca} or $K_{Ca}2$) and intermediate (IK_{Ca} or $K_{Ca}3.1$) conductance activated by NS309 are involved in both nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) type relaxation in large and small rat mesenteric arteries. Segments of rat superior and small mesenteric arteries were mounted in myographs for functional studies. NO was recorded using NO microsensors. SK_{Ca} and IK_{Ca} channel-currents, and mRNA expression were investigated in human umbilical vein endothelial cells (HUVECs), and calcium concentrations both in HUVEC and mesenteric arterial endothelial cells. In both superior (~1093 μm) and small mesenteric (~300 μm) arteries, NS309, evoked endothelium- and concentration-dependent relaxations. In superior mesenteric arteries, NS309 relaxations and NO release were inhibited both by asymmetric dimethylarginine (ADMA, 300 μM), an inhibitor of NO synthase, and by apamin (0.5 μM) plus TRAM 34 (1 μM), blockers of SK_{Ca} and IK_{Ca} channels, respectively. In small mesenteric arteries, NS309 relaxations were slightly reduced by ADMA, whereas apamin plus an IK_{Ca} channel blocker almost abolished the relaxation. Iberiotoxin did not change NS309 relaxation. HUVECs expressed mRNA for SK_{Ca} and IK_{Ca} channels, and NS309 induced increases in calcium, outward current, and NO release that was blocked by apamin and TRAM 34 or charybdotoxin. These findings suggest that opening of SK_{Ca} and IK_{Ca} channels leads to endothelium-dependent relaxation that is mainly mediated by NO in large mesenteric arteries and by EDHF-type relaxation in small mesenteric arteries. NS309-induced calcium influx appears to contribute to formation of NO.

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Introduction

The vascular endothelium plays a major role in the regulation of blood flow. Through the release of vasoactive agents, the endothelium may decrease the contraction level of the vascular smooth muscle and hence increase blood flow. The vasoactive agents released by the endothelium include nitric oxide (NO), prostacyclin and factors involved in the endothelium-dependent hyperpolarizing factor (EDHF)-type relaxation (Feletou and Vanhoutte, 1988;Feletou and Vanhoutte, 2009). The production of endothelium-dependent relaxing factors generally involves an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. An increase in endothelial $[Ca^{2+}]_i$ will open calcium-activated potassium channels of small (SK_{Ca} or $K_{Ca}2$) and intermediate (IK_{Ca} or $K_{Ca}3.1$) conductance thereby hyperpolarizing the endothelial cell. These channels have been acknowledged as a required component for the activation of the EDHF-type relaxation (Burnham, et al., 2002;Bychkov, et al., 2002;Eichler, et al., 2003). In vivo studies in mice show that suppression of the $SK_{Ca}3$ channel (Taylor, et al., 2003), knockout of the IK_{Ca} channel (Si, et al., 2006), and deficit of both $SK_{Ca}3$and IK_{Ca} channels (Brahler, et al., 2009) leads to elevated blood pressure. In addition, opening SK_{Ca} and IK_{Ca} channels decreases myogenic tone, increases acetylcholine-induced relaxation in rat cremaster arterioles (Sheng, et al., 2009), and restores the attenuated EDHF-type relaxation in mesenteric small arteries from Zucker diabetic fatty (ZDF) rats (Brondum, et al., 2009). Moreover, opening IK_{Ca} channels decreases mean arterial blood pressure in angiotensin II-induced hypertensive mice (Sankaranarayanan, et al., 2009) and conscious dogs (Damkjaer, et al., 2011). These results support the findings that SK_{Ca} and IK_{Ca} channels are involved in controlling blood pressure and organ blood flow and this is often attributed to EDHF type relaxation. However, recent studies suggest that SK_{Ca} and IK_{Ca} channel-opening is also associated with activation of NO synthase and NO production (Stankevicius, et al., 2006;Sheng and Braun, 2007;Sheng, et al.,

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2009;Brahler, et al., 2009;Dalsgaard, et al., 2010a). In addition, the mechanisms involved in SK_{Ca} and IK_{Ca} channel activation leading to vasodilation are largely unknown.

Therefore, the present study investigated the effects of opening endothelial SK_{Ca} and IK_{Ca} channels by use of 6,7-dichloro-1*H*-indole-2,3-dione 3-oxime (NS309) in a large artery and small artery, and hypothesized that opening of endothelial SK_{Ca} and IK_{Ca} channels would lead to NO induced relaxation in large arteries and EDHF-type relaxation in small arteries. NS309 is an activator of SK_{Ca} and IK_{Ca} channels in human epithelial kidney cells, possibly acting through increasing the channel sensitivity for Ca^{2+} (Strobaek, et al., 2004). Therefore, we chose to characterize the effects of the compound in human umbilical vein endothelial cells HUVECs by patch clamp studies and to address how the compound may lead to NO release by measuring NS309-induced changes in $[Ca^{2+}]_i$ in HUVECs and freshly isolated mesenteric arterial endothelial cells.

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Material and methods

Rat mesenteric arteries

Adult male Wistar rats (12 weeks old) were killed in accordance with a protocol to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The used protocol was approved by The Danish Ministry of Justice (permission 2005/561-964). The mesenteric vascular bed was isolated and the superior mesenteric artery and third order small mesenteric arteries, were dissected, isolated and transferred into cold physiological saline solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, glucose 5.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 1.6, and ethylenediaminetetraacetic acid (EDTA) 0.026. The solution was equilibrated with bioair of the following composition; 5% CO_2, 21% O_2 and 74% N_2 to maintain pH at 7.4.

Isometric tension recordings. Superior mesenteric arteries (internal diameter 1093±9 µm, n=39) were mounted on two 100 µm wires in a wire myograph (model 310A, DMT, Aarhus, Denmark) for isometric tension recordings and normalized as previously described (Simonsen, et al., 1999). In brief, arterial segments were stretched to their optimal lumen diameter for active tension development, i.e., to an internal circumference of 90% of that achieved when the vessels were exposed to a passive tension yielding a transmural pressure of 100 mmHg (Simonsen, et al., 1999). Segments were discarded if noradrenalin-induced (5 µM) contraction was below 10 kPa or if acetylcholine-induced (ACh, 10 µM) relaxation on noradrenalin-induced (5 µM) contraction was less 75%.

To investigate the role of opening SK_{Ca} and IK_{Ca} channels on tension, arterial segments were incubated with ADMA (300 µM), the SK_{Ca} channel blocker, apamin (0.5 µM), and the IK_{Ca} channel blocker, 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM 34, 1 µM). Unless otherwise

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stated, arterial segments were always incubated with the cyclooxygenase inhibitor, indomethacin (3 μM). After 30 min of incubation with inhibitor and/or blocker, segments were contracted with norepinephrine (0.5 μM) to constrict the vessel to 60-70% of the maximum response. When contraction was stable, cumulative concentration-response curves for NS309 (0.01-10 μM) were constructed and changes in tension were recorded.

Isobaric tension recordings. Third order mesenteric small arteries (internal diameter 300±30 μm, n=34) were mounted in a myograph (model 110P, DMT, Aarhus, Denmark) where pressure and flow could be adjusted as previously described (Thorsgaard, et al., 2003). In brief, the arterial segment was mounted on two micropipettes and viewed through an inverted microscope equipped with a CCD video camera linked to a PC via a frame grabber. Hydrostatic pressures of both inlet and outlet reservoirs were measured by pressure transducers connected to the perfusion line on the inlet and outlet side, respectively, and the segment was pressurized to 50 mmHg. The inclusion criteria for the arterial segments were as follows: 1) The contractile response induced by 11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619, 10 nM) should reduce vessel diameter with at least 25%, 2) relaxation evoked by ACh (10 μM) should be above 50% of U46619-induced constriction, and 3) if an air bubble passed through the arterial segment, the experiment was excluded. Unless otherwise stated, arterial segments were always incubated with indomethacin (3 μM).

To investigate the role of opening SK_{Ca} and IK_{Ca} channels on vessel diameter, arterial segments were incubated with ADMA (300 μM), apamin (0.5 μM), the IK channel blockers, TRAM-34 or charybdotoxin (ChTX, 0.1 μM), and the large conductance calcium-activated potassium channel (BK_{Ca} or $K_{Ca}1.1$) blocker, iberiotoxin (IbTX, 0.1 μM). After 30 min of incubation with inhibitor/blocker, segments were contracted with U46619 (10-40 nM) to constrict

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the vessel to 60 % of resting diameter. When contraction was stable, cumulative concentration-response curves for NS309 (0.001-1 μM) were constructed and changes in diameter were recorded.

Simultaneous measurements of force and NO. Superior and third order small mesenteric arteries (internal diameter 238±26 μm, n=20) were mounted on two 40 μm wires in a wire myograph (model 310A, DMT, Aarhus, Denmark) for isometric tension recordings with normalization procedure and inclusion criteria as described above. For simultaneous measurement of NO concentration and force, a NO-sensitive microsensor either ISONOP3020 (diameter of 30 μm) or ISONOP007 (diameter of 7 μm) (World Precision Instruments, Stevenage, UK) was first calibrated by use of NO gas in solution and selectivity was confirmed by the absence of response to sodium nitrite (10 μM) and noradrenaline (1 μM). Changes in NO concentration were recorded using a NO meter (ISO-NO Mark II, World Precision Instruments, Stevenage, UK). The sensor was then introduced into the lumen of the artery mounted in the myograph. Unless otherwise stated, arterial segments were always incubated with indomethacin (3 μM).

To investigate the role of opening SK_{Ca} and IK_{Ca} channels on NO production and relaxation, segments were incubated with ADMA (300 μM), the NO scavenger, oxyhemoglobin (10 μM), apamin (0.5 μM) and TRAM 34 (1 μM). After 30 minutes of incubation with the inhibitors and/or blockers, segments were contracted with noradrenalin (0.5 μM). When contraction was stable, NS309 (1 μM) was added to the organ bath, and NO concentration simultaneously with force was recorded.

Membrane potential measurements. Segments from small mesenteric arteries were mounted on 40 μm wires in a myograph (model 410A, DMT, Aarhus, Denmark) with normalization procedure and inclusion criteria as described above. Microelectrodes (glass, AS100F, WPI, Sarasota, FL, USA)

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were prepared on a horizontal puller (Sutter P-97, Novato, CA, USA), filled with 3 M KCl and connected to an amplifier (Intra 767, WPI). Electrodes with stable resistances (>30 MΩ) were used to measure membrane potential as described previously (Mulvany, et al., 1982). Recordings from smooth muscle cells in small mesenteric arterial segments were made by advancing the electrode through the adventitia into the media. Unless otherwise stated, the segments were always incubated with indomethacin (3 μM). Effect of NS309 (1 μM) on membrane potential was investigated in the presence of ADMA (300 μM) alone and in combination with ChTX (0.1 μM) and apamin (0.5 μM).

Studies in isolated endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (Ostergaard, et al., 2007). In brief, pregnant women attending routine antenatal care at the Department of Obstetrics and Gynecology, Skejby Hospital, Denmark, were invited to participate in the study. Written consent was obtained from those who agreed to participate (n=20). The investigation conformed to the principles outlined in the Declaration of Helsinki, and was approved by the local ethics committee of Aarhus (Reference number: 20040154). The umbilical cords were obtained immediately after delivery. HUVECs were isolated by rinsing the veins with Dulbecco´s Modified Eagle´s Medium, DMEM, (Sigma Aldrich, St. Louis, MO, USA) to remove as much blood as possible. Then 10 mL of 0.1% collagenase solution was flushed through the vein and the umbilical cord was closed at both ends. After incubating for one hour at 37°C the umbilical cord was washed with 20 mL DMEM with 10% fetal calf serum. The cells were spun and the pellet resuspended in growth medium containing 2% serum, epidermal growth factor, hydrocortisone, vascular endothelial growth factor, fibroblast growth factor, insulin growth factor, ascorbic acid, heparin, amphotericin B, and gentamicin (Endothelial Cell Growth Medium 2 with supplement pack, Promocell, Heidelberg, Germany). Cells were then plated out in gelatin-coated flasks or

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dishes and allowed to adhere overnight. Growth medium was changed every second day and the cells were passaged with 0.25% trypsin when confluent. In all experiments cells were used at passage one or two. The endothelial phenotype was confirmed using phase-contrast microscopy (i.e. cuboidal, cobblestone-appearing monolayer of cells) and positive immunofluorescence staining for anti-von Willebrand factor (1:4000, Dako Denmark A/S, Glostrup, Denmark).

Isolation of endothelial cells from superior mesenteric artery was carried out using previously described protocols (Brahler, et al., 2009). In brief, 1-2 cm segment of superior mesenteric artery were dissected from the mesenteric bed and cleaned from fat and connective tissue. Segment was mounted on a small glass capillary and filled with a trypsin (0.25%)/EDTA-buffer (Biochrom KG, Berlin, Germany). After filling, superior mesenteric artery was sutured and incubated for 45 min at 37°C. Thereafter, the vessel was cut open and the luminal surface was gently scrapped with a 10 µl pipette tip. Detached single endothelial cells and endothelial cell clusters were aspirated and transferred to a culture dish containing DMEM medium supplemented with 10% fetal calf serum and penicillin/streptomycin (Biochrom KG, Berlin, Germany), and cover slips. Cells were allowed to settle down for 2-4 hrs and used for calcium measurements within 24 hours.

Quantitative PCR. RNA extraction from HUVECs was performed using Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Total RNA was then treated with DNAse I (Qiagen Nordic, Copenhagen, Denmark). The concentration of the RNA was estimated by optical density measurements at 260 nm. Total RNA (100 µg/mL) was then reverse transcribed using Oligo-dT primer and Superscript^{TM} SIII reverse transcriptase (Invitrogen, San Diego, CA, USA).

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Expression of $SK_{Ca,}$ $IK_{Ca,}$ and BK_{Ca} channels was assessed by TaqMan quantitative PCR (QPCR). 200 ng RNA was used in a 25 μL reaction and QPCR conducted using the Ex Taq™ (TaKaRa Bio Inc, Shiga, Japan). The following cycles were run: 1 cycle at 95ºC for 2 minutes, 40 cycles at 95ºC for 15 seconds, 55ºC for 1 minute and 70ºC for 15 seconds on a Stratagene Mx3000P machine (Agilent Technologies, Santa Clara, CA, USA). Primer and fluorogenic probes were designed using PerlPrimer (MWG-Biotech AG, Ebersberg, Germany). The probe contained the reporter dye FAM (6-carboxyflorescein) at the 5'end and the Blackhole Quencher 1 (BHQ1) dye at the 3' end. Following primers and probes were used: GAPDH (BC_023632) forward (237-259): 5'-AAA TCC CAT CTT CCA GG-3', reverse (338-355): 5'-AGC CCC AGC CTT CTC CA-3' and probe (290-317): 5'-ATG CTG GCG CTG AGT ACG TCG TGG AGT-3'. BK_{Ca} (U13913) forward (3010-3030): 5'-TTC CTC AGC AAT CAG AGC CTC-3', reverse (3121-3141): 5'-ACA GCA TTT GCC GTC AGT GTC-3' and probe (3067-3099): 5'-AAT ATC CTC ACC CTG ATA CGG ACC CTG GTG ACC-3'. IK_{Ca} (NM_002250) forward (1368-1390): 5'-GTT CTA CAA ACA TAC TCG CAG GA-3', reverse (1572-1592): 5'-GCG TGT CAA TCT GTT TCT CAA-3' and probe (1439-1467): 5'- TCA ACG CGT TCC GCC AGG TGC GGC TGA AA-3'.SK_{Ca}3 (NM_002249) forward (2087-2107): 5'- GAT TGA CCA TGC CAA AGT GAG-3', reverse (2210-2230): 5'-ACA TGA CAT TCT GCA TCT TGG-3' and probe (2125-2149): 5'- TCC TCC AAG CTA TCC ACC AGT TGA G-3'.

Patch clamp. HUVECs were dispersed using 0.25% trypsin or non-enzymatic cell dissociation solution (Sigma Aldrich, St. Louis, MO, USA) and seeded onto glass coverslips ~1h prior to start of experiments. Once cells had attached, patch clamp experiments were performed at room temperature (~20 °C). Patch pipettes were fabricated from borosilicate glass and had a resistance of 3-6 MΩ after being fire polished. Membrane currents were measured using the conventional whole-

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cell patch clamp configuration with an EPC 10 patch clamp amplifier controlled by Pulse Trace 8.65 (HEKA Elektronik, Lambrecht, Germany) connected to a computer. Currents were recorded using an extracellular solution containing (mM): NaCl 105, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, HEPES 10, EGTA 0.026, glucose 5.5, and $CaCl_2$ 1.6 (pH 7.4). In some experiments $CaCl_2$ was omitted from the solution (Ca-free extracellular solution). The pipette solution contained (mM): KCl 30, K aspartate 100, $MgCl_2$ 1, EGTA 10, Na_2ATP 3, HEPES 5, $CaCl_2$ 8.5 (pH 7.2). Free $[Ca^{2+}]$ in the pipette solution was estimated to be 1 μM and free $[Mg^{2+}]$ was 50 μM (calculated by Winmaxc32 v2.50 [http://www.stanford.edu/~cpatton/maxc.html] using the constants in CMC0204E.TCM). In some experiments $CaCl_2$ was omitted from the solution (Ca-free pipette solution). Currents were sampled at 2 kHz and analyzed using Patchmaster Software (HEKA Elektronik, Lambrecht, Germany). Series resistances were <5MΩ and were not compensated for. Current-voltage (I/V) relationships were constructed using a holding membrane potential of – 60 mV and by stepping from -120 mV to 120 mV in 40 mV increments for 200 msec. Currents measured at a step holding potential of 80 mV were used to compare the effects of NS309 (0.1 and 1 μM), in the absence and presence of apamin (0.3 μM), ChTX (0.1 μM), TRAM 34 (1 μM), IbTX (0.1 μM), and glibenclamide (0.1 μM).

Measurements of NO concentration in HUVECs. HUVECs were seeded onto glass coverslips coated with gelatin (0.5 %). After cells reached confluence of 50-70%, the coverslips were transferred in to an organ bath and a NO-sensitive microsensor with a diameter of 30 μm (ISONOP30, World Precision Instruments, Stevenage, UK) was placed above the HUVECs and changes in NO concentration were recorded as described above.

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Measurements of intracellular Ca^{2+}. For measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) HUVECs were seeded onto gelatin-coated glass coverslips (0.5%) and loaded with FURA-2 AM (4 μM) for 30 min. After loading, cells were transferred in to a perfusion chamber (Warner instruments, Hamden, CT, USA). Ratio images of 340/380 nm were used for measurements of increases in $[Ca^{2+}]_i$ as previously described (Ostergaard, et al., 2007). To investigate the effects of opening of SK_{Ca} and IK_{Ca} channels on $[Ca^{2+}]_i$, NS309 was applied in three different concentrations (0.01, 0.1 and 1 μM) in the absence and the presence of TRAM 34 (1 μM) and compared to the effect of histamine (1 μM). Maximum $[Ca^{2+}]_i$ was estimated by addition of ionomycin (1 μM).

For calcium measurements, the mesenteric endothelial cells were loaded with a buffer consisting of the visible light excitable calcium indicator; Oregon Green BAPTA 1-AM (7.24 mM), the nonoinic surfactants; Cremophor EL [0.066% (w/v)], and Pluronic F-127 (10.66 mM), and dimethylsulphoxide (DMSO, 0.32% (w/v)) at 37°C for 20 min in PSS. The solutions were equilibrated with bioair of the following composition: 5% CO_2, 21% O_2 and 74% N_2. Image acquisition of endothelial cell calcium was performed with an inverted confocal microscope (model LSM 510 Exciter, Carl Zeiss Inc, Oberkochen, Germany) equipped with a 63 x NA = 0.75 long working distance objective excited at a wavelength of 488 nm using the Zeiss LSM Image Browser software program (Zeiss Inc, Oberkochen, Germany). To investigate the effects of opening of SK_{Ca} and IK_{Ca} channels on $[Ca^{2+}]_i$, NS309 (1 μM) was applied in the absence and the presence of TRAM 34 (1 μM) and extracellular Ca^{2+} (PSS0.0) and compared to the effect of ACh (10 μM). Maximum $[Ca^{2+}]_i$ was estimated by addition of ionomycin (1 μM).

Drugs

ACh, ADMA (N^G, N^G-asymmetric dimethyl-L-arginine), IbTX, glibenclamide, indomethacin, noradrenalin, TRAM 34 (1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole),

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Cremophor EL, Pluronic F-127, DMSO (dimethylsulfoxide), and U46619 (9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$) were purchased at Sigma Aldrich, St. Louis, MO, USA. Apamin and ChTX were purchased at Latoxan (Valence, France). FURA-2-AM and Oregon Green BAPTA 1-AM were obtained from Invitrogen (San Diego, CA, USA). NS309 was kindly donated by Dr. Joachim Demintz (Neurosearch A/S, Ballerup, Denmark). Indomethacin was prepared in PSS without $CaCl_2$. NS309 (6,7-dichloro-1*H*-indole-2,3-dione 3-oxime) and glibenclamide stock solutions were dissolved in DMSO and further diluted in distilled water. Total amount of DMSO never exceeded 1% in organ baths and did not affect any of the results. All other drugs were prepared in distilled water.

Data and statistical analysis

For the isometric tension recordings, the stable tension after each addition of NS309 was expressed as percentage of the active tension, where the active tone was defined as the level of contraction after addition of noradrenalin, minus the level of contraction after the normalization procedure. For the NO measurements, NO concentrations and relaxation were measured 3 min after exposure to SNAP, ACh, or NS309. For the calcium measurements, the relative increase in fluorescence intensity ($\Delta F/F_0$) of FURA-2-AM or Oregon Green BAPTA 1-AM were used for measurements of increases in $[Ca^{2+}]_i$. For QPCR measurements, the amount of cDNA was normalized to the reference gene by using the threshold Ct values: ΔCt =Ct(target) - Ct(GAPDH) and the results were analyzed as a ratio to GAPDH expression: Ratio = $2^{-\Delta Ct}$. For the patch clamp studies, the changes in current were expressed relative to the capacitance of the cell. Results are expressed as mean±S.E.M. and analyzed by a Student's t-test or a two-way ANOVA as appropriate; $p<0.05$ was considered significant.

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Results

Effects of an opener of SK_{Ca} and IK_{Ca} channels, NS309 in rat superior mesenteric arteries

To investigate the effect of opening SK_{Ca} and IK_{Ca} channels on relaxation and the relative contribution from NO and EDHF-type relaxation in a larger sized artery, NS309-induced NO release and relaxation in superior mesenteric arterial segments were investigated. NS309 (0.01-10 μM) induced concentration-dependent relaxations (EC_{50}: 1.4±1.2 μM, n=7) that were endothelium-dependent. Inhibition of NO synthase with ADMA (300 μM) and blocking IK_{Ca} channels with TRAM 34 (1 μM) reduced NS309-induced relaxation (EC_{50}: 5.6±1.4 μM, and 15.3±3.1 μM, n=7, respectively), whereas blocking SK_{Ca} channels with apamin (0.3 μM) had no effect on NS309-induced relaxation (EC_{50}: 1.7±1.4 μM, n=5). NS309-induced relaxation was not further decreased when both SK_{Ca} and IK_{Ca} channels were blocked simultaneously (EC_{50}: 17.4±4.8 μM, n=7) compared to blocking only the IK_{Ca} channels. Inhibition of NO synthase and blocking both IK_{Ca} and SK_{Ca} channels reduced NS309-induced relaxation to the same level as removal of the endothelium (Figure 1A). Blocking large conductance calcium-activated K channels (BK_{Ca}) reduced NS309 relaxation in rat superior mesenteric artery (Figure 2A), and rightward shifted concentration-response curves for the NO donor, S-nitroso-N-acetylpenicillamine (results not shown).

Simultaneous measurements of NO release and relaxation in superior mesenteric arteries showed that ACh (10 μM) increased NO release to 22.9±2.1 nM (n=5), which was accompanied by relaxation of 87.9±5.2 % (n=5). Inhibition of NO synthase by ADMA (300 μM) reduced ACh-induced increases in NO and relaxation to 3.1±0.5 nM, and 65±7%, n=5, respectively. Similar, NS309 (1 μM) increased NO release to 20.1±1.7 nM resulting in 22.9 ± 4 % relaxation (n=6). ADMA reduced NS309-induced NO release to 4.1±1.6 nM and abolished NS309-induced relaxation (n=6). In the presence of apamin (0.5 μM) and TRAM 34 (1 μM), the NS309-induced

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release of NO and relaxation was reduced to 4.4±1.3 nM and 4.3±2.5 % respectively (n=5). Finally, in the presence of the NO scavenger oxyhemoglobin (OxHb, 10 μM) NS309 failed to release NO and cause relaxation (Figure 1B and 1C, n=6).

***Effects of an opener of SK_{Ca} and IK_{Ca} channels, NS309* in small mesenteric arteries**

To investigate the effect of opening SK_{Ca} and IK_{Ca} channels on relaxation and the relative contribution from NO and EDHF-type relaxation in a smaller sized artery, the NS309-induced release of NO and relaxation in small mesenteric arterial segments was investigated. NS309 (0.001-1 μM) induced potent concentration-dependent relaxations (EC_{50}: 39±12 nM, n=7). Inhibition of NO synthase with ADMA (300 μM) reduced NS309-induced relaxations only at the highest concentration (1 μM) (EC_{50}: 72±16 nM, n=7), whereas, blocking SK_{Ca} channels with apamin (0.5 μM) (EC_{50}: 0.62±0.13 μM, n=7), or blocking the IK_{Ca} channels with TRAM 34 or ChTX (0.1 μM) (EC_{50}: not available, n=4 and n=7, respectively) led to a much higher reduction of NS309 relaxation. Inhibition of NO synthase, and blocking both SK_{Ca} and IK_{Ca} channels abolished NS309-induced relaxations (n=7) (Figure 3A). Blocking BK_{Ca} channels with IbTX (0.1 μM), had no effect on NS309-induced relaxations in mesenteric small arteries (Figure 2B).

In small mesenteric arteries, ACh (10 μM) hyperpolarized the smooth muscle cell membrane (ΔV_m: -25.3±2.5 mV, n=5). ACh-induced hyperpolarizations were reduced in the presence of ADMA (300 μM) (ΔV_m: -17.3±2.0 mV, n=5) and abolished by simultaneous inhibition of NO synthase with ADMA (300 μM), blocking SK_{Ca} channels with apamin (0.5 μM), and blocking IK_{Ca} channels with ChTX (0.1 μM) (ΔV_m: 0.6±1.5 mV, n=4). NS309 (1 μM) also hyperpolarized smooth muscle cell membrane (ΔV_m: -17.7±2.3 mV, n=6). Treatment with ADMA alone had no effect on membrane potential (ΔV_m: -11.7±2.3 mV, n=4), whereas the combination of

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ADMA, apamin, and ChTX abolished NS309-induced hyperpolarizations (ΔV_m: -0.4±1.9 mV, n=4) (Figure 3B).

Simultaneous measurements of NO release and relaxation in small mesenteric arteries showed that ACh (10 μM) increased NO concentration with 8.8±2.0 nM and relaxed the arteries with 100±1 % (n=4). Inhibition of NO synthase by ADMA (300 μM) markedly reduced NO release (Fig. 3C), but only reduced the peak relaxation induced by ACh to 91±5% ($P< 0.05$, n=3). Addition of NS309 (1 μM) increased the luminal NO concentration with 10.1±2.0 nM and relaxed the arteries 57±10% (Figure 3C).

Effects of an opener of SK_{Ca} and IK_{Ca} channels, NS309 in isolated endothelial cells

In HUVECs, $SK_{Ca}3$ mRNA showed a ~ 30 times higher expression than IK_{Ca} mRNA, whereas no expression of BK_{Ca} mRNA was found (figure 3A). NS309 (0.1 and 1 μM) increased the outward current in HUVECs. The NS309-induced increase in outward current was abolished in the absence of intra- and extra cellular Ca^{2+} (Figure 3B). Investigating the involvement of different potassium channels in the NS309-induced increase in current, ChTX (0.1 μM) or TRAM 34 (1 μM), abolished the increase in current, apamin (0.3 μM), reduced the increase in outward current, whereas IbTX (0.1 μM) or glibenclamide (0.1 μM), had no effect on the increase in current (Figure 4C-D).

In HUVECs, histamine (1 μM) increased the release of NO to 8.95±0.2 nM, n=6. In the presence of apamin (0.5 μM) and TRAM 34 (1 μM) the response to histamine was reduced to 6.36±0.1 nM, n=7 (Figure 5A and 5B). Replacing TRAM 34 with ChTX (0.1 μM), the histamine-induced release of NO was reduced to 1.9±2.1 nM (n=6). In the presence of ADMA (300 μM), apamin, and ChTX, the histamine-induced release of NO was reduced to 0.7±0.4 nM (n=6). NS309

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(1 μM) increased the NO concentration to 12.9 ± 1.8 nM (n=5), which was reduced to 6.4±2.0 nM (n=5), in the presence of apamin (0.5 μM) and TRAM 34 (1 μM) (Figure 5A and 5B).

In HUVECs loaded with FURA 2-AM, histamine (1 μM) increased whole cell $[Ca^{2+}]_i$ by 0.36±0.03 (n=6), while stimulation with NS309 (0.01 μM) increased $[Ca^{2+}]_i$ by 0.06±0.02 (n=6). Increasing the concentration of NS309 to 0.1 μM and 1 μM had no further effect on whole cell $[Ca^{2+}]_i$ (Figure 5C). TRAM 34 (1 μM) decreased basal $[Ca^{2+}]_i$ by 0.27±0.03 (n=6), reduced histamine-induced increase in $[Ca^{2+}]_i$ to 0.26±0.03 (n=6), and abolished the NS309-induced increase in $[Ca^{2+}]_i$ (Figure 5C).

In mesenteric arterial endothelial cells loaded with Oregon Green BAPTA 1-AM, both ACh (10 μM) and NS309 (1 μM) increased whole cell $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (0.27±0.04 and 0.10±0.01, (n=5-6), respectively). In contrast, in the absence of extracellular Ca^{2+} NS309 (1 μM) failed to change $[Ca^{2+}]_I$ (0.02±0.02, n=3), whereas ACh (10 μM) induced a transient increase (0.07±0.03, n=3) (Figure 5D). In the presence of TRAM 34 (1 μM), ACh and NS309 induced less increase in $[Ca^{2+}]_i$ (0.16±0.01 and 0.04±0.01, n=4, respectively), and ionomycin-induced increases in calcium were unaltered (0.66±0.08 and 0.68±0.16, n=5, respectively) (Figure 5D).

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Discussion

The main findings of the present study are that NS309 induces endothelium-dependent relaxations that are largely mediated by release of NO in superior mesenteric arteries, whereas NS309 induces hyperpolarization and an EDHF-type relaxation in small mesenteric arteries. In HUVECs NS309 increased current sensitive to SK_{Ca} and IK_{Ca} channel blockers, but the current remained unaltered in the presence of a blocker of BK_{Ca} channels, iberiotoxin. Moreover, NS309-induced calcium influx appears to contribute to formation of NO.

Contribution of NO and EDHF to NS309-induced relaxation

NS309 induced more potent relaxations in small compared to superior mesenteric arterial segments in the present study. Compared to isometric preparations membrane potential of pressurized arteries is more depolarized (Schubert, et al., 1996), and together with the observations that smooth muscle hyperpolarization induced by an endothelium-dependent vasodilator e.g. ACh is attributable to SK_{Ca} channels, whereas IK_{Ca} channels play an important role during the ACh-induced repolarization phase following depolarization (Crane, et al., 2003), we cannot exclude this may contribute to the different potency of NS309 observed in large versus small mesenteric arteries in the present study. However, calcium-activated K channels were suggested to play a role for the regional heterogeneity in ACh-induced relaxation in the rat mesenteric vascular bed (Hilgers, et al., 2006), and that may also contribute to the different potency of NS309 in large versus small arteries. Both in the superior and small mesenteric arteries NS309 relaxation was markedly inhibited in the presence of blockers of IK_{Ca} channels and further reduced by the combined inhibition of IK_{Ca} and SK_{Ca} channels suggesting that the endothelium-dependent relaxations induced by NS309 involve IK_{Ca} and SK_{Ca} channels. In contrast to the small mesenteric arteries, the BK_{Ca} selective blocker, iberiotoxin also reduced NS309 relaxation, but it also reduced relaxations to the NO donor SNAP

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suggesting, the effect of iberiotoxin on NS309 relaxation can probably be attributed to smooth muscle BK_{Ca} channels activated by NO in rat superior mesenteric artery.

We have in previous studies found that endothelial SK_{Ca} and IK_{Ca} channels contribute to ACh-induced endothelial hyperpolarization and NO-mediated relaxation in large arteries (Stankevicius, et al., 2006). Moreover, in an endothelial cell line (EA.hy926) derived from human umbilical vein inhibition of SK_{Ca} and IK_{Ca} channels reduced while openers of SK_{Ca} and IK_{Ca} channels were found to increase NO-sensitive DAF-FM fluorescence (Sheng, et al., 2009). In the present study, an opener of SK_{Ca} and IK_{Ca} channels, NS309, increased NO concentration in superior mesenteric arteries and primary cultured HUVECs providing direct evidence that opening of SK_{Ca} and IK_{Ca} channels are involved in release of NO in large arteries.

Opening endothelial SK_{Ca} and IK_{Ca} channels is thought to be pivotal for EDHF-type relaxation, and inhibition of these channels by the combination of apamin and ChTX has been considered as a unique characteristic of the non-NO nonprostanoid-type relaxations (Edwards, et al., 1998;Buus, et al., 2000), and hyperpolarization evoked by agonists in small arteries (Edwards, et al., 1998;Zygmunt and Hogestatt, 1996;Yamamoto, et al., 1999). Confirming these previous studies, this study found that ACh-induced hyperpolarizations and relaxations were also blocked when apamin and ChTX were added together with an inhibitor of NO synthase in rat mesenteric small arteries (Figure 3). This was also the case for NS309-induced hyperpolarizations and relaxations in rat mesenteric small arteries suggesting that in this preparation, an EDHF is the main contributor to NS309-induced hyperpolarizations and relaxations. However, NO has previously been suggested to contribute to the EDHF-type relaxation and hyperpolarization induced by ACh in rat mesenteric arteries (Chauhan, et al., 2003). In the present study, ADMA also inhibited ACh-induced hyperpolarizations and abolished increases in NO concentration in rat small mesenteric arteries, hence providing further support that NO contributes to EDHF-type vasodilation in this

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preparation. Therefore, in addition to contributing to a main EDHF component, NO also contributes to the sustained ACh-induced relaxation in rat small mesenteric arteries.

In porcine retinal arteries, we found that NS309 as well as a selective opener of SK_{Ca} channels, CyPPA, induced apamin-sensitive NO-mediated relaxations and potentiation of bradykinin relaxations, suggesting that SK_{Ca} channels are coupled to release of NO from the endothelial cell layer (Dalsgaard, et al., 2009;Dalsgaard, et al., 2010a). Moreover, downregulation of the $SK_{Ca}3$ channel in mice appears to inhibit the L-NAME sensitive bradykinin relaxation in carotid arteries (Brahler, et al., 2009). However, in the present study TRAM 34 reduced both release of NO and abolished endothelium-dependent relaxation induced by NS309 in rat superior mesenteric artery, suggesting that mainly IK_{Ca} channels are coupled to the release of NO in this preparation. Thus, depending on vascular bed and/or the specific endothelial cell compartmentalization either SK_{Ca} or IK_{Ca} channels may be coupled to the release of NO.

Cellular mechanisms involved in responses to NS309

In the present study, we found that in primary HUVECs both $SK_{Ca}3$ and IK_{Ca} channels were expressed, whereas BK_{Ca} channel expression was undetectable. These findings agree with previous work in early passage HUVECs (Kestler, et al., 1998), and our previous observations that an opener of BK_{Ca} channels, NS11021, does not change voltage-current relationship in early passage HUVECs (Kun, et al., 2009). Furthermore, our patch clamp studies in HUVEC indicated that the increased potassium conductance induced by NS309 was dependent on the presence of $[Ca^{2+}]_i$ and that it was partially inhibited by apamin and abolished by ChTX and TRAM 34, while it remained unaltered in the presence of iberiotoxin. In conjunction with the more marked inhibition of vasodilation to NS309 by blockers of IK_{Ca} channels (Figure 1), these data suggest that IK_{Ca}

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channels may play a more prominent role in the response to NS309 in HUVECs and isolated arteries.

Opening of potassium channels will draw the membrane potential towards the equilibrium potential for potassium and lead to hyperpolarization. In endothelial cells, hyperpolarization is thought to increase the driving force for influx of Ca^{2+} via cation channels belonging to transient receptor potential ion channels (e.g. TRPCs and TRPV4) and thereby prolongs and strengthens the activating Ca^{2+} signal (Nilius and Droogmans, 2001;Earley and Brayden, 2010). Inhibition of calcium-activated potassium channels is associated with a decrease in $[Ca^{2+}]_i$ (Nilius and Droogmans, 2001;Sheng and Braun, 2007). Moreover, studies in an endothelial cell line (EA.hy926) derived from human umbilical veins suggested that opening SK_{Ca} channels was associated with increase in Ca^{2+} and NO formation (Sheng and Braun, 2007;Sheng, et al., 2009). In the present study, both histamine and NS309 increased $[Ca^{2+}]_i$ and NO release in HUVECs and in both cases the increase in NO was reduced by incubation with TRAM 34 and apamin. These results suggest that opening of SK_{Ca} and IK_{Ca} channels is involved in the effects of histamine and NS309 and is therefore consistent with the view that membrane hyperpolarization by opening of SK_{Ca} and IK_{Ca} channels leads to increased influx of extracellular Ca^{2+}. Moreover, the findings that in freshly isolated mesenteric arterial cells the increase in $[Ca^{2+}]_i$ induced by NS309 was markedly reduced by TRAM 34 and completely abolished in the absence of extracellular calcium suggest that opening of IK_{Ca} channels are coupled to calcium influx.

In contrast to the isolated endothelial cell studies, openers of SK_{Ca} and IK_{Ca} channels e.g. NS309, fails to change Ca^{2+} in intact segments of retinal (Dalsgaard, et al., 2010a) and rat small mesenteric arteries (Brondum, et al., 2009), and a combination of apamin plus ChTX fails to change agonist-induced increases in endothelial cell Ca^{2+} in rat superior mesenteric artery and small mesenteric arteries (Ghisdal and Morel, 2001;McSherry, et al., 2005;Stankevicius, et al., 2006).

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Despite comparable increases in NO concentration, the increase in $[Ca^{2+}]_i$ was much more marked with histamine compared to NS309 in HUVECs and with ACh compared to NS309 in mesenteric arterial endothelial cells in the present study. These findings suggest that also in isolated endothelial cells, it is questionable whether the rise in $[Ca^{2+}]_i$ can completely account for the increase in NO seen with NS309. Thus, these findings suggest that Ca^{2+}-independent events may also couple SK_{Ca} and IK_{Ca} channel opening to changes in release of NO as proposed either through membrane potential regulation of superoxide production and/or L-arginine uptake (Dalsgaard, et al., 2010b). Moreover, it was recently suggested that potassium efflux resulting in an acute increase of potassium in the physiological range swells the endothelial cell and, hence may increase the release of NO (Oberleithner, et al., 2009). However, further studies are required to clarify how activation of these mechanisms is coupled to increased formation of NO in endothelial cells.

Conclusion and perspectives

In conclusion, in superior mesenteric arteries, the NS309-induced relaxation was mainly mediated by NO, whereas in small mesenteric arteries, the NS309-induced relaxation was mainly mediated by EDHF. NS309-induced calcium influx appears to contribute to formation of NO. These results suggest that treatment with SK_{Ca} and IK_{Ca} channel openers in atherosclerotic or hypertensive disease involving a reduction in blood flow, will not only improve blood flow in vascular beds, where the relative contribution from the EDHF-type vasodilation is high such as in coronary vasodilation from enhanced perfusion pulsatility (Paolocci, et al., 2001), but may also improve blood flow in vascular beds, where the relative contribution from NO to vasodilation is high.

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Authorship contributions

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Footnotes

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Legends for Figures

Figure 1. Effect of blocking NO synthase, SK_{Ca} and IK_{Ca} channels on NS309-induced release of NO and relaxation in rat superior mesenteric arteries. A) Isometric tension recordings showing the relaxing effect of NS309 in the absence and presence of ADMA (300 μM), apamin (0.5 μM), and TRAM 34 (1 μM). All experiments were performed in the presence of indomethacin (3μM). Results are mean±S.E.M. n=5-7. Two-way ANOVA. *P<0.05 from control. B) Original trace showing NS309-induced NO release and relaxation in a rat superior mesenteric artery contracted with noradrenaline (0.5 μM). C) Average increase in NO concentration evoked by NS309 in the absence and presence of apamin (0.5 μM), TRAM 34 (1 μM), ADMA (300 μM), and oxyhemoglobin (OxHb, 10 μM). All experiments were performed in the presence of indomethacin (3 μM). Results are means±S.E.M. n=5-6. Student's t-test. *P < 0.05 from control.

Figure 2. Effect of blocking large-conductance calcium activated K (BK_{Ca}) channels on NS309 relaxation in lage and small rat mesenteric arteries. A) Isometric tension recordings in rat superior mesenteric artery and B) isobaric diameter recordings in small mesenteric arteries showing the relaxing effect of NS309 in the absence and presence of a blocker of BK_{Ca} channels (0.1 μM). All experiments were performed in the presence of indomethacin (3 μM). Results are means±S.E.M. n=5-6. Two-way ANOVA. *P<0.05 from control.

Figure 3. Effect of blocking NO synthase, SK_{Ca} and IK_{Ca} channels on NS309-induced release of NO and relaxation in small rat mesenteric arteries. A) Isobaric diameter recordings showing the relaxing effect of NS309 in the absence and presence of ADMA (300 μM), apamin (0.5 μM), charybdotoxin (ChTX, 0.1 μM), TRAM 34 (1 μM), and apamin plus ChTX. All experiments were performed in the presence of indomethacin (3μM). Results are means±S.E.M. n=7. Two-way

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ANOVA. *P<0.05 from control. B) Smooth muscle cell hyperpolarizations induced by ACh (10 μM) and NS309 (1 μM) in the absence and presence of apamin (0.5 μM), ChTX (0.1 μM), and ADMA (300 μM). All experiments were performed in the presence of indomethacin (3μM). Results are mean±S.E.M. n=4-6. Student's t-test: *P<0.05 from control. C) Average trace (n=3) showing SNAP- (10 μM), ACh- (10 μM), and NS309- (1 μM) induced NO release and relaxation in the absence and presence of indomethacin (3 μM) and ADMA (300 μM) in small mesenteric arteries contracted with noradrenaline (0.5 μM).

Figure 4. SK_{Ca} and IK_{Ca} channel currents and expression in HUVECs. A) Quantitative PCR showing the expression of SK_{Ca} 2.3, IK_{Ca} and BK_{Ca} RNA. B-C) Current voltage relationships derived from whole cell patch clamp experiments showing the effect of NS309 on whole cell currents in the presence and absence of intracellular Ca^{2+}, apamin (0.3 μM) and ChTX (0.1 μM). Results are mean±S.E.M. n=4-7. Two-way ANOVA. *P<0.05 from control with calcium #P<0.05 from NS309 (0.1 μM, -Ca^{2+}). $P<0.05 from NS309 (1 μM). D) Relative NS309-induced increase in current at -80 mV after addition of various potassium channel blockers; Apamin (0.3 μM), ChTX (0.1 μM), TRAM 34 (1 μM), IbTX (0.1 μM), and glibenclamide (0.1 μM). Results are means±S.E.M. n=4-7. Student's t-test. * P < 0.05 from NS309.

Figure 5. NS309-induced release of NO and increase in intracellular Ca^{2+} in isolated endothelial cellss. A) Original recording showing NS309 (1 μM)-induced NO release in HUVEC. (B) Average increase in NO concentration induced by NS309 (1 μM) in the absence and presence of apamin (0.5 μM) and TRAM 34 (1 μM). Results are mean±S.E.M. n=5-7. Student's t-test. *P<0.05 from control. C) Histamine- (1 μM) and NS309 (1 μM)-induced increase in intracellular Ca^{2+} concentration in HUVECS the absence and presence of TRAM 34 (1 μM). Results are

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means±S.E.M. n=6. Student's t-test. *P<0.05 from histamine control. Two-way ANOVA. *P<0.05 from NS309 controls. (D). Relative increase in rat mesenteric endothelial cell calcium after addition of acetylcholine (ACh, 10 μM), NS309 (1 μM), and ionomycin (1 μM) in the absence and presence of TRAM 34, an IK_{Ca} channel blocker, and for acetylcholine (ACh, 10 μM), and NS309 (1 μM) in the absence of extracellular calcium. Results are means±S.E.M. (n=3-5). Student's t-test. *P<0.05 from control.

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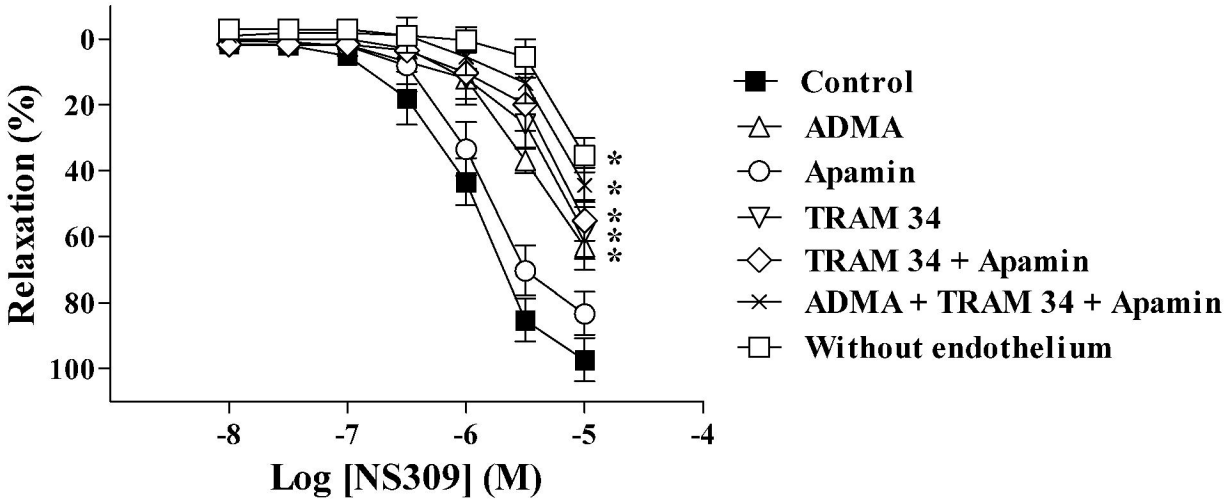

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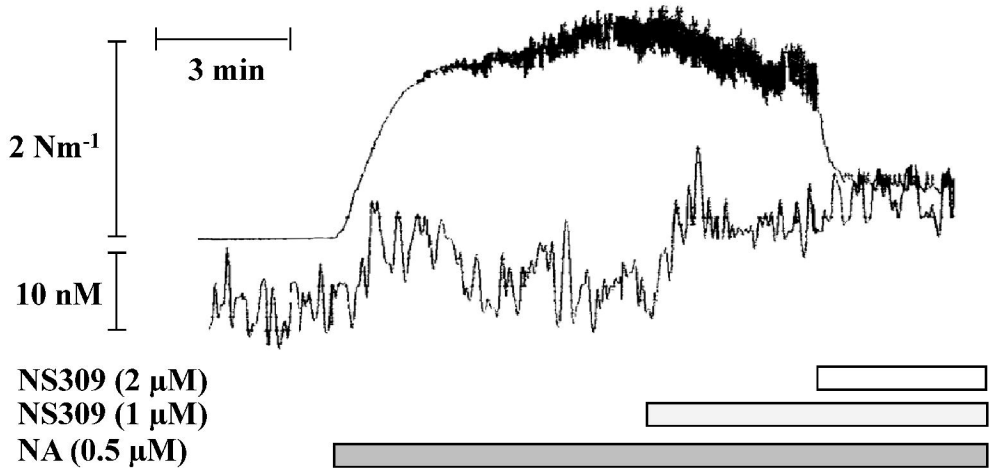

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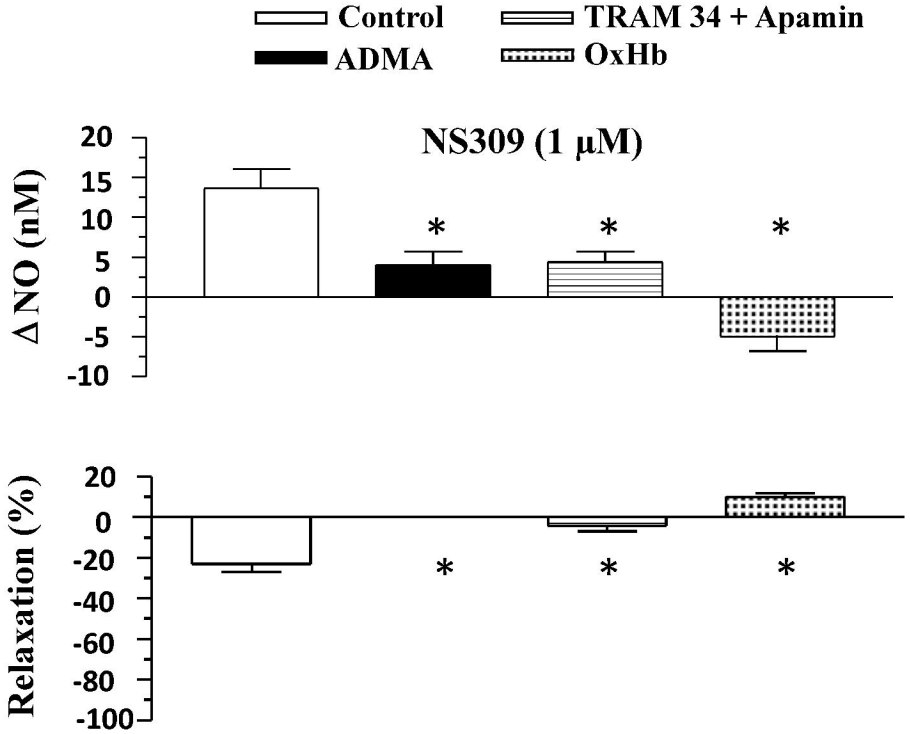

Figure 1

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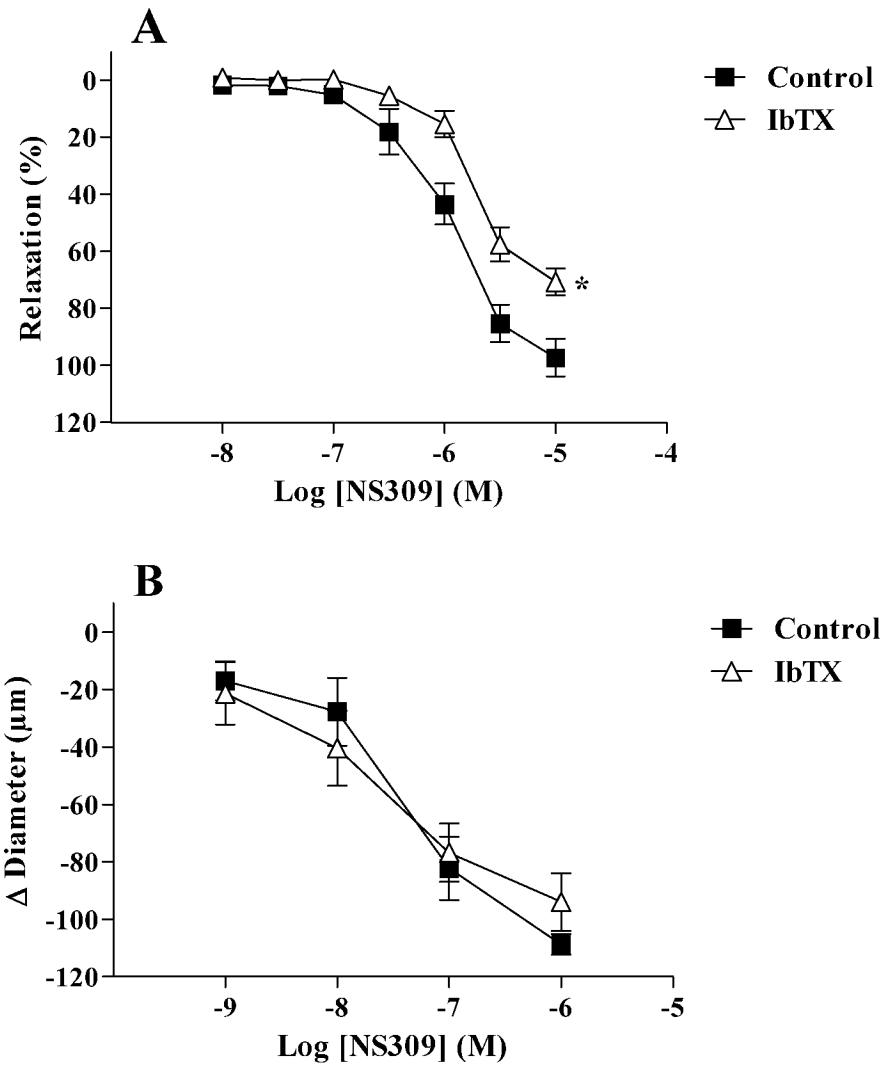

Figure 2

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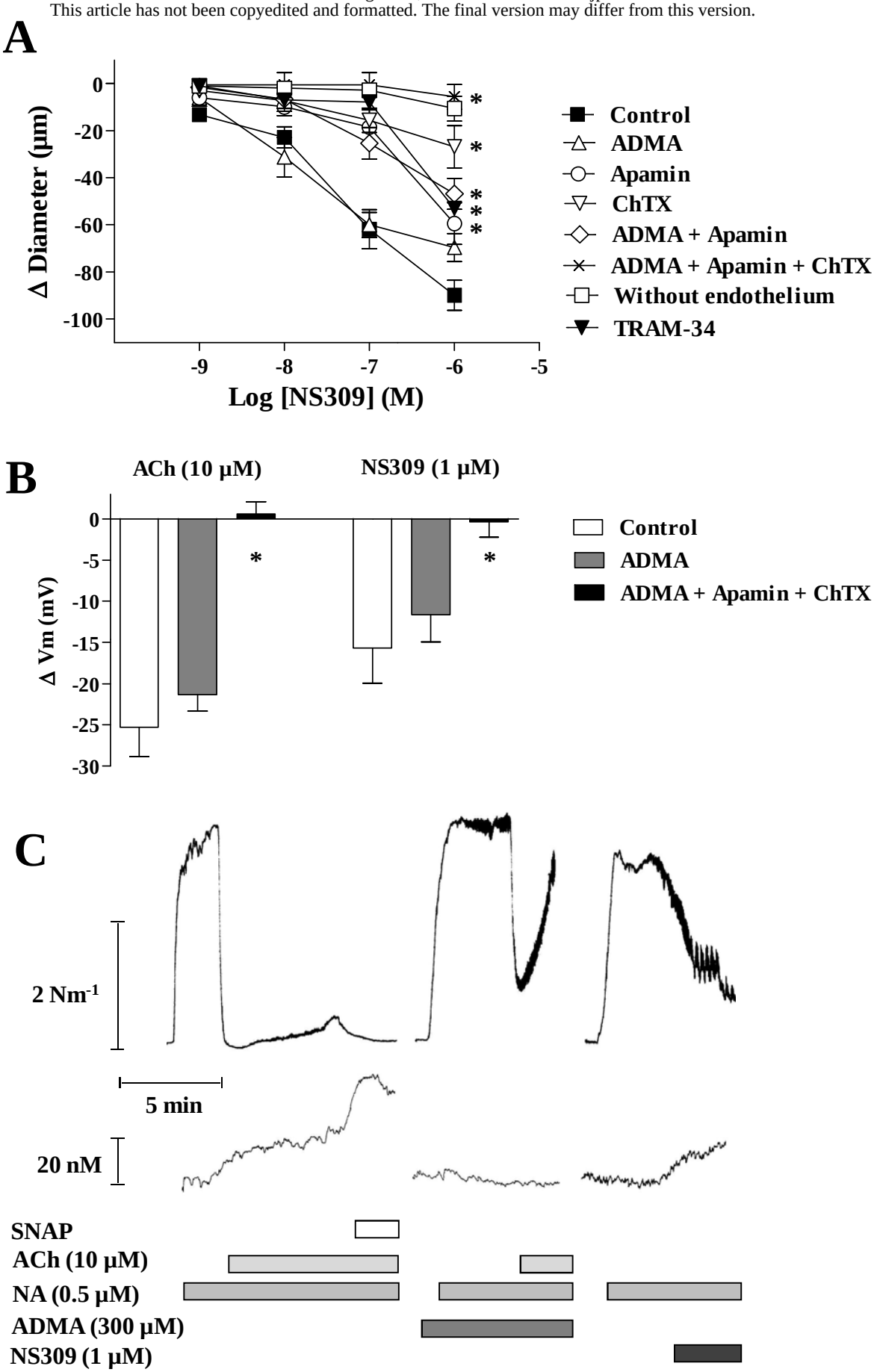

Figure 3

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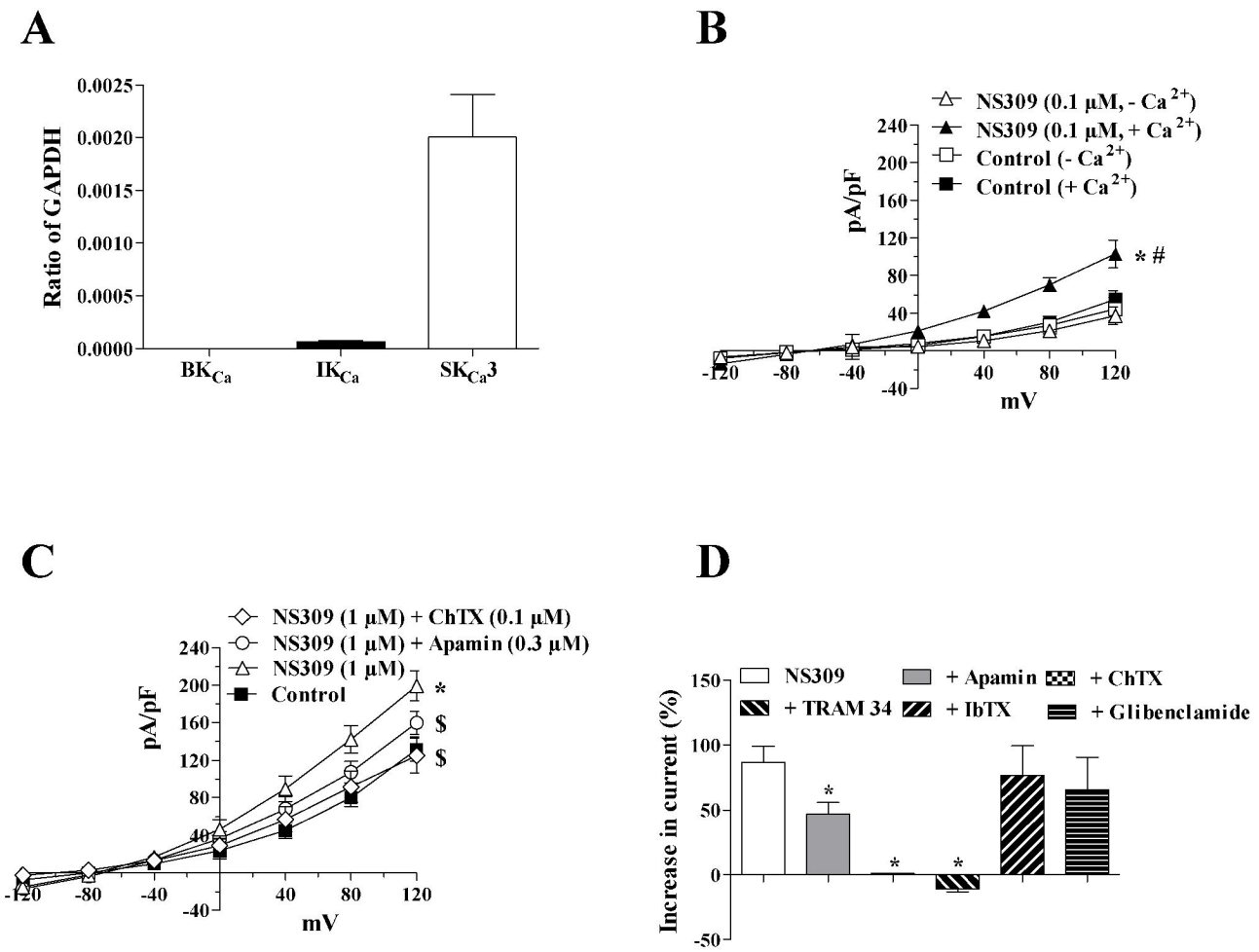

Figure 4

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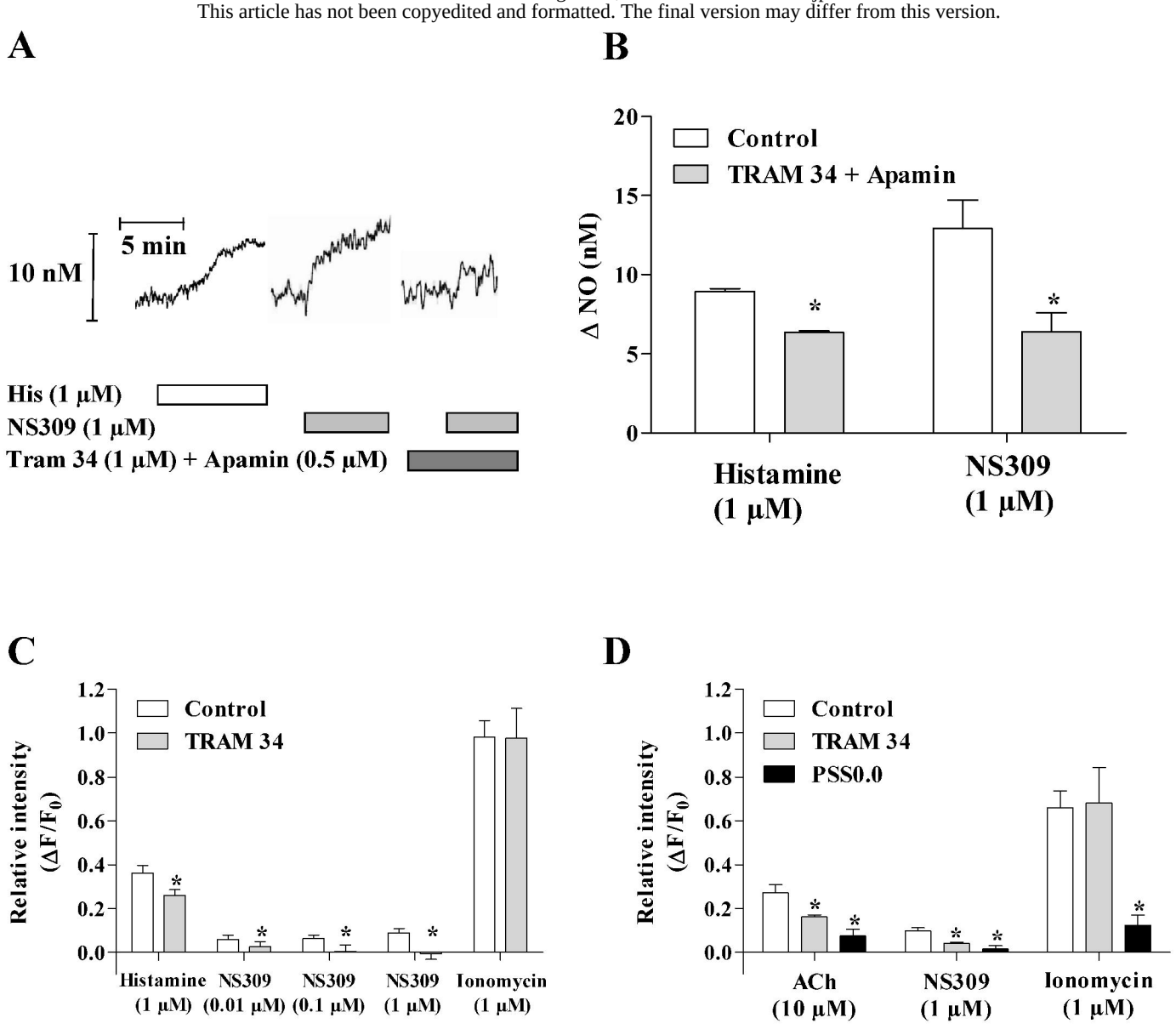

Figure 5