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RAPID COMMUNICATION

Cytosolic Free Calcium Response to Angiotensin II in Aortic VSMC Isolated from Male and Female SHR

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Summary

Previous *in vitro* studies have shown that vascular smooth muscle cells (VSMC) isolated from the aortae of male spontaneously hypertensive rats (SHR) proliferate more rapidly than those obtained from female SHR. Sex-dependent differences of cytosolic free calcium concentration ($[Ca^{2+}]_i$) were therefore studied in VSMC under basal conditions and after the stimulation by different concentrations of angiotensin II (Ang II). No significant difference in basal $[Ca^{2+}]_i$ was found in VSMC from male and female SHR. Angiotensin II significantly increased $[Ca^{2+}]_i$ in VSMC from both genders. This $[Ca^{2+}]_i$ rise elicited by 10^{-7} and 10^{-9} M Ang II was more pronounced in cells isolated from males than in those from females. This difference may be attributed to greater mobilisation of intracellular calcium stores in male VSMC. It can be concluded that the cytosolic free calcium response to angiotensin II is augmented in VSMC of male SHR, which also grow more rapidly in response to this peptide hormone.

Key words
Spontaneously hypertensive rat – Gender – Primary culture – VSMC – Angiotensin II – Cytosolic free calcium

Intracellular calcium as a key factor controlling vascular tone plays an important role in the pathogenesis of primary hypertension (Erne and Hermsmeyer 1989). Several abnormalities in cell calcium handling have been observed in SHR (Sugiyama *et al.* 1986; Bendhack *et al.* 1992).

Free cytosolic calcium serves as a mediator of many intracellular signalling pathways stimulated by different hormones, neurotransmitters and mitogenic factors. Angiotensin II, a potent vasoconstrictor, leads to a cascade of several intracellular events, which result in a $[Ca^{2+}]_i$ increase and smooth muscle contraction (Bodin *et al.* 1993; Van Renterghem and Lazdunski 1994; Haller *et al.* 1994; Touyz *et al.* 1994). Furthermore, angiotensin II has also been shown to have a mitogenic effect on aortic VSMC isolated from SHR (Paquet *et al.* 1990; Hadrava *et al.* 1989).

The experimental data obtained in SHR

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(Bačáková and Kuneš 1995; Loukotová *et al.* 1998) have demonstrated that aortic smooth muscle cells isolated from males proliferate more rapidly than those isolated from females. In order to find the mechanisms responsible for this sex-dependent VSMC growth *in vitro*, we searched for sex differences in cytosolic free calcium concentrations in VSMC isolated from male and female SHR. Intracellular $[Ca^{2+}]_i$ was measured by image analysis in single myocytes loaded with Fura-2 under basal conditions and in response to different Ang II concentrations.

Male (n=5) and female (n=6) SHR aged 10 weeks were obtained from the breeding colony of our Institute. The rats were killed by decapitation and VSMC were obtained by enzymatic digestion from their thoracic aortae. Briefly, thoracic aortae were aseptically excised and cleaned of connective tissue and adherent fat. The adventitia was removed by stripping the aortae with a pair of dissecting forceps. The isolated arteries were cut longitudinally and the endothelium was removed. The medial segments were rinsed with DMEM (Dulbecco's Modified Eagle's Medium, SEVAC, Prague) plus gentamycin, minced into very small pieces and digested using collagenase 2 mg/ml (Sigma), elastase 0.12 mg/ml (Sigma) and bovine serum albumin 2 mg/ml (Sigma) at 37 °C for 60 min (Touyz *et al.* 1994). Isolated cells were centrifuged and resuspended in DMEM + 20 % FCS (foetal calf serum, Bioclot, Germany) + gentamycin. A monolayer of VSMC was grown on 22 mm^2 glass coverslips coated with poly-L-lysine (Sigma). After seeding the cells, Petri dishes containing the glass coverslips were maintained at 37 °C in a humidified incubator in 95 % air to 5 % CO_2. Male and female VSMC were always matched for the duration of *in vitro* cultivation.

Measurements of $[Ca^{2+}]_i$ in cultured VSMC were performed using Fura-2. Only primary cultures were used, always one aorta in each coverslip. The cells were loaded in 5 μM Fura-2-AM with pluronic acid for 30 min. Afterwards the cells were washed three times with buffer A (in mmol/l: NaCl 135, KCl 5, Na_2HPO_4 1, NaH_2PO_4 0.25, $MgCl_2$ 1.5, $CaCl_2$ 1.5, glucose 5, HEPES 5, pH 7.4 at 37 °C) and allowed further 30 min to enable the deesterification of Fura-2-AM. Both loading and washing procedures were carried out at 37 ° C.

In order to perform a dose-response experiment on a single coverslip, four greased teflon rings were sealed to the surface of the coverslip. Each ring was filled with 50 μl of buffer A. The coverslip was placed under an inverted microscope (Olympus IX50-S1F) and than the $[Ca^{2+}]_i$ response to a given Ang II concentration was measured in each ring. The concentrations of 10^{-9}, 10^{-7} and 10^{-5} M angiotensin II were used. $[Ca^{2+}]_i$ response to Ang II was monitored for about 80 s. The measurements were usually performed on 8–12 cells in each ring.

The cells were alternatively excited by 340 and 380 nm wavelengths. The emission wavelength was set to 510 nm. $[Ca^{2+}]_i$ was calculated as described by (Grynkiewicz *et al.* 1985)

$$[Ca^{2+}]_i = [(R - R_{min})/(R_{max} - R)] \times (F_{min}/F_{max}) \times K_D$$

where R is the measured ratio of 340/380 nm fluorescence, R_{max} is the 340/380 ratio in the presence of saturating calcium concentration (10^{-2} M), R_{min} is the 340/380 ratio in Ca^{2+}-free buffer A with 0.25 mM EGTA and K_D is the dissociation constant of Fura-2 for calcium ions, taken to be 224 nmol/l. F_{min} is the fluorescence intensity of 380 nm wavelength at minimum free calcium and F_{max} is the fluorescence intensity at the same wavelength at maximum free calcium. Fluorescence intensities were corrected for the background. Calibration was performed individually for each cell with calcium (10^{-2} M) and EGTA (0.25 mM), both in the presence of ionomycin (2 μM).

Student's t-test was used for statistical evaluation of differences between both genders. The paired t-test was used for assessing the time course of the Ang II-induced $[Ca^{2+}]_i$ response.

Table 1 compares the levels of cytosolic free calcium in aortic VSMC isolated from SHR of both genders before and after stimulation with different concentrations of angiotensin II. Basal $[Ca^{2+}]_i$ values found in both genders did not differ significantly. All the studied concentrations of angiotensin II increased the cytosolic free calcium concentration in cells from both genders. Ang II (both 10^{-7} and 10^{-5} M) induced a typical $[Ca^{2+}]_i$ rise. In contrast, Ang II 10^{-9} M elicited a moderate slow $[Ca^{2+}]_i$ elevation which reached a plateau in the last 40 s of our measurements (Table 1). Ang II (10^{-7} M) induced a higher $[Ca^{2+}]_i$ rise in male than in female VSMC (Fig. 1, Table 1). This was also true for 10^{-9} M Ang II, which increased $[Ca^{2+}]_i$ by +91 ± 18 % in male and by +32 ± 5 % in female VSMC ($p<0.02$).

Table 1. Effect of different angiotensin II (Ang II) concentrations on free cytosolic calcium level ($[Ca^{2+}]_i$) in VSMC isolated from male and female SHR.

Ang II	**Males** Baseline $[Ca^{2+}]_i$	Angiotensin II-induced Average	Early $[Ca^{2+}]_i$ rise	Late	**Females** Baseline $[Ca^{2+}]_i$	Angiotensin II-induced Average	Early $[Ca^{2+}]_i$ rise	Late
10^{-9} M	76.0±5.3 n = 24	138.7±13.7	135.2±17.8	141.7±16.6	79.1±4.2 n = 26	105.3±8.6	99.8±7.2	110.5±10.2
10^{-7} M	87.3±9.9 n = 33	423.9±37.9**	550.8±55.2***	311.8±25.2#	69.3±4.7 n = 51	274.0±30.1	309.9±36.5	237.2±24.7#
10^{-5} M	83.5±9.1 n = 40	272.3±26.8*	350.5±41.1	205.2±16.6***#	86.6±6.5 n = 57	360.7±22.5	423.8±34.5	299.2±16.0#

*Data (in nmol/l) are expressed as means ± S.E.M., n represents number of cells measured. Early and late angiontensin II-induced $[Ca^{2+}]_i$ rises correspond to the first 40 s and the last 40 s of the measurement covering a period of about 80 s after peptide administration. *p<0.05, **p<0.01, ***p<0.001 vs. females; #p<0.001 vs. early $[Ca^{2+}]_i$.*

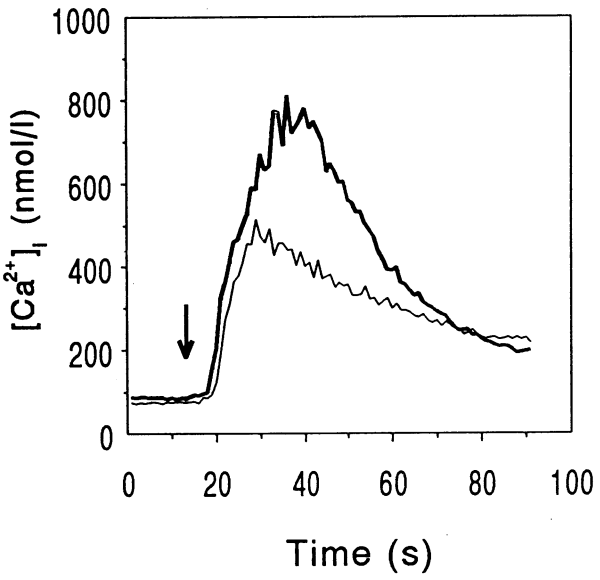

Fig. 1. *The time course of $[Ca^{2+}]_i$ changes induced by angiotensin II (10^{-7} M) in vascular smooth muscle cells isolated from male (thick line) and female (thin line) SHR.*

Our study did not disclose any significant differences in basal $[Ca^{2+}]_i$ values between male and female VSMC. Thus the enhanced *in vitro* proliferation of VSMC isolated from aortae of male SHR could not be ascribed to increased $[Ca^{2+}]_i$. On the other hand, the increased $[Ca^{2+}]_i$ evoked by 10^{-7} M Ang II was substantially greater in male compared to female VSMC (Fig. 1). In female cells, similar degree of $[Ca^{2+}]_i$ elevation was reached by 10^{-5} M Ang II. A greater mobilisation of intracellular calcium stores in male VSMC *via* inositol trisphosphate mechanism might be responsible for this sex difference in Ang II-induced $[Ca^{2+}]_i$ response. Although the threshold Ang II concentration for VSMC of both genders is not known, it seems to be close to 10^{-9} M concentration which elicited almost threefold higher $[Ca^{2+}]_i$ increase in aortic VSMC isolated from male SHR compared to $[Ca^{2+}]_i$ response of female cells.

At present, it is rather premature to speculate about the possible relationship between enhanced $[Ca^{2+}]_i$ and growth responses of male VSMC to Ang II, encountered in this and a parallel study (Loukotová *et al.* 1998). It has, however, been demonstrated that proliferating VSMC cultures have reduced the $[Ca^{2+}]_i$ response to Ang II due to markedly decreased calcium stores compared to confluent and/or postconfluent cultures (Cortes *et al.* 1996). The reduction of the Ang II-dependent $[Ca^{2+}]_i$ rise in proliferating SHR VSMC was ascribed to the impairment of Ca^{2+}-induced Ca^{2+} release from Ang II- and ionomycin-releasable calcium stores through ryanodine-sensitive calcium channels

(Cortes *et al.* 1997). Our data would be compatible with the above findings providing that the more rapid proliferating male VSMC (Bačáková and Kuneš 1995; Loukotová *et al.* 1998) would attain the confluent state earlier than slowly growing female VSMC. Consequently, the higher Ang II-induced $[Ca^{2+}]_i$ response in aortic VSMC isolated from male SHR would reflect more advanced transition of primary culture from the proliferative phase into confluence. However, this hypothesis requires further experiments.

Taken together with the results on VSMC proliferation obtained in a parallel study (Loukotová *et al.* 1998), aortic VSMC from male SHR are characterised by augmentation of both growth and $[Ca^{2+}]_i$ response to Ang II administration. The enhanced reactivity of cultured male VSMC to angiotensin II is in agreement with the higher vascular reactivity to this peptide in male than in female SHR (Collis and Vanhoutte 1977).

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Reprint requests

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