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Apelin Reduces Nitric Oxide-Induced Relaxation of Cerebral Arteries by Inhibiting Activation of Large Conductance, Calcium-Activated K Channels

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Abstract

Activation of the apelin/APJ receptor signaling system causes endothelium- and nitric oxide (NO)-dependent relaxation in several peripheral arteries. The effects of apelin in cerebral arteries are unknown; however, apelin inhibits voltage-dependent increases in large-conductance, calcium-activated K channel (BK_{Ca}) currents in cerebral artery smooth muscle cells. Since NO-induced relaxation of cerebral arteries is mediated, in part, by activation of BK_{Ca} channels, the goals of the present study were to determine the net effect of apelin in cerebral arteries, as well as test the hypothesis that the actions of apelin in cerebral arteries are secondary to stimulation of APJ receptors. Immunoblot and RT-qPCR analyses detected APJ receptors in cerebral arteries of male Sprague-Dawley rats, and immunofluorescence studies using confocal microscopy confirmed APJ receptor localization in smooth muscle cells. In myograph studies, apelin itself had no direct vasomotor effect, but inhibited relaxations to the NO-donor, DEA NONOate, and to the endothelium-dependent vasodilator, bradykinin. These effects of apelin were mimicked by the selective BK_{Ca} channel blocker, iberiotoxin, and suppressed by the APJ receptor antagonist, F13A. Apelin also inhibited relaxations evoked by the BK_{Ca} channel openers, NS1619 and BMS 191011, but had no effect on relaxation to levcromakalim, a selective K_{ATP}-channel opener. Apelin had no effect on DEA NONOate- or bradykinin-induced increases in cyclic GMP levels. Patch clamp recordings demonstrated that apelin and iberiotoxin each suppressed the increase in BK_{Ca} currents induced by DEA and NS1619 in freshly isolated cerebral artery smooth muscle cells. The results demonstrate that apelin inhibits NO-induced relaxation of cerebral arteries through a mechanism involving activation of APJ receptors and inhibition of BK_{Ca} channels in cerebral arterial smooth muscle cells.

Keywords

apelin; APJ receptors; BK_{Ca} channels; cerebral arteries; nitric oxide

INTRODUCTION

Apelin is an endogenous peptide that is emerging as an important signaling molecule in several organ systems, including the cardiovascular system (1). Apelin binds to a single G-

protein-coupled receptor, termed APJ, which is widely expressed in peripheral organs and tissues as well as the central nervous system (2, 3). With respect to the cardiovascular system, APJ receptors are found in heart (4, 5), blood vessels (5, 6), and cardiovascular regulatory centers in the brain (3, 7, 8).

A growing body of evidence supports a role for the apelin/APJ receptor signaling system in the regulation of blood vessel diameter. Studies indicate that apelin is a powerful vasodilator with an efficacy that exceeds that of hydralazine or nitrates (9, 10). This action, along with its inotropic effects on the heart, has led to the proposition that modulating the apelin/APJ system may offer a novel and rational therapeutic strategy for treating certain cardiovascular disorders, including myocardial ischemia, pulmonary arterial hypertension, and heart failure (10, 11).

A confounding factor, however, is that the effect of APJ receptor activation on vascular tone is dependent on the particular vascular bed being studied. While APJ receptor stimulation leads to endothelium- and NO-dependent vasodilation in several blood vessels (12, 13), it can also result in vascular smooth muscle contraction in others (12, 14). These divergent vasomotor effects of apelin are consistent with the widely recognized heterogeneity that exists among blood vessels from different origins in terms of their reactivity to vasoactive agents (15). Therefore, whereas systemic administration of apelin may have an overall beneficial hypotensive effect, it could also result in dangerous vasoconstriction in specific vascular beds.

Our knowledge of the vasomotor actions of apelin in specific blood vessels is rather limited at the present time. One important vascular bed that has not been rigorously investigated with respect to the apelin/APJ system is the cerebral vasculature; however, a potential vasoconstrictor role for apelin in the regulation of cerebral arterial tone is suggested by our recent finding that apelin inhibits large conductance, calcium-activated K (BK_{Ca}) channel currents evoked by voltage-dependent increases in membrane potential in cerebral artery smooth muscle cells (16). Thus, in cerebral arteries the net effect of apelin on blood vessel diameter could be the result of the ability of APJ receptor activation on the endothelium to evoke NO-dependent relaxations on one hand versus the effect of APJ receptor activation on the smooth muscle to inhibit relaxation via interference with NO-induced increases in BK_{Ca} currents. In the present study we directly assessed the question of the net effect of apelin in cerebral arteries, as well as the hypothesis that the actions of apelin in cerebral arteries are indeed secondary to stimulation of APJ receptors.

MATERIALS AND METHODS

Animals and Tissue Preparation

Experiments were performed on tissues isolated from 12-week-old male Sprague-Dawley rats (Envigo RMS, Indianapolis, IN). Rats were housed at 22 ± 2°C on a 12 h–12 h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee. Brains were isolated from animals anesthetized with isoflurane and placed into ice-cold physiological salt solution (PSS) with the following composition (in mM): NaCl 119mM,

NaHCO₃ 15mM, KCl 4.6mM, MgCl₂ 1.2mM, NaH₂PO₄ 1.2mM, CaCl₂ 1.5mM and glucose 5.5mM. Middle cerebral arteries were dissected and cleaned of surrounding tissue.

Western Immunoblotting

After collection, cerebral arteries were immediately frozen in liquid nitrogen. Tissues were homogenized in lysis buffer and supplemented with a protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C using an IKA Ultra-Turrax T8 homogenizer (IKA Works Inc., Wilmington, NC). Tissue homogenates were kept on ice for 10 min and then centrifuged at 10000g for 10 min. Supernatant was collected and protein determination was performed using a Pierce BCA protein estimation kit (ThermoFisher Scientific, Waltham, MA). Aliquots of supernatant containing equal amounts of protein (40 µg) were separated on 7.5–10% polyacrylamide gel by SDS–polyacrylamide gel electrophoresis, and proteins were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS, pH 7.4) and incubated overnight at 4°C with a primary antibody specific for APJ receptors using a dilution of (1:200) (Santa Cruz Biotechnology Inc.). Membranes were washed three times for 10 min using TBS-Tween 20 and incubated with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc.). To ensure equal loading, the blots were analyzed for β-actin expression using an anti-actin antibody (Santa Cruz Biotechnology Inc.). Immuno-detection was performed using an enhanced chemiluminescence light detection kit (ThermoFisher Scientific).

RT-qPCR

Freshly isolated arteries were immediately frozen in liquid nitrogen and total RNA was isolated using an RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Germantown, MD). The concentration and purity of RNA was determined using a spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized using 50 ng of RNA and an iScript cDNA synthesis kit (Bio-Rad). A Sybr Green expression assay was used to determine expression of APJ receptors, with β-actin used as a housekeeping gene (Bio-Rad). The following primers (synthesized by Invitrogen, Carlsbad, CA) were used: APJ receptor – forward: 5'-ACAAGACATGTGCCATTGGA-3'; reverse: 5'-TCTCCAGAAAGCCTCCTACA-3'; β-actin – forward: 5'-GTCGTACCACTGGCATTGTG-3'; reverse: 5'-TCTCAGCTGTGGTGGTGAAG-3'. Real-time RT-PCR reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s.

Immunofluorescence Microscopy

Freshly isolated cerebral arteries were fixed in 10% formalin solution, processed and embedded in paraffin blocks. Paraffin blocks were sectioned at 7 µm and mounted onto ProbeOn Plus microscopic slides (ThermoFisher Scientific). Sections were fixed by heating at 60°C for 30 min. Nonspecific antibody binding was blocked with normal serum [10% w/v; in TBS] for 1 h at room temperature. Sections were incubated overnight at 4°C in 1% serum containing a primary antibody against APJ receptors (Abcam, Cambridge, MA) and/or smooth muscle actin (Santa Cruz Biotechnology). For co-localization of APJ receptor with smooth muscle actin, double immunofluorescent staining was performed by incubating

the tissue sections with more than one primary antibody at the same time. Detection of the primary antibodies against the APJ receptor and smooth muscle actin was accomplished using Texas red-conjugated (goat anti-rabbit IgG; Invitrogen, Carlsbad, CA) and Alexa Fluor-488 (goat anti-mouse IgG; Santa Cruz Biotechnology) secondary antibodies, respectively. Rat lung tissue was used as positive control for APJ receptor detection (7). Negative controls were incubated overnight at 4°C with 1% serum containing a primary isotype control antibody (nonimmune IgG; Abcam) from the same species (rabbit) as that of the APJ receptor antibody, followed by secondary antibodies. All dilutions and thorough washes between stages were performed using TBS containing Triton X-100 [0.3% (v/v)] unless otherwise stated. Sections were drained by blotting with filter paper, and a drop of mounting medium containing an anti-fade reagent (Vector Laboratories, Burlingame, CA) was added to the slides. Images of the sections were obtained using an Olympus confocal laser-scanning microscope (Olympus, Tokyo, Japan). The images were generated using Olympus FluoView FV300 (v. 4.3) confocal software.

Vascular Function Studies

Arterial rings (80–100 µm; 1.2 mm in length) were suspended in wire myographs (DMT, Aarhus, Denmark) for isometric tension recording. The myograph chambers were filled with PSS (5 ml), which was continuously aerated with 95% O₂/5% CO₂ and maintained at 37°C throughout the experiment. The vessel rings were stretched to an initial tension of 2 mN and then allowed to stabilize for 30–40 min with intermittent washings. Vessel reactivity was confirmed by evoking a response to KCl (60 mM) (17). In some vessels, the endothelium was removed by gently rubbing the intimal surface with a human hair. The presence or absence of endothelium was confirmed by measuring relaxation in response to the endothelium-dependent vasodilator, bradykinin (10⁻⁷ M). Responses to the vasodilators used in this study were obtained in arterial rings contracted with 5-HT (10⁻⁷ M). Relaxation responses were obtained with: 1) DEA NONOate, an exogenous NO-donor (18); 2) bradykinin, which, in rat cerebral arteries, causes relaxation by releasing endothelium-derived (endogenous) NO (19, 20); 3) the BK_{Ca} channel openers, NS1619 and BMS 191011 (21, 22); and 4) the K_{ATP} channel opener, levcromakalim (23). Inhibitors were added to the myograph chamber 20 min prior to contraction with 5-HT, with the exception of apelin (10⁻⁷ M), which was added 5 min after the contraction had stabilized in order to minimize desensitization of APJ receptors. This concentration of apelin was selected because in previous studies it produced the greatest inhibitory effect on BK_{Ca} channel currents in rat cerebral smooth muscle cells (16). Other inhibitors used included: 1) iberiotoxin, a selective BK_{Ca}-channel blocker (24); 2) F13A, a selective APJ receptor antagonist (25); and 3) the nitric oxide synthase inhibitor, nitro-L-arginine (NLA). After addition to the myograph chamber, the inhibitors remained in contact with the tissues for the remainder of the experiment. Experiments with inhibitors were conducted in parallel with untreated control rings taken from the same animal.

Cerebral Artery Smooth Muscle Cell Isolation

Enzymatic isolation of single vascular smooth muscle cells was carried out as described previously, with brief modification (26). Arteries were placed in cell isolation solution of the following composition (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl₂, 10 glucose,

and 10 HEPES (pH 7.2). Arterial segments were initially incubated in 1.2 mg/ml papain (Worthington) and 2.0 mg/ml dithioerythritol (Sigma Aldrich) for 17 min at 37°C and then in 0.8 mg/ml type II collagenase (Worthington) for 12 min at 37°C. The digested segments were washed three times in ice-cold cell isolation solution and incubated on ice for 30 min. After this incubation period, vessels were triturated to liberate smooth muscle cells and stored in ice-cold cell isolation solution for use. Smooth muscle cells were studied within 6 h following isolation.

Electrophysiological Recording

Isolated smooth muscle cells were placed into a recording chamber (Warner Instruments, Hamden, CT) and allowed to adhere for 20 min at room temperature. Whole cell currents were recorded using an AxoPatch 200B amplifier equipped with an Axon CV 203BU headstage (Molecular Devices). Current data were collected and analyzed with pCLAMP 10.0 software (Molecular Devices, Sunnyvale, CA). The patch electrodes (3–4 M Ω) were fabricated from 1.5-mm borosilicate glass capillaries. Smooth muscle cells were superfused at a rate of 2.0 ml/min with a solution containing (in mM) 145 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, 10 glucose; pH 7.4 (NaOH). Patch pipettes were filled with internal pipette solution containing (in mM) 145 KCl, 5 NaCl, 0.37 CaCl₂, 2 MgCl₂, 10 HEPES, 1 EGTA, 7.5 Glucose; pH 7.2 (KOH). All drugs were diluted in fresh bath solution and perfused into the experimental chamber. Voltage-activated currents were filtered at 2 kHz and digitized at 10 kHz, and capacitive and leakage currents were subtracted digitally. Series resistance and total cell capacitance were calculated from uncompensated capacitive transients in response to 10 ms hyperpolarizing step pulses (5 mV), or obtained by adjusting series resistance and whole-cell capacitance using the Axopatch 200B amplifier control system. Standard recording conditions for BK_{Ca} currents were achieved by stepping in 10 mV increments from a holding potential of –60 to +80 mV. BK_{Ca} currents were expressed as current density (current divided by its capacitance).

Cyclic GMP Estimation

A competitive ELISA method was used to measure cyclic GMP levels in cerebral arteries. Arterial segments were equilibrated in PSS for at least 20 min at 37°C. After the equilibration period, arterial segments were treated with bradykinin (10^{–7} M, 5 min) or DEA NONOate (DEA; 10^{–6} M, 5 min). In some experiments, arterial segments were pretreated with apelin (10^{–7} M) for 5 min, before being exposed to bradykinin or DEA NONOate. After drug treatments, segments were immediately frozen in liquid nitrogen and homogenized with an IKA Ultra, Turrax-T8 homogenizer (IKA Works) at 4°C in 0.1 M hydrochloric acid. Tissue homogenates were centrifuged for 10 min at 600 g. Cyclic GMP and total protein content were determined in the supernatant as described in the direct cyclic GMP enzyme immunoassay kit (Assay Design, Ann Arbor, MI) and Pierce BCA protein assay kit (ThermoFisher Scientific), respectively. Cyclic GMP levels were expressed as picomoles per microgram of protein.

Data Analysis

Relaxation responses are expressed as a percent of the initial tension induced by 5-HT (10^{–7} M). EC₅₀ values (drug concentration that produced 50% of its own maximal response) were

determined, converted to their negative logarithms, and expressed as $-\log$ molar EC_{50} (pD_2). RT-qPCR data were analyzed to quantify relative gene expression by C_t relative to β -actin. All results are expressed as means $\pm$ SEM, and n refers to the number of animals from which blood vessels were taken. Values were compared by Student's t -test or one-way ANOVA using Tukey's test as post-hoc analysis for paired or unpaired observations, as appropriate, to determine significance between groups. Values were considered significantly different when $p < 0.05$.

Drugs

The following drugs were used: bradykinin, diethylamine NONOate (DEA NONOate), diltiazem, 5-hydroxytryptamine (5-HT), levcromakalim, nitro-L-arginine (NLA), and NS1619 (Sigma Chemical, St. Louis, MO); BMS 191011, and iberiotoxin, (Tocris, Ellisville, MO); apelin-13, and F13A (H-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Ala-OH trifluoroacetate salt) (Bachem, Torrance, CA). Drug solutions were prepared fresh daily, kept on ice, and protected from light until used. All drugs were dissolved in double-distilled water with the exception of BMS 191011 and NS1619, which were dissolved initially in DMSO prior to further dilution in double-distilled water. Drugs were added to the myograph chambers in volumes not greater than 0.02 ml. Drug concentrations are reported as final molar concentrations in the myograph chamber.

RESULTS

APJ Receptor Expression in Cerebral Arteries

Western immunoblot analysis demonstrated expression of APJ receptor protein in rat cerebral arteries. A strong immunoreactive band at 42 kDa corresponding to APJ receptors was detected in immunoblots of cerebral artery homogenates and in rat lung used as a positive control (Figure 1A) (7). RT-qPCR analysis confirmed the expression of mRNA transcripts in rat cerebral arteries (Figure 1B). In immunofluorescence studies, APJ receptor protein was immunodetected in rat cerebral arteries and localized to the smooth muscle cell layer and endothelium (Figure 1C.1). Smooth muscle cell actin (SMCA) was detected in the smooth muscle cell layer (Figure 1C.2), and APJ receptors were co-localized with SMCA in the smooth muscle cell layer of the cerebral artery (Figure 1C.3). Incubation of arteries with an isotype negative control antibody, followed by the secondary antibody, demonstrated a lack of non-specific binding of secondary antibody in the absence of primary APJ receptor antibody (Figure 1C.5). Similar results were obtained in rat lung, which demonstrated immunostaining for APJ receptors on bronchial epithelium (Figure 1C.6).

Vascular Relaxation Studies

In cerebral arteries contracted with 5-HT (10^{-7} M), addition of increasing concentrations of apelin (10^{-8} – 3×10^{-6} M) to the preparations had no effect on vasomotor tone in rings with or without endothelium (Figure 2). Likewise, these concentrations of apelin had no effect on resting tone in quiescent cerebral arterial rings (data not shown).

DEA NONOate (10^{-9} – 10^{-5} M) caused concentration-dependent relaxation of endothelium-denuded cerebral arteries contracted with 5-HT (10^{-7} M) (Figure 3). The concentration-

response curve to DEA NONOate was inhibited in the presence of apelin (10^{-7} M) (Figure 3A); the pD_2 values for DEA NONOate were 6.92 ± 0.20 vs. 6.13 ± 0.08 , in the absence and presence of apelin, respectively ($p < 0.05$). Iberiotoxin (10^{-7} M) had an inhibitory effect on the response to DEA NONOate that was similar to that observed with apelin (Figure 3B). The corresponding pD_2 values for DEA NONOate were 6.98 ± 0.21 vs. 6.14 ± 0.13 , without and with iberiotoxin, respectively ($p < 0.05$). Treatment with a combination of apelin (10^{-7} M) plus iberiotoxin (10^{-7} M) had no greater inhibitory effect on DEA NONOate-induced relaxation of isolated cerebral arteries ($pD_2 = 6.19 \pm 0.07$; $n=6$) than either apelin or iberiotoxin alone. F13A (10^{-7} M), which had no effect of its own on the response to DEA NONOate, abolished the inhibitory effect of apelin on DEA NONOate-induced relaxation (Figure 3C).

Bradykinin-induced relaxation of endothelium-intact cerebral arteries was also inhibited in the presence of apelin (10^{-7} M), as evidenced by a significant difference in pD_2 values for bradykinin (7.76 ± 0.20 vs. 7.03 ± 0.12 , in the absence and presence of apelin, respectively; $p < 0.05$) and a reduction in the maximal relaxation response (Figure 4A). Iberiotoxin (10^{-7} M) had an inhibitory effect on the bradykinin concentration-response curve that was similar to that observed with apelin (Figure 4B); the pD_2 values for bradykinin were 8.02 ± 0.21 vs. 7.19 ± 0.22 in the absence and presence of iberiotoxin, respectively ($p < 0.05$). The effect of combined treatment with apelin (10^{-7} M) plus iberiotoxin (10^{-7} M) on bradykinin-induced relaxation of isolated cerebral arteries was similar to that observed with either inhibitor alone ($pD_2 = 7.15 \pm 0.17$; $n=6$). In the presence of nitro-L-arginine (3×10^{-5} M), the response to bradykinin was nearly abolished and neither apelin nor iberiotoxin had any effect on bradykinin-induced relaxations (Supplementary Figure 1). As observed in the experiments with DEA NONOate, F13A (10^{-7} M) had no effect on the response to bradykinin but abolished the inhibitory effect of apelin on bradykinin-induced relaxation (Figure 4C).

Apelin inhibited the responses to equieffective concentrations of NS1619 and BMS 191011 (Figure 5A and 5B), but had no effect on levromakalim-induced relaxation of similar magnitude in endothelium-denuded cerebral arteries (Figure 5C).

Electrophysiology Studies

DEA NONOate (10^{-5} M) caused a significant increase in whole-cell BK_{Ca} channel current measured in freshly isolated cerebral arterial smooth muscle cells (Figure 6). The DEA NONOate-induced increase in BK_{Ca} channel current was nearly abolished by apelin (10^{-7} M) (Figure 6A–E); however, in the presence of F13A (10^{-7} M), apelin (10^{-7} M) had no effect on the DEA NONOate-induced increase in BK_{Ca} channel current (peak current density (+80 mV) = 79.3 ± 7.2 with DEA NONOate alone versus 77.7 ± 5.5 pA/pF with DEA NONOate in the presence of apelin plus F13A; $p > 0.05$; $n=6$). As was observed in the vascular relaxation studies, the inhibitory effect of apelin on the response to DEA NONOate was mimicked by iberiotoxin (10^{-7} M) (Figure 6F). Likewise, apelin (10^{-7} M) also suppressed the NS1619-induced increase in BK_{Ca} channel current in isolated cerebral arterial smooth muscle cells (Figure 6G).

Cyclic GMP Measurements

DEA NONOate caused a significant increase in intracellular cyclic GMP levels in cerebral arteries. The basal level of cyclic GMP was 0.05 ± 0.01 pmol/ μ g protein and increased to 0.28 ± 0.06 pmol/ μ g in the presence of DEA NONOate (10^{-6} M) ($P < 0.05$; $n = 6$). Cyclic GMP levels in the presence of DEA NONOate plus apelin (10^{-7} M) were 0.26 ± 0.03 pmol/ μ g and did not differ significantly from those observed with DEA NONOate alone ($P > 0.05$; $n = 6$). Bradykinin also caused a significant increase in intracellular cyclic GMP levels in cerebral arteries. The basal level of cyclic GMP was 0.06 ± 0.02 pmol/ μ g protein and increased to 0.28 ± 0.05 pmol/ μ g in the presence of bradykinin (10^{-6} M) ($P < 0.05$; $n = 5$). In the presence of apelin (10^{-7} M), the bradykinin-induced increase in cyclic GMP levels was 0.23 ± 0.04 pmol/ μ g and did not differ significantly from that observed with bradykinin alone ($P > 0.05$; $n = 5$).

DISCUSSION

The major finding of the present study is that receptors for apelin (i.e. APJ receptors) are expressed in cerebral arteries and that activation of these receptors on vascular smooth muscle cells inhibits NO-induced relaxation. This conclusion, which identifies a previously unrecognized, novel action of apelin in blood vessels, is based on the findings that: 1) APJ receptor mRNA and protein are expressed in cerebral arteries; 2) APJ receptor protein co-localizes with smooth muscle cell actin in the arterial wall; 3) apelin inhibits relaxation of cerebral arteries in response to endothelium-derived NO, as well as an NO-donor; and 4) the inhibitory effects of apelin on NO-induced relaxation are abolished by an APJ receptor antagonist. Moreover, the findings indicate that apelin inhibits relaxation of cerebral arteries to NO by attenuating NO-induced increases in BK_{Ca} current in arterial smooth muscle cells. When taken together with the observation that apelin failed to cause relaxation in cerebral arteries, these actions of apelin could be expected to result in a net increase in cerebral vasomotor tone.

The results of the present study demonstrate for the first time that APJ receptor gene transcripts and proteins are expressed in cerebral arteries. Moreover, immunofluorescence co-localization studies indicate that APJ receptors are present on both vascular smooth muscle and endothelial cells in the cerebral arterial wall, suggesting the possibility that APJ receptors may be involved in the local regulation of cerebral artery vasomotor tone. Evidence for a functional role for APJ receptors in regulating vascular tone is supported by both *in vivo* and *in vitro* studies. Intravenous administration of exogenous apelin has been reported to cause both an increase and a decrease in mean arterial blood pressure (9, 27, 28), and, in humans, apelin increases forearm and coronary blood flow (29, 30). In isolated arterial rings, apelin causes endothelium-dependent relaxations under normal conditions (12, 13), whereas vascular smooth muscle contraction is observed in blood vessels in which the endothelium has been removed or damaged (12, 14). Nonetheless, apelin failed to cause either contraction or relaxation of isolated cerebral arteries, regardless of the status of the endothelium and despite the presence of APJ receptors on vascular smooth muscle and endothelial cells in these arteries. These results demonstrate that apelin itself does not

directly alter vasomotor tone in cerebral arteries, in contrast to its actions reported in other arteries studied thus far.

A key finding of the present study is that despite having no measurable direct effect on cerebrovascular tone, apelin inhibited relaxations in response to bradykinin (endogenous NO) (19, 20) and to DEA NONOate (exogenous NO) (18). Since APJ receptors are expressed on cerebral artery endothelial cells, one potential site of action for apelin, at least with regard to endogenously produced NO, could be inhibition of endothelial nitric oxide synthase (eNOS); however, the only published reports regarding the regulation of eNOS by apelin indicate that the peptide increases, rather than inhibits, eNOS activity (31, 32). Although an effect of apelin on eNOS in cerebral arteries cannot be ruled out by the present study, the observation that apelin also inhibits NO-induced relaxation in response to DEA NONOate, which acts independently of the endothelium, suggests a site of action other than or in addition to eNOS. These results, together with data from the molecular studies indicating that cerebral arterial smooth muscle cells express APJ receptors, are consistent with an inhibitory effect of apelin at the level of the vascular smooth muscle, which is the primary site of action for the relaxing effect of NO (33, 34). Moreover, that the inhibitory effects of apelin on both the functional and electrophysiological responses to NO were blocked by pharmacologic inhibition with F13A, an APJ receptor antagonist (25), strongly suggests that activation of APJ receptors on smooth muscle cells mediates this effect of apelin in cerebral arteries.

The finding that apelin inhibits NO-induced relaxation in cerebral arteries stands in contrast to its putative beneficial vasodilator effects reported in several peripheral arteries. Given the considerable heterogeneity known to exist among blood vessels from different origins in terms of their reactivity to vasoactive agents it is not necessarily surprising that cerebral arteries may differ from peripheral arteries in their response to apelin. In the rat, for example, the pineal hormone, melatonin, is a potent vasoconstrictor in isolated cerebral arteries (35), but causes relaxation of isolated aorta and caudal artery (36, 37). The reason(s) for the divergent responses to apelin among different blood vessels are not yet readily apparent, but possible explanations could include: 1) differences in the subtype of APJ receptor expressed in smooth muscle of cerebral vs peripheral arteries; however, it should be noted that there are no reports in the literature of multiple APJ receptor subtypes; 2) differences in the intracellular signaling pathways coupled to APJ receptor activation in cerebral versus peripheral arteries; and 3) differences in the role of BK_{Ca} channels in NO-induced relaxation among blood vessels.

The apelin/APJ receptor-signaling axis is generally considered to play a protective role in the cardiovascular system, with suggested benefits in disorders such as ischemia-reperfusion injury, heart failure, and pulmonary hypertension (10, 11, 38). Although apelin may have protective vasodilator effects in certain arteries (e.g. pulmonary vascular bed), it is evident from the present study that, in cerebral arteries, the net effect of APJ receptor activation could adversely affect cerebrovascular function. This may present an important caveat when exploring the cardiovascular efficacy of systemically administered APJ receptor agonists. Indeed, apelin-like analogs are now in preclinical development as potential therapeutic agents for myocardial infarction and ischemia-reperfusion injury (38), and apelin itself is

currently being administered to patients in clinical trials because of its putative beneficial effects on cardiac function and the pulmonary circulation (30, 39). The cerebrovascular consequences of such drugs may be particularly significant in certain patients, inasmuch as a single nucleotide polymorphism in the gene encoding the human APJ receptor, which results in increased apelin/APJ receptor signaling, has recently been identified and is associated with an increased risk of brain infarction (40). Given the potential of sex hormones in regulating mechanisms of vasodilatation, it will also be important to determine whether the effects of apelin on NO-induced relaxation reported in male arteries in the present study are also observed in cerebral arteries from females. Such information may have significant implications for drug development with regard to possible adverse effects in specific end organs, such as brain. Since impaired vasodilation is a hallmark characteristic of the vascular dysfunction that occurs in many forms of cardiovascular disease (34), it is interesting to note that endogenous apelin levels are elevated in conditions such as obesity and diabetes (41–43), which are major risk factors for stroke and other cerebrovascular disorders (44).

The molecular mechanism by which APJ receptor activation causes impaired relaxation to NO is likely due to inhibition of NO-induced increases in BK_{Ca} channel current in cerebral smooth muscle cells. In cerebral arteries of the rat, NO-induced relaxation is mediated in part by increased BK_{Ca} channel activity and in part by increased formation of intracellular cyclic GMP resulting from activation of soluble guanylyl cyclase (45–47). Since apelin had no effect on the increase in cyclic GMP levels evoked by either bradykinin or DEA NONOate it is unlikely that inhibition of guanylyl cyclase underlies the ability of apelin to reduce NO-induced relaxation in cerebral arteries. On the other hand, a role for BK_{Ca} channels in the effect of apelin is supported by several pieces of evidence, including: 1) apelin inhibited the increases in BK_{Ca} current induced by DEA NONOate and NS1619 in cerebral arterial smooth muscle cells; 2) the effects of apelin on both the functional and electrophysiological responses to NO were mimicked by iberiotoxin, a potent and selective BK_{Ca} channel blocker (24); 3) combined treatment with apelin plus iberiotoxin had no greater effect on NO-induced relaxation than either inhibitor alone, consistent with a shared mechanism of action for apelin and iberiotoxin; and 4) apelin inhibited relaxation of endothelium-denuded cerebral arteries in response to the chemically distinct BK_{Ca} channel activators, BMS 191011 and NS1619 (21, 22), without affecting the response to the K_{ATP} channel opener, levcromakalim (23). Taken together, the data indicate that the effect of apelin on BK_{Ca} channels is sufficient to meaningfully impair cerebral arterial relaxation evoked by NO as well as other BK_{Ca} channel-activating molecules. Though the primary focus in the present study was to identify the mechanism(s) by which apelin inhibits NO-induced relaxation of cerebral arteries, it will be important to identify the downstream intracellular signaling pathway(s) linking APJ receptor activation to inhibition of NO-induced increases in BK_{Ca} current in cerebral arterial smooth muscle. In this regard, a possible role for the PI3-kinase/Akt pathway is suggested by our recent findings that apelin increases Akt phosphorylation in cerebral arterial smooth muscle cells, and inhibition of PI3-kinase markedly attenuates the inhibitory effect of apelin on voltage-dependent increases in BK_{Ca} channel current (16).

In summary, apelin activates APJ receptors in cerebral arterial smooth muscle cells, resulting in inhibition of BK_{Ca} currents and reduced relaxation to endogenous and exogenous NO in

cerebral arteries from male rats. Whether the ability of apelin to regulate vasomotor tone by inhibiting NO-induced relaxation is unique to the cerebral circulation is not yet clear, but given the pivotal role of NO in controlling vascular function, it will be important to determine if this novel effect of apelin is observed in other blood vessels. The potential for adverse effects on the cerebral circulation to occur with the use of APJ receptor-activating drugs merits further consideration in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression and localization of APJ receptors

(A) Representative immunoblot showing expression of APJ receptor (APJR) protein in homogenates from rat cerebral arteries (Lane 1) and lung tissue (Lane 2); β -actin was used as a loading control. The immunoblots shown are representative of data obtained from five different animals. (B) Bar graph showing relative mRNA expression of APJ receptor, represented as Δ Ct values relative to β -actin. Data are presented as mean $\pm$ SEM (n=3). (C) Representative immunofluorescence images of: (1) APJ receptors (red fluorescence), (2) smooth muscle cell actin (green fluorescence), (3) merged image showing co-localized areas in smooth muscle cells (yellow fluorescence) and endothelium (red fluorescence) in rat cerebral artery; (4) differential interference contrast image of rat cerebral artery; and (5) isotype negative control (nonimmune IgG) for APJ receptor antibody with nuclear staining (blue fluorescence); (6) APJ receptors (red fluorescence) with nuclear staining (blue fluorescence) in rat bronchial epithelium (positive control). The images are representative of those obtained from three different animals. Scale bar, 20 μ m.

Figure 2. Lack of direct effect of apelin on rat isolated cerebral arteries

(A) Representative original tracings of isometric tension recordings from rat isolated cerebral arteries (with and without endothelium) in response to cumulative addition of increasing concentrations of apelin, followed by bradykinin (BK; 10^{-7} M) and diltiazem (10^{-5} M). (B) Mean data demonstrating a lack of vasomotor effect of apelin on cerebral arteries with (E+) and without (E-) endothelium. Data are expressed as a percentage of the initial increase in tension induced by 5-HT (10^{-7} M). Each point represents the mean $\pm$ S.E.M. (n=5).

Figure 3. DEA NONOate-induced relaxation of isolated cerebral arteries is inhibited by apelin and iberiotoxin

DEA NONOate-induced relaxation of endothelium-denuded cerebral arteries in the absence and presence of: (A) apelin (10^{-7} M); and (B) iberiotoxin (IBTx; 10^{-7} M). (C) Bar graph representing the effect of apelin (10^{-7} M) and F13A (10^{-7} M), alone or in combination, on DEA NONOate (10^{-6} M)-induced relaxation of cerebral arteries. Experiments with inhibitors were conducted in parallel with untreated control rings taken from the same animal. Data are expressed as a percentage of the initial increase in tension induced by 5-HT

(10^{-7} M). Each point represents the mean $\pm$ S.E.M. (n=5–10), *p<0.05 vs. DEA NONOate response in the absence of inhibitor.

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Figure 4. Bradykinin-induced, endothelium-dependent relaxation of isolated cerebral arteries is inhibited by apelin and iberiotoxin

Bradykinin-induced relaxation of endothelium-intact cerebral arteries in the absence and presence of: (A) apelin (10^{-7} M); and (B) iberiotoxin (IBTx; 10^{-7} M). (C) Bar graph representing the effect of apelin (10^{-7} M) and F13A (10^{-7} M), alone or in combination, on bradykinin (10^{-6} M)-induced relaxation of cerebral arteries. Experiments with inhibitors were conducted in parallel with untreated control rings taken from the same animal. Data are expressed as a percentage of the initial increase in tension induced by 5-HT (10^{-7} M). Each point represents the mean $\pm$ S.E.M. (n=6). *p<0.05 vs. bradykinin response in the absence of inhibitor.

Figure 5. Apelin selectively inhibits relaxation of cerebral arteries in response to BK_{Ca} channel activators

Bar graph representing the effects of apelin (10^{-7} M) on relaxation of cerebral arteries in response to: (A) NS1619 (10^{-6} M); (B) BMS 191011 (3×10^{-6} M); and (C) levcromakalim (3×10^{-6} M). Each point represents the mean $\pm$ S.E.M. (n=6), *p<0.05 vs. NS1619 or BMS 191011 in the absence of apelin.

Figure 6. Apelin inhibits DEA NONOate- and NS1619-induced increases in whole cell BK_{Ca} currents

Whole cell BK_{Ca} currents were recorded in freshly isolated vascular smooth muscle cells in response to successive voltage pulses of 800 ms duration, increasing in 10 mV increments from -60 mV to +80 mV. Currents were recorded before and after treatment with DEA NONOate (DEA, 10⁻⁵ M) or NS1619 (10⁻⁵ M), with and without apelin (10⁻⁷ M) or iberiotoxin (IBTx, 10⁻⁷ M). **A-D:** Representative tracings depicting the sequential current recordings from a single smooth muscle cell in **(A)** control conditions, followed by **(B)** DEA (10⁻⁵ M, 2 min), **(C)** apelin (10⁻⁷ M, 5 min), and **(D)** combination of DEA (10⁻⁵ M) and apelin (10⁻⁷ M). **E:** Bar graph summarizing the effect of apelin (10⁻⁷ M, 5 min) on DEA (10⁻⁵ M)-induced average current density (pA/pF) at +80mV. **F:** Bar graph summarizing the effect of iberiotoxin (10⁻⁷ M, 5 min) on DEA (10⁻⁵ M)-induced average current density at +80mV. **G:** Bar graph summarizing the effect of apelin (10⁻⁷ M, 5 min) on NS1619 (10⁻⁵ M)-induced average current density at +80mV. Values are represented as mean ± SEM (n=6). *p<0.05 vs control current density; #p<0.05 vs DEA NONOate- or NS1619-induced current density.