Urocortin II Inhibits the Apoptosis of Mesenteric Arterial Smooth Muscle Cells Via L-type Calcium Channels in Spontaneously Hypertensive Rats

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Key Words

Urocortin (UCN) II • Apoptosis • Whole-cell patch clamp
• L-type calcium channels • Mesenteric arterial smooth muscle cells (MASMC)

Abstract

Urocortin (UCN) II, a newly isolated corticotropin-releasing-factor (CRF) related peptide, has been found to have potent cardiovascular protective effects. To investigate the mechanisms of its vascular protective effects, we exposed mesenteric arterial smooth muscle cells (MASMC) from spontaneously hypertensive rats (SHR) to UCN II to observe the change in cell apoptosis using TUNEL assay and measured intracellular calcium concentration ($[Ca^{2+}]_i$) using confocal laser scanning microscope. In addition, effects of UCN II on L-type calcium currents ($I_{Ca,L}$) were also measured using whole-cell patch clamp. Our results showed that UCN II concentration-dependently, but time-independently inhibited cell apoptosis. Astressin 2B, a special CRF 2 receptor antagonist, had no influence on this inhibition. Hypoxia or Bay K8644, the L-type calcium channel activator, induced the apoptosis of MASMC from SHR. Pretreatment of the cells with UCN II diminished the effects of hypoxia or Bay K8644. UCN II was also observed to reduce

$[Ca^{2+}]_i$ increase induced by KCl or Bay K8644. UCN II concentration-dependently inhibited $I_{Ca,L}$, which was not affected by astressin 2B. It did not affect the activation of $I_{Ca,L}$, but markedly shifted the inactivation curve to the left. In conclusion, UCN II inhibits the apoptosis of MASMC from SHR via inhibiting L-type calcium channels.

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Introduction

Four corticotropin-releasing-factor (CRF) related peptides have been found in mammals and are known as CRF, urocortin (UCN), stresscopin related peptide (SRP) or UCN II, and UCN III or also known as stresscopin (SCP) [1]. UCN II, the newly isolated member of CRF family, has been reported to widely distribute in central nervous system and peripheral tissues. It was originally cloned from rat and thereafter the human brain and has receptors also expressed in peripheral tissues, including the heart and vasculature [2]. Previous reports showed that UCN II had a number of cardiovascular physiological properties, such as causing a dose-dependent increase in heart rate, cardiac output and reducing systemic arterial pressure [3], etc.

In cardiac system, it was demonstrated that UCN II could protect neonatal rat cardiac myocytes *in vitro* when administered before hypoxia or at the point of reoxygenation and protect the adult rat heart *ex vivo* where UCN II reduced the infarct size of a perfused intact rat heart exposed to regional ischemia [4]. UCN and UCN II were found to have an inhibitory effect on the apoptosis in cardiomyocytes during hypoxia/reoxygenation [5, 6]. Since hypoxia or ischemia damage is highly associated with Ca^{2+} -overload, these cardiovascular effects of UCN II suggest that L-type calcium channels may play some roles. Our previous results showed that UCN exerted an inhibitory effect on the L-type calcium currents directly instead of via binding firstly to its CRF receptors (CRFR) in adult rat ventricular myocytes [7].

In vascular system, UCN was reported to have hypotensive and other vasoactive effects [3, 8] in spontaneously hypertensive rats (SHR). UCN II was observed to induce both endothelium-dependent and -independent vascular relaxation [9], highly implying that UCN II may influence on the vascular smooth muscle cells (VSMC) directly. As we know, VSMC play a key role in vascular pathology, which is often caused by apoptosis, proliferation, hypertrophy, and migration [10] of VSMC. In addition, it has been well established that vascular function and VSMC apoptosis are highly associated with intracellular calcium [11, 12], which mainly influxes via L-type calcium channels in VSMC. In the present study, our hypothesis is that UCN II may inhibit VSMC apoptosis via L-type calcium channels in SHR.

In the present study, we aim to investigate the effects of UCN II on the apoptosis in mesenteric arterial smooth muscle cells (MASMC) from SHR and its mechanisms for further understanding the role of UCN II in cardiovascular protection.

Materials and Methods

Animal care and handling conformed to the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health, and the study was approved by the local institutional ethical committee.

Drugs and reagents

Urocortin II, Bay K8644, astressin 2B, pancreatin, and nifedipine were obtained from Sigma. Fetal bovine blood serum was purchased from HYCLONE and DMEM was purchased from GIBCO. The culture medium contains: DMEM 13.4 g/L,

NaHCO_3 3.7 g/L, 10% Fetus bovine blood serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin; pH was adjusted with HCl or NaOH to about 7.3. The external solution for L-type calcium currents ($I_{\text{Ca,L}}$) recording was composed of (in mM): Tetraethylammonium (TEA) 120, HEPES 10, MgCl_2 1.0, CsCl 10, glucose 10, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.0, pH adjusted to 7.4 with CsOH. The electrode internal solution for $I_{\text{Ca,L}}$ recording was composed of (in mM): CsCl 20, Cs-Methane-Sulfonate 90, EGTA 10, HEPES 10, CaCl_2 3, Mg-ATP 4, Tris-GTP 0.4, pH adjusted to 7.2 with CsOH.

Isolation of mesenteric arteries smooth muscle cells (MASMC) from SHR

The MASMC were dissociated from the male 16-week-old spontaneously hypertensive rats (Shanghai SLAC Laboratory Animal Co. Ltd., China) by a modified method described previously [13]. After the SHR rats were euthanized by intraperitoneal injection with sodium pentobarbital (50 mg/kg), the secondary branches (150-200 μm in diameter) of mesenteric artery were carefully dissected under a microscope. The arteries were cleaned of connective tissue and stored at 4 °C in oxygenated Krebs solution. The arteries were minced and transferred to a digestion tube containing collagenase (1 mg/ml) and elastase (0.25 mg/ml). The tissues were incubated with the enzymes in a 37 °C bath for 45 min and carefully triturated to liberate free smooth muscle cells. The resulting smooth muscle cell suspension was stored at 4 °C and used within 8 h. Individual cells were allowed to adhere to the glass bottom of a 1-ml recording chamber.

TUNEL assay

To detect DNA strand breaks, TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) was performed by using the apoptotic detection kit (Promega, Madison, WI, USA) as described previously [14]. MASMC cultured on coverslips were fixed in 4% paraformaldehyde at 4 °C for 1 h. After permeabilization in 0.1% Triton X-100 (Sigma) for 20 min and several rinses in PBS, the cells were incubated with the TUNEL reaction mixture for 1 h at 37 °C in the dark. The mesenteric arteries muscle cells were rinsed in PBS and then mounted on a slide and viewed under a fluorescent microscope (Olympus, Tokyo, Japan). MASMC were treated with Bay K8644 (1 μM), astressin 2B (1 μM), verapamil (2.5 μM), hypoxia for 24 h, or different concentrations of UCN II, respectively.

Measurement of nitrite oxide (NO)

NO production was determined by measuring the nitrite content of the culture medium. Nitrite content was evaluated as previously described. Briefly, the nitrite level was quantified colorimetrically after addition of 100 μl Griess reagent (1 part 0.11 % naphthylethylenediamine dihydrochloride to 1 part 1 % sulfanilamide in 5 % phosphoric acid) to 100 μl of sample. The absorbance was measured at 540 nm. The nitrite concentration was determined by interpolation of a calibration curve of standard sodium nitrite concentration against absorbance. Nitrite levels were corrected by protein measurement of the mesenteric arteries muscle cell, and data were shown as nmol/mg protein.

Fig. 1. Effects of UCN II on the apoptosis of mesenteric arterial muscle cells in SHR. (A) Concentration-dependent effects of UCN II on cell apoptosis (n=6). (B) Time-course of UCN II on the cell apoptosis (n=6). Values were represented as mean $\pm$ S.E.M. *P<0.05 vs. control; #P<0.01 vs. control.

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measurements

$[\text{Ca}^{2+}]_i$ was measured with the ratiometric fluorescent dye Fluo-3/AM utilizing an LSM 510 invert confocal laser scanning microscope (Carl Zeiss Jena, Germany) with an argonkrypton laser (488 nm) excitation source. MASMCM were seeded on a sterile glass coverslip at an appropriate density to allow imaging of 10 to 20 single cells. These attached cells were loaded with Fluo-3/AM (2 mM) for 40 min in a dark place at room temperature. The dye-loaded cells were gently washed three times with a medium containing (in mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10 glucose, 1 CaCl_2 , 0.5 NaH_2PO_4 , and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). The cells were kept in medium for a further 20 min to allow the hydrolysis of Fluo-3/AM into Ca^{2+} -sensitive free acid form (Fluo-3) by cell esterases. The coverslip with attached cells was then transferred to a 1-ml thermoregulated chamber (22°C) on the stage of a ZEISS LSM 510 inverted microscope. The excitation wavelength was selected by a dichroic mirror (FT510). The fluorescence intensities were detected at wavelengths greater than 515 nm using an additional cut-off filter (LP515) in front of the detector. Fluorescence images were scanned and stored as a time series. Cells were pre-exposed to UCN II for 48 h. Bay K8644 or KCl was applied to the cells 1 minute after scanning began. Regions of interest (ROI's) were subsequently selected for determination of the fluorescence intensities in the cytosol and the nucleus. These data were stored as ASCII files and computed off-line. Different solutions were used as the extracellular medium (in mM): NaCl 140, KCl 4.6, CaCl_2 2, glucose 10; These media and active compounds offered various possibilities of investigating changes in the intracellular Ca^{2+} signal.

Transient expression of human Cav1.2 in HEK293 Cells

In a 35-mm Petri dish, 1.65 μg of $\alpha_1\text{C77-WT}$ (Subcloned in pcDNA3 expression vector) together with 1.25 μg of rat β_{2a} , 1.25 μg of α_{28} , and 0.25 μg of T-antigen were co-transfected into CRFR2-deficient HEK293 cells [15] using the standard

calcium phosphate transfection method [16]. The parental full-length human $\text{Ca}_v1.2$ α_1 -subunit ($\alpha_1\text{C77-WT}$) in pBluescript vector was kindly provided by Dr. Roger Zuhlke (Swiss Agency for Therapeutic Products, Switzerland). The β_{2a} and α_{28} clones were provided by Dr. Terry Snutch (University of British Columbia). I_{Ca} was recorded at room temperature using the whole-cell patch clamp technique 48-72 h after transfection.

Whole-cell patch clamp recording

Cells (MASMC or transfected HEK293 cells) were placed in a 0.5 ml chamber mounted on an inverted microscope (IX70, Olympus), and superfused at room temperature (19-23°C). Complete replacement of external solution 2ml/min in the chamber was achieved within 2-3 minutes. The currents were recorded by a patch clamp amplifier (Axon 200B, Inc.), Digidata 1322A, and p-Clamp software 8.2 (Axon Instrument, Foster City, CA, USA). Borosilicate glass electrodes were pulled using a level puller (Sutter Instruments, Model P-97) and had a resistance of 2 to 4 M Ω when filled with the electrode internal solution. Cell capacitance was measured by integrating the area of the capacitive transient. Data acquisition, storage and analysis running on a personal computer were accomplished with p-Clamp 8.2 software (Axon Instrument).

Voltage-activated $\text{I}_{\text{Ca,L}}$ were evoked by a 200 ms-long depolarizing step pulse from the holding potential of -40 mV to 0 mV at the frequency of 1 Hz. Contaminating K^+ currents were eliminated by K^+ substitution both in the pipette and the superfusion solutions, and Na^+ currents were eliminated by substituting extracellular NaCl with tetraethylammonium chloride. Current-voltage (I-V) relationship was obtained by applying pulses for 200 ms from -40 mV to 80 mV in 10 mV increments. The current density of $\text{I}_{\text{Ca,L}}$ was calculated by normalizing the peak amplitude of current with cell capacitance (pA/pF). For estimating the steady-state activation, $\text{I}_{\text{Ca,L}}$ conductances were plotted against test potentials. The derived activation curve was fitted to data with a Boltzmann equation: $G/G_{\text{max}} = \{1 + \text{EXP}[(V_{0.5} - V)/k]\}^{-1}$, where G/G_{max} is the relative conductance normalized by the maximal conductance, $V_{0.5}$ is

the potential required for half-activation of the current, and k is the Boltzmann coefficient. The steady-state inactivation of $I_{Ca,L}$ was examined using a double-pulse protocol. Steady-state inactivation curves were obtained by applying test pulses for 200 ms from -40 mV to 0 mV, 5 ms after 2-s conditioning pulses ranging from -60 mV to 40 mV and were fitted with the Boltzmann equation: $I/I_{max} = 1/\{1 + \exp[(V - V_{0.5})/\kappa]\}$, where $V_{0.5}$ and κ are the half-maximum inactivation potential and the slope factor, respectively.

Data analysis

Data were expressed as means $\pm$ S.E.M. Data were compared statistically by an analysis of variance (ANOVA) with repeated measures completed by the corrected Dunnett's t-tests. The threshold of significance retained was $P < 0.05$ or $P < 0.01$.

Results

Inhibitory effects of UCN II on the apoptosis of MASM from SHR

As shown in the Fig.1A, UCN II significantly inhibited the apoptosis of MASM from SHR. UCN II at 0.001 μ M, and 0.01 μ M, did not significantly affect the cell apoptosis. When the concentration was raised to 0.1 μ M, UCN II markedly inhibited the cell apoptosis about $19.4 \pm 1.3\%$ ($n=6$, $P < 0.05$). Treatment of cells with UCN II at 0.1 μ M for 1 h, 6 h, 12 h, 24 h and 48 h, respectively, resulted in the same inhibition of the apoptosis (Fig.1B). These results suggest that the effects of UCN II on the apoptosis show no time dependence within 48 h.

Effect of astressin 2B, Bay K8644, verapamil, or hypoxia on UCN II's inhibition

When MASM were pre-exposed to astressin 2B (1 μ M), the CRFR2 antagonist, UCN II at 0.1 μ M inhibited the apoptosis about 19.1% ($n=6$, $P < 0.05$) by 48 h, which showed no difference from the inhibition in the absence of astressin 2B (Fig.2A). Bay K8644 (1 μ M), a special L-type calcium channel activator, increased the cell apoptosis including vascular smooth muscle cells (VSMC), which was consistent with previous reports [17]. Pretreatment of the cells with UCN II could significantly reduce the effect of Bay K8644 ($n=6$, $P < 0.05$). Verapamil (2.5 μ M), a L-type calcium channel blocker, reduced the apoptosis of VSMC, as it was previously documented [18]. However, in the presence of UCN II at 0.1 μ M, verapamil did not further reduce the apoptosis of the cells (Fig.2B). Pre-treatment the cells with hypoxia for 24 h could increase the cell apoptosis of the MASM from SHR. UCN II (0.1 μ M to 10 μ M) significantly diminished

Fig. 2. (A) Effects of astressin 2B (1 μ M) on the cell apoptosis of mesenteric arterial muscle cells in SHR. * $P < 0.05$ vs. control, # $P > 0.05$ vs. UCN II, $n=6$. (B) Effects of Bay K8644 (1 μ M), verapamil (2.5 μ M) on UCN II's inhibition on the cell apoptosis. # $P < 0.05$ vs. control, * $P < 0.05$ vs. Bay K8644 (1 μ M), $n=6$. (C) Effects of UCN II on the cell apoptosis during hypoxia. # $P < 0.05$ vs. control, * $P < 0.05$ vs. Hypoxia, $n=6$.

Fig. 3. Effects of UCN II (0.01 μ M to 1 μ M) on nitrite oxide synthesis in time course (n=6). Values were represented as mean $\pm$ S.E.M. There was no significant difference in nitrite accumulation between UCN II groups with the control or among the three different concentrations of UCN II.

Fig. 4. Effects of UCN II (0.1 μ M) on the intracellular Ca^{2+} levels in mesenteric arterial muscle cells of SHR. (A) In the presence of Bay K8644 (1 μ M) or nifedipine (1 μ M) (* P <0.01 vs control; # P <0.05 vs. Bay K8644 1 μ M). (B) In the presence of KCl (40 mM) (* P <0.01 vs. control, # P <0.05 vs. KCl 40 mM) (n=6).

the hypoxia-induced cell apoptosis (Fig.2C).

Effects of UCN II on nitrite oxide synthesis

To interpret the mechanisms of UCN II's inhibitory effects on the apoptosis, we measured nitrite accumulation under UCN II at different concentrations in the culture medium. As shown in Fig.3, UCN II (0.01 μ M, 0.1 μ M, or 1 μ M) had no significant influence on nitrite oxide accumulation compared to the control within 48 h.

Inhibition of UCN II on intracellular Ca^{2+} of MASMC from SHR

Bay K8644 (1 μ M), the L-type calcium channel activator, can significantly increase the intracellular Ca^{2+} levels. When the cells were pre-exposed to nifedipine, the special L-type voltage-gated calcium blockers, this effect of Bay K8644 was remarkably diminished, which confirmed that the Ca^{2+} influx took place through L-type voltage-activated calcium channels (Fig.4A, upper panel).

Fig. 5. Effects of UCN II on $I_{Ca,L}$ in mesenteric arterial muscle cells of SHR. (A) Original records of the currents evoked by a series of depolarizing pulses from a holding potential of -40 mV to test potentials between -40 and +60 mV, in 10-mV increments, and effects of Bay K8644 (1 μ M) or nifedipine (1 μ M) on the currents (from the holding potential of -40 mV to 0 mV). (B) Concentration-dependent effects of UCN II on $I_{Ca,L}$ (* P <0.05 vs. control, ** P <0.01 vs. control, n =6). (C) Current traces of $I_{Ca,L}$ obtained in the absence or presence of UCN II 0.1 μ M, and washout. (D) Effect of UCN II 0.1 μ M and UCN II 0.1 μ M + astressin 2B 1 μ M on $I_{Ca,L}$ (* P <0.05 vs. control, n =6). $I_{Ca,L}$ were evoked by a 200 ms-long depolarizing step pulse from the holding potential of -40 mV to 0 mV. (E) Time-course of UCN II effect on $I_{Ca,L}$. The control current was marked with ($\diamond$), and 0.1 μ M with ($\blacktriangle$), 1 μ M with ($\blacklozenge$), and 10 μ M with ($\blacksquare$), the currents show time-dependence (n =6).

The agonistic effect of BayK8644 was reduced increasingly by higher concentrations of UCN II from 0.1 μ M to 10 μ M (Fig.4A, lower panel). UCN II could also attenuate the increase in Ca^{2+} concentration induced by KCl (40 mM) (Fig.4B). The inhibitory effects of UCN II on intracellular Ca^{2+} increase induced by KCl or Bay K8644 were clearly shown to be statistically significant.

Effects of UCN II on $I_{Ca,L}$ in MASMC from SHR

A series of evoked current traces were recorded when the single cell was given designed depolarizing test pulses. The current was increased by 1 μ M Bay K8644 (n =5), but completely blocked by 1 μ M nifedipine (n =4), the L-type calcium channel blocker, indicating the currents' L-type characteristics (Fig.5A).

Fig. 6. Effects of UCN II on electrophysiological properties of $I_{Ca,L}$. (A) Effects of UCN II at different concentrations on current-voltage (I-V) relation. Control is marked with (◆), UCN II at 0.1 μ M with (■), and UCN II at 1 μ M with (▲). (B) Effects of UCN II on activation and inactivation kinetics of $I_{Ca,L}$ in VSMC. (a) Inactivation kinetics of $I_{Ca,L}$. Control is marked with (■) and UCN II at 0.1 μ M with (◆). (b) Activation kinetics of $I_{Ca,L}$. Control is marked with (▲) and UCN II at 0.1 μ M with (×).

Exposure of cells to UCN II at higher than 0.1 μ M, the current was significantly reduced with a maximum inhibitory effect at 10 μ M. UCN II at 10 μ M reduced the peak current density of $I_{Ca,L}$ from 6.6 ± 0.7 pA/pF to 2.7 ± 0.5 pA/pF ($n=6$, $P<0.01$). UCN II at 0.01, 0.1, 0.5, 1, 5, 10 μ M markedly inhibited $I_{Ca,L}$ by $5.8 \pm 0.5\%$ ($n=6$), $21.5 \pm 1.4\%$ ($n=6$, $P<0.05$), $26.2 \pm 4.5\%$ ($n=6$, $P<0.05$), $40.5 \pm 7.1\%$ ($n=6$, $P<0.01$), $56.3 \pm 6.3\%$ ($n=6$, $P<0.05$), and $61.1 \pm 5.2\%$ ($n=6$, $P<0.01$), respectively (Fig.5B). After washout, the peak of $I_{Ca,L}$ partially returned (Fig.5C). In the presence of the CRFR2 antagonist, astressin 2B (1 μ M), UCN II (0.1 μ M) inhibited the calcium currents at 5th min by about 23.5%, from 6.4 ± 0.3 pA/pF to 5.1 ± 0.6 pA/pF ($n=6$, $P<0.05$) (Fig.5D), which showed no difference from the inhibition in the absence of astressin 2B.

Fig.5E shows the time course of UCN II effects on $I_{Ca,L}$ in MASM. When the cells were held at -40 mV, and given a 200 ms, 1 Hz depolarizing pulse, $I_{Ca,L}$ reached its maximum. The current rundown at the 5th min was below 10% ($n=6$). After exposure of the cells to UCN II at 0.1 μ M, 1 μ M, and 10 μ M for 5 min, the currents were reduced by about 21%, 41%, and 61%, respectively at the same time point.

Effects of UCN II on electrophysiological properties of $I_{Ca,L}$

As demonstrated in Fig.6A, it showed that UCN II significantly up-shifted the I-V curve concentration-dependently. The current density at 20 mV was declined

from 6.8 ± 0.9 pA/pF to 5.3 ± 0.8 pA/pF ($n=6$, $P<0.05$) and 3.9 ± 0.7 pA/pF ($n=6$, $P<0.05$) in the presence of UCN II at 0.1 and 1 μ M, respectively.

UCN II (0.1 μ M) did not influence the activation curve much (half activation potential $V_{0.5}$ from about -13.8 $\pm$ 0.6 to -14.6 $\pm$ 1.3 mV, and the slope factor (κ) from about 5.1 $\pm$ 0.5 to 4.9 $\pm$ 1.8 mV) ($n=6$). However, after exposure to UCN II (0.1 μ M), the $V_{0.5}$ of the inactivation curve was shifted to the left from -19.1 $\pm$ 3.2 to -29.5 $\pm$ 3.7 mV, and τ value from 6.9 $\pm$ 0.7 to 12.2 $\pm$ 1.3 mV ($n=6$, $P<0.05$) (Fig.6B).

Effects of UCN II on $Ca_v1.2$ calcium channel currents in HEK293 cells

HEK293 cells transfected with α_1C77 -WT were used to investigate the effects of UCN II on $Ca_v1.2$ calcium channels. As shown in Fig.7A, $Ca_v1.2$ calcium channel current traces were obtained in the absence or presence of UCN II. UCN II at 1 μ M markedly inhibited $Ca_v1.2$ calcium channel currents at 5th min by about 43.2%, from 7.9 ± 0.7 pA/pF to 4.6 ± 0.9 pA/pF ($P<0.05$, $n=6$) (Fig.7B).

Discussion

As mentioned above, UCN II possesses a wide spectrum of physiological and pharmacological actions in an autocrine or paracrine way [19, 20]. The effects of

Fig. 7. Effects of UCN II on $\text{Ca}_v1.2$ calcium currents in $\alpha_1\text{C77-WT}$ transfected HEK293 cells. (A) Current traces of $\alpha_1\text{C77-WT}$ calcium channel currents obtained in the absence or presence of UCN II 1 μM . (B) Inhibition of $\text{Ca}_v1.2$ ($\alpha_1\text{C77-WT}$) calcium channel currents by UCN II (1 μM) (* $P < 0.05$ vs. control, $n = 6$).

UCN II on cardiovascular and other systems, such as brain, immune system, digestive system [21], have been extensively investigated. UCN II could induce both endothelium-dependent and -independent vasorelaxation [9], implying an association of the UCN II with vascular smooth muscle cells (VSMC), and hence vasculopathy.

It is well established that apoptosis of VSMC [22, 23] is a key factor in the formation of vasculopathy [24]. We observed that UCN II concentration-dependently inhibited the apoptosis of MASMC from SHR by TUNEL assay. Our results hence suggest that UCN II is an endogenous beneficial vasoactive protective agent.

As well known, nitric oxide is the important agent in vascular relaxation and cell apoptosis. In this investigation, UCN II had no obvious effects on nitric oxide releasing. Pretreatment of the cells with CRFR2 blocker, astressin 2B, did not affect the inhibitory effect of UCN II on the apoptosis of MASMC from SHR as well. Hypoxia, the pathological condition associated with intracellular Ca^{2+} overload in cardiovascular disease such as hypertension, or Bay K8644, the L-type calcium channel activator, can increase the apoptosis of VSMC from SHR [13]. Pre-exposure of the cells to UCN II significantly diminished the effects of Bay K8644 or hypoxia. Furthermore, we found that UCN II could attenuate the increase in the intracellular Ca^{2+} fluorescence intensity induced by Bay K8644 or KCl. As we know, Ca^{2+} plays a very important role in the effect of cell apoptosis [25]. Therefore, these

results suggest that UCN II may exert the inhibitory effects on apoptosis of MASMC from SHR via L-type calcium channels directly instead of via binding firstly to its CRFR2 or nitrite oxide pathway.

Our results also showed that UCN II could directly inhibit $I_{\text{Ca,L}}$ in MASMC from SHR in a concentration-dependent manner. Under the present experimental conditions, Ca^{2+} was the only charge carrier for inward current [26], which was also confirmed by the result that $I_{\text{Ca,L}}$ was completely blocked by 1 μM nifedipine, a specific L-type calcium channel blocker, and activated by Bay K 8644 significantly, indicating that it was not contaminated by K^+ and Na^+ currents [27]. Rundown of ionic currents is always a concern in whole-cell patch clamp recording. We minimized time-dependent changes in $I_{\text{Ca,L}}$ by using high resistance pipettes filled with Mg-ATP 4 mM and beginning the experiments within 5 min after membrane rupture [28]. Furthermore, after washout, the currents partially recovered, indicating that the effect of UCN II on $I_{\text{Ca,L}}$ was not the consequence of the rundown of $I_{\text{Ca,L}}$. UCN II concentration-dependently inhibited $I_{\text{Ca,L}}$, up-shifted the I-V curve, and shifted the inactivation curve to the left. Pretreatment of the cells with CRFR2 blocker, astressin 2B, did not influence the inhibitory effects of UCN II on the currents, suggesting that UCN II may exert the inhibitory effect directly on the L-type calcium channel instead of via binding firstly to its CRFR2. This result was supported by the fact that, in $\alpha_1\text{C77-WT}$

transfected CRFR2-deficient HEK293 cells, UCN II could also inhibit the $\text{Ca}_v1.2$ calcium channel currents, the main calcium channel type in VSMC.

The data are in line with our *in vivo* investigation, showing that UCN, the homologous peptide of UCN II in CRF family, similar to the L-type calcium channel blocker, verapamil, significantly diminished the infarction size of adult rat hearts [29]. As reported, vascular relaxation, remodeling and VSMC apoptosis are highly associated with intracellular Ca^{2+} [30], and in VSMC, L-type calcium channels are the main resource of intracellular Ca^{2+} . Therefore, this $\text{I}_{\text{Ca,L}}$ and intracellular Ca^{2+} decreasing effect of UCN II may contribute greatly to its VSMC apoptosis inhibition. However, our results were inconsistent with some previous reports. It was reported that UCN II exerted its anti-inflammation effect by inducing macrophage apoptosis via CRFR2 [31]. Previous reports also showed that UCN II exerted its protective effects via MAPK signaling pathway [6, 24], cAMP/PKA pathway [32], or PKC α activation [33] in cardiac

myocytes. The interpretation for the differences remains to be explored, which might be attributed to the variety of UCN II's action mechanisms or the different cell species/ages used for the experiments.

In conclusion, our results provide convincing evidence of a possible link between the apoptosis-inhibitory effects of UCN II and L-type calcium channels in SHR. Our data demonstrated that UCN II inhibits $\text{I}_{\text{Ca,L}}$, intracellular Ca^{2+} and thus MASMC apoptosis. Based on the theory that VSMC apoptosis plays a key role in hypertensive vasculopathy, these findings highly imply that UCN II might be a beneficial agent in vascular pathology where Ca^{2+} -overload plays an important role.

Acknowledgements

This work was supported by grants from Natural Science Foundation of China (No. 30371646) and Key Subject Fund of China Ministry of Education (No. 03047).

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