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Angiotensin II causes endothelial-dependent increase in expression of Ca_v1.2 protein in cultured arteries

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Abstract

Examination was made of the direct vascular effects of the hypertension-inducing pressor hormone angiotensin II on expression and activity of the voltage-gated calcium channel Ca_v1.2. Freshly dissected rat superior mesenteric artery beds were maintained in organ culture unpressurized for 24 h in the presence or absence of angiotensin II. Relative to controls, angiotensin II increased Ca_v1.2 protein expression and tension-inducing activity but not Ca_v1.2 message. The increase in Ca_v1.2 protein expression by angiotensin II was abrogated by damaging the endothelium. Thus, the endothelium is involved in regulating Ca_v1.2 expression in the vascular wall.

Keywords

Angiotensin II; Ca_v1.2 Ca²⁺ channel; cultured artery; endothelium; vascular smooth muscle

1. Introduction

Several reports have indicated that Ca_v1.2 protein expression is increased in resistance arteries of both spontaneously hypertensive rats (SHR) and rats rendered hypertensive by banding of the aorta between the renal arteries (Pratt et al., 2002; Pesic et al., 2004; Wang et al., 2006). However, it is difficult to perform interventions *in vivo* to study the mechanisms involved in the regulation of channel expression. *In vitro* models are significantly easier to manipulate experimentally and better suited to determine direct effects on vasculature.

Angiotensin II is an octapeptide well known for its ability to vasoconstrict (Bohr and Uchida, 1967) and to elevate blood pressure when infused into rats or mice (Gerzanich et al., 2003). It also has been reported to release Ca²⁺ from intracellular stores (Grover et al., 1994) and to generate increased vascular L-type Ca current (Ohya and Sperelakis, 1991). Angiotensin converting enzyme inhibitors, angiotensin II receptor blockers and Ca²⁺ channel blockers all share an ability to reduce blood pressure when administered chronically. However, it is relatively difficult to interpret effects on angiotensin II-induced signaling in such infused animal studies, particularly in light of evidence suggesting neural contributions to angiotensin II-induced hypertension (Li et al., 1996). We were intrigued by a recent report that angiotensin II could downregulate expression of potassium channels in a cerebral artery culture model *in*

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vitro (Amberg et al., 2004). The aim of the present study was to employ a similar culture model utilizing mesenteric resistance arteries (Kleppisch et al., 1996) to assess if angiotensin II directly increases vascular Ca_v1.2 channel expression.

2. Materials and Methods

2.1. Primary culture of rat mesenteric artery vascular smooth muscle cells (VSMC)

Isolation of mesenteric arteries from freshly euthanized rats was conducted with protocols approved by our institutional animal care and use committee. VSMC were isolated from rat superior mesenteric arteries by digestion with papain (1 mg/ml) and dithiothreitol (DTT) (0.5 mg/ml) for 30 min at 37°C in 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 0.05 mM CaCl₂, and 10 mM HEPES, pH 7.35 containing 2.5 mg/ml bovine serum albumin (BSA) and 0.25 mg/ml DTT. This was followed by another 10–15 min in papain (0.5 mg/ml), collagenase (1 mg/ml), trypsin inhibitor (0.5 mg/ml), and elastase (0.25 mg/ml). Freshly isolated VSMC were cultured in DMEM supplemented with antibiotics and 10% fetal bovine serum (FBS), passaged twice and then synchronized in serum free medium for 24 h. VSMC were treated in DMEM plus 0.1% FBS for 24 h with or without 1 μM angiotensin II.

2.2. Arterial culture

The superior mesenteric artery of 300–400 g male Sprague Dawley rats was dissected, cleaned of superficial fat, cut into approximately 6 pieces, placed in DMEM containing 0.1% fetal bovine serum in 35 mm diameter culture dishes. It was maintained in tissue culture at 37°C and 5% CO₂ with and without angiotensin II in DMEM with 0.1% FBS and antibiotics for 24 h. After this time, arteries were subjected to one (or more) of the following procedures.

2.3. Western blotting

Arterial tissue was minced and homogenized on ice in lysis buffer (50 mM NaCl, 25 mM TrisCl, 0.5% Na deoxycholate, 1% IGEPAL CA-630, 1 mM PMSF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, pH 7.4). The homogenate was differentially centrifuged at 13,000 × g for 10 min to yield a supernatant containing the channels. Protein concentration in the supernatant was determined by a BCA protein assay kit (Pierce, Rockford, IL), and 20–40 μg protein applied to each lane. Cultured cells were directly lysed with lysis buffer, and proteins extracted. Western blots were run using Tris Acetate gradient gels (Invitrogen, Carlsbad, CA), transferred onto PVDF membranes. The blots were blocked and probed for Ca_v1.2 with a primary antibody from Alomone (Jerusalem, Israel) vs the II–III linker and probed for α-actin as a control for loading.

2.4. Realtime RT-PCR

Total RNA was isolated from blood vessels using RNeasy Fibrous Tissue (Qiagen, Valencia, CA), which involves treatment with DNase I for 10 min at 25°C. The RNA was reverse transcribed into cDNA by using Applied Biosystems High Capacity cDNA Archive Kit using random primers for 2 h at 37°C prior to any PCR. The cDNA produced from 1 μg total RNA was then utilized for realtime RT-PCR using a forward primer ccgcagtgtaagccaacgtc (1834 in GenBank [M59786](#)) and reverse primer gagcgagtgagtcagcaa (2227, generating a 394 bp product). Reactions were run in a Stratagene Mx3000P instrument with a Stratagene SYBR green-based real-time QPCR kit and measurement temperatures ≥80°C to differentiate specific PCR products from nonspecific primer-dimers. PCR products were verified by gel electrophoresis and melting curve analysis, demonstrating a melting temperature of 86°C. Certain samples were obtained at culture points of 3, 6, and 12 h.

2.5. Functional assessment of Ca_v1.2 activity

Tension determinations were performed by cutting 4 mm rings of mesenteric vessels and mounting them on force transducers at their optimal length-tension. Rings of the primary branches of the artery were equilibrated in a tissue bath for 1 h prior to measuring tension responses to elevated KCl or the L-type Ca²⁺ channel agonist FPL 64176. Rings were equilibrated 1 h in Krebs-Henseleit (K-H) solution (mM): NaCl 118, KCl 5.4, NaH₂PO₄ 1.4, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 18, Glucose 11 bubbled with 95% O₂ and 5 % CO₂ (pH 7.4). Basal tension was adjusted to 1.5 g. Rings were then exposed to increasing concentrations of Ca²⁺ channel activator FPL 64176 or KCl. Results were normalized by weight of each ring.

2.6. Endothelial damage

Endothelial damage was generated by intra-arterial perfusion for 35 s with ~ 1 ml K-H solution containing 0.3% CHAPS (Randall et al., 1988) followed by a 30 s wash perfusion with K-H solution without CHAPS. Another method to damage the endothelium with gentle mechanical rubbing of the intimal layer with an appropriate cotton swab was utilized to confirm the results obtained from endothelium damage by CHAPS.

2.7. Reagents

DMEM culture medium was purchased from GIBCO. All other chemicals were purchased from Sigma.

2.8. Statistical analysis of data

Data are presented as mean ± S.E.M. and the statistical significance was determined by student's *t*-test. A *P* value < 0.05 was considered significant.

3. Results

Angiotensin II caused increased expression and activity of Ca_v1.2 protein. Culture of arteries for 24 h in the presence and absence of 0.5 μM angiotensin II revealed a very reproducible increase in expression of Ca_v1.2 protein by Western blot (Fig. 1A). Tension measurements were employed to determine whether the increase in Ca_v1.2 expression resulted in any increase in functional Ca_v1.2 activity. As seen in Fig. 1B, tension responses in the presence of the L-type Ca²⁺ channel agonist FPL 64176 (Fan et al., 2000) were noticeably greater in rings from arteries cultured in the presence of angiotensin II than in rings from arteries cultured in its absence.

Angiotensin II did not cause increased expression of Ca_v1.2 message. The upregulation of Ca_v1.2 protein was not accompanied by an increase in expression of Ca_v1.2 transcript, as revealed by realtime RT-PCR (Fig. 1C). Because an increase in Ca_v1.2 message might have been temporary, the determinations were also performed at shorter time points, but in every case the results were negative. An increase in protein levels without a corresponding increase in transcript levels would be compatible with an increase in translation of a constant amount of message or with a decrease in degradation of Ca_v1.2 protein.

Angiotensin II-induced increases in Ca_v1.2 expression required functional endothelium. Similar increases in Ca_v1.2 expression from primary cultures of rat mesenteric artery VSMC were not observed (not shown). In order to determine the reason, we functionally removed the endothelium from mesenteric arteries prior to culture. This was accomplished by a brief 35 s perfusion with solution containing 0.3% CHAPS or with mechanical rubbing of the intimal layer. As observed in Fig. 2A), endothelium damage with 0.3% CHAPS perfusion virtually obliterated endothelial-dependent acetylcholine-induced relaxation while still preserving robust responses to KCl and phenylephrine. Culture of such de-endothelialized arteries for 24

h no longer preserved the ability of angiotensin II to upregulate $\text{Ca}_v1.2$ expression (Fig. 2B and C). After culture in a de-endothelialized state, angiotensin II has no effects on the $\text{Ca}_v1.2$ protein expression (Fig. 2D) or tension responses induced by $\text{Ca}_v1.2$ channels activator FPL 64176 (Fig. 2E). Endothelium damage with a mechanical method confirmed the results obtained with endothelium damage using CHAPS (Fig. 2F).

4. Discussion

We describe a cultured artery preparation usable for demonstrating that angiotensin II can directly increase the protein expression of vascular $\text{Ca}_v1.2$. Since mRNA levels were not increased at any time point examined, it does not appear that increased transcription of the $\text{Ca}_v1.2$ gene is involved. The effect is not manifest with either vascular smooth muscle cells alone or in arteries with the endothelium damaged. Thus, the cultured artery preparation is probably the simplest that can be utilized to examine the direct effects of Angiotensin II on vascular $\text{Ca}_v1.2$ expression and function. The preparation is certainly not susceptible to central nervous system effects which have indicated roles for central angiotensin II receptors in blood pressure regulation (Li et al., 1996; Yamakawa et al., 2003).

Endothelial dysfunction is associated with cardiovascular diseases such as hypertension, atherosclerosis, diabetic vasculopathy and heart failure (Ray et al., 2004; Ogita et al., 2005). Endothelial function and integrity determines Ca^{2+} channel activity in vascular smooth muscle (Simard et al., 2000; Gerzanich et al., 2003). Our findings provide new and direct evidence that endothelium is involved in the angiotensin II-induced regulation of this vascular smooth muscle Ca^{2+} channel.

We anticipate that this preparation will be very useful in examining angiotensin II signaling relating to long-term pressor and other effects at the level of the vasculature. Angiotensin II has previously been shown to be involved in development of hypertension and increased $\text{Ca}_v1.2$ activity (Gerzanich et al., 2003). Our *in vitro* results are confirmatory of angiotensin II-mediated increased $\text{Ca}_v1.2$ activity. Our results would seem to indicate that angiotensin II can increase $\text{Ca}_v1.2$ expression and activity without first elevating blood pressure, although the angiotensin II concentrations are quite high. Pesic *et al* (2004) have indicated that $\text{Ca}_v1.2$ can be upregulated *in vitro* by depolarization and that increases in $\text{Ca}_v1.2$ also are associated with depolarization *in vivo* in another rat model of hypertension. Our *in vitro* result is clearly independent of blood pressure and raises the possibility that elevation of angiotensin II *in vivo* may promote $\text{Ca}_v1.2$ expression in small arteries. Whether angiotensin II mediates this event by depolarizing the vascular smooth muscle cells remains to be determined.

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Fig. 1.

Ca ν 1.2 protein expression (A) and function (B) but not mRNA (C) are enhanced by exposure of rat superior mesenteric artery to 0.5 μ M angiotensin II for 24 h in culture. (A) Typical Western blot demonstrating Ca ν 1.2 protein expression with quantification shown underneath. Numbers in the graph indicate number of separate experiments performed. * $P < 0.0001$ compared with control. (B) Ca ν 1.2 protein expression enhancement by angiotensin II is accompanied by increased indication of Ca ν 1.2 function. Tension responses of arterial rings to challenges by increasing concentrations of L-type Ca $^{2+}$ channel agonist FPL 64176 indicate greater tension from rings exposed to 0.5 μ M angiotensin II for 24 h (right) than control rings maintained in culture in its absence (left). All tension responses were rapidly inhibited by application of 1 μ M nifedipine. Below traces are shown graphical representation of results from these and 9 other similar experiments from 3 different arteries. * $P < 0.05$, ** $P < 0.01$ with respect to control. (C) Ca ν 1.2 message expression is not enhanced by exposure of rat superior mesenteric artery to 0.5 μ M angiotensin II for 3–24 h in culture. Realtime RT-PCR results indicated no difference in Ca ν 1.2 mRNA levels after 24 h in culture. Earlier time points were then utilized in case an increase in mRNA was only transient and preceded the increase in Ca ν 1.2 protein. No increases were found at any time point examined.

Fig. 2.

Ca_v1.2 protein expression enhancement by angiotensin II requires functional endothelium. (A) Brief perfusion of mesenteric arteries with 0.3% CHAPS solution for 35 s abrogates relaxation induced by 1 μM acetylcholine but not constriction of mesenteric arterial rings to either 80 mM KCl or 0.5 μM phenylephrine (PE). (B) Brief perfusion of mesenteric arteries with 0.3% CHAPS solution for 35 s prior to placement in culture completely blocks the enhanced expression of Ca_v1.2 protein induced by angiotensin II exposure. (C) Western results quantification for results in (B). Numbers in the graph indicate numbers of separate observations. * $P < 0.05$ compared with control. (D) After endothelial damage with CHAPS, angiotensin II has no effects on the Ca_v1.2 protein expression compared with arteries after endothelial damage alone and (E) arteries incubated with or without angiotensin II after endothelial damage with 0.3% CHAPS showed similar tension responses to Ca_v1.2 channel activator FPL 64176 (1 μM), and these effects were blocked by Ca_v1.2 channel blocker nifedipine (1 μM). Similar results were seen in three different experiments. (F) After endothelial damage by mechanical rubbing, angiotensin II also failed to increase Ca_v1.2 protein expression.