Effects of rat urotensin II on coronary flow and myocardial eNOS protein expression in isolated rat heart[1]

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KEY WORDS urotensin II; nitric oxide synthase; nitric oxide; heart function tests; coronary circulation; rats

ABSTRACT

AIM: To examine the effects of urotensin II, a recently discovered endogenous peptide, on coronary flow (CF), cardiac function, and endothelial nitric oxide synthase (eNOS) expression in isolated rat hearts. **METHODS:** Heart was isolated and perfused retrogradely via the aorta in Langendorff mode. Rat urotensin II was administered in the perfusion solution. The eNOS content in myocardium was determined by Western blot. **RESULTS:** Rat urotensin II had no effect on the heart rate, left ventricular systolic pressure, left ventricular end-diastolic pressure, or $\pm dp/dt_{max}$. While rat urotensin II dose-dependently increased CF. CF was increased by 11.43 %, 6.67 %, 6.62 %, 6.56 %, 6.36 %, and 5.86 % respectively in a time-dependent manner at 5, 10, 15, 20, 25, and 30 min after injection of rat urotensin II 6.66×10^{-2} μg. The maximal effect on CF was found at 5 min following urotensin II administration. N^G-nitro-*L*-arginine methyl ester (*L*-NAME) did not prevent the increased CF in response to urotensin II. Rat urotensin II dose-dependently increased the cardiac eNOS protein expression and this effect was not inhibited by *L*-NAME. **CONCLUSION:** Rat urotensin II did not alter cardiac function but increased CF and the amount of myocardial eNOS protein in the isolated rat heart. The increased CF was independent of the involvement of eNOS.

INTRODUCTION

Urotensin II is a cyclic dodecapeptide, originally isolated from the urophysis of the teleost fish, and recently cloned in several mammalian species, including humans[1-5]. The orphan receptor GPR14, a G-protein-coupled receptor first cloned from rat, has been defined as the specific receptor for urotensin II[6-11]. Positive immunoreactive staining for human urotensin II and GPR14 mRNA were detected in both atrial and ventricular tissues[8-9,12]. These studies indicate that urotensin II might act as one of endogenous modulators of cardiac function.

Systemic administration of human urotensin II to monkeys elicited a fall in cardiac output, severe myocardial contractility depression, and fatal circulatory collapse[8]. A recent study has revealed a direct effect of human urotensin II on human cardiac myocytes, and demonstrated that urotensin II was the most potent positive inotropic agent[13]. However, little is known about the effects of rat urotensin II on cardiac function and coronary flow (CF).

It is noteworthy that nitric oxide synthase (NOS) inhibitor, N^G-nitro-*L*-arginine methyl ester (*L*-NAME), attenuated the *in vivo* systemic depressor actions of

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human urotensin II in the rat and augmented the vascular contractile actions of human urotensin II in rat isolated pulmonary arteries[14-16]. The NOS inhibitor increased the maximum constriction of the coronary artery and inhibited the later vasodilatation response to human urotensin II in the perfused rat hearts[17,18]. These results suggested that urotensin II might modulate vascular tone through releasing factors such as nitric oxide (NO). NO has been demonstrated to be an important determinant of cardiac function[19]. The endothelial NOS (eNOS) expression has been documented in cardiac sinoatrial, atrioventricular, and ventricular myocytes from several rodent species[19-22]. Therefore, the present work was designed to investigate the possible effects of rat urotensin II on cardiac function, CF, and myocardial eNOS in the isolated perfused rat heart.

MATERIALS AND METHODS

Materials Male Sprague-Dawley (SD) rats (weighing 280-340 g) were purchased from the Sino-British SIPPR/BK Lab Animal Ltd and allowed at least one week to acclimation. Rats were housed with controlled temperature (21±2 ºC) and lighting (8:00-20:00) and with free access to standard rat chow and water. All procedures were in accordance with institutional animal care guidelines.

L-NAME, rabbit polyclonal eNOS antibody, and horseradish-labeled goat anti-rabbit immunoglobulin secondary antibody were purchased from Sigma (St Louis, MO, USA). ECL was gained from Amersham. Rat urotensin II was obtained from the Peptide Institute, Inc (Osaka, Japan). All chemicals used were of analytical grade.

Perfusion of hearts *In vitro* heart perfusion was performed as described by Nelissen-Vrancken *et al*[23]. Briefly, under anesthesia with sodium pentobarbital (50 mg/kg, ip) heart was rapidly excised and rinsed in ice-cold Krebs-Henseleit buffer containing (in mmol/L): NaCl 118.0; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.0; glucose 11.0. After removal of the lung and fatty tissues, heart was connected to the aortic cannula of a Langendorff apparatus and was perfused with Krebs-Henseleit buffer bubbled with 95 % O_2 and 5 % CO_2 (37 ºC) at a constant pressure of 100 cm H_2O. The left ventricle was inserted with a water-ballon connected to a pressure transducer to measure intraventricular pressure. Three silver wire electrodes (0.1-mm in diameter) were placed in the right ventricle, right atrium, and left atrium to record electrocardiogram. The coronary venous effluent was collected from the pulmonary artery for determining the coronary flow every five minutes.

Western blot analysis Tissues were homogenized in 5 volumes of homogenization buffer [Tris-HCl 500 mmol/L (pH 7.5), NaCl 150 mmol/L, 1 % Triton-X 100, 1 % sodium deoxycholate, 0.1 % SDS]. After sonication and centrifugation at 4 ºC (10 min, 10 000×*g*), the supernatant was used for determination of protein concentrations, and the equal amounts of total solubilized proteins were eluted by heating with SDS-PAGE sample buffer and separated by 6 % SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulode membrane. The membrane was blocked at room temperature with 5 % non-fat dry milk in Tris-buffered saline with 0.1 % (v/v) Tween-20 (TBST) and incubated with rabbit polyclonal eNOS antibody (1:2500 dilution) for 2 h. After washing, the membrane was incubated for 1 h with horseradish-labeled goat anti-rabbit immunoglobulin secondary antibody (1:1000 dilution) for 1 h. After five times of additional washes in TBST, the membrane was incubated with an enhanced chemiluminescence detecting reagents according to the manufacturer's protocols and exposed to X-ray film. The amount of eNOS protein was quantified by densitometric analysis of the autoradiograms using a scanning laser densitometer.

Experimental protocol Hearts were randomly divided into the following groups: control, rat urotensin II 6.66×10^{-5}, 6.66×10^{-4}, 6.66×10^{-3}, 6.66×10^{-2}, and 0.67 μg groups. Hearts were perfused for 20 min prior to drug administration. Rat urotensin II were injected as a bolus within 1 min into the rubber tube connected to the aortic cannula. The volume of a single bolus injection was less than 0.3 mL. The coronary flow, electrocardiogram, and intraventricular pressure were monitored during perfusion period. At the end of the experiment, cardiac tissues were homogenized for Western blotting study. Two additional groups were used for the eNOS determination: treatment with *L*-NAME (30.6 μg) and co-treatment with *L*-NAME (30.6 μg) and rat urotensin II (6.66×10^{-2} μg).

Statistical analysis All data were expressed as the mean±SD. Comparisons among the groups were made by an ANOVA analysis and followed by unpaired *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Effects of rat urotensin II on cardiac functions There was no significant difference in heart rate between control and urotensin II-treated groups. Left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), +dp/dt_{max}, and -dp/dt_{max} were not modified by rat urotensin II (Tab 1).

Effects of rat urotensin II on coronary flow CF reached its maximum at 5 min after injection of rat urotensin II and then decreased gradually, but it was still higher than the control value. CF was increased by 11.43 %, 6.67 %, 6.62 %, 6.56 %, 6.36 %, and 5.86 % respectively in a time-dependent manner at 5, 10, 15, 20, 25, and 30 min after injection of rat urotensin II 6.66×10^{-2} μg (Fig 1A). CF was increased by 7.8 %, 8.2 %, 9.8 % ($P<0.05$), 11.4 % ($P<0.05$), and 13.7 % ($P<0.05$) at 5 min after pre-treatment with urotensin II 6.66×10^{-5}, 6.66×10^{-4}, 6.66×10^{-3}, 6.66×10^{-2}, and 0.67 μg, respectively as compared with the control group (Fig 1B).

Effects of *L*-NAME on rat urotensin II-induced cornary flow increase There was a significant decrease in CF following *L*-NAME perfusion. However, treatment with *L*-NAME did not prevent urotensin II-induced increase in CF (6.66×10^{-2} μg) (Fig 2).

Effects of rat urotensin II on eNOS content The content of eNOS was determined by Western blot in the cytosolic fraction prepared from ventricular tissue. Treatment with rat urotensin II (6.66×10^{-3}-0.67 μg) significantly increased the content of eNOS (Fig 3A, B). The maximal increase in eNOS content was found at 15 min after treatment with rat urotensin II (6.66×10^{-2} μg) but declined in the following 30 min (Fig 3C, D). Treatment with *L*-NAME inhibited the amount of eNOS by about 50 %. Treatment with *L*-NAME did not prevent the increase in eNOS induced by 6.66×10^{-2} μg urotensin II (Fig 4).

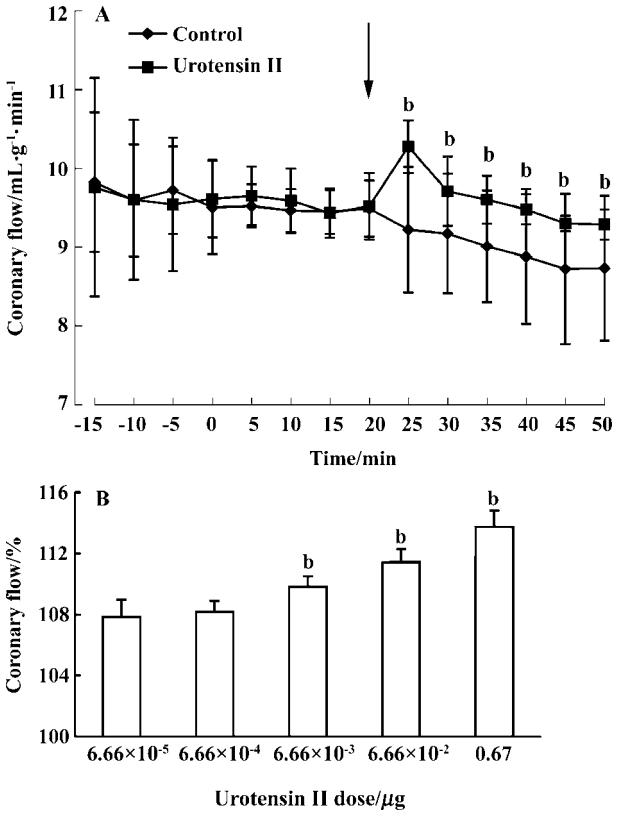

Fig 1. Effect of rat urotensin II on coronary flow in isolated perfused rat hearts. (A) Line chart of different time dots of coronary flow between urotensin II (6.66×10^{-2} μg) and control groups during perfusion period. Arrow indicates urotensin II injection. (B) Dose-dependent effects of urotensin II on coronary flow at 5 min after urotensin II injection. n=9. Mean±SD. $^bP<0.05$ *vs* control.

DISCUSSION

Tab 1. Heart rate and heart function at 5 min after pre-treatment of rat with urotensin II. n=9. Mean±SD.

Dose (μg)	HR (beats/min)	LVSP (mmHg)	LVEDP (mmHg)	+dp/dt_{max} (mmHg/s)	-dp/dt_{max} (mmHg/s)
Control	221±14	83±7	6.4±0.6	2640±150	1463±128
6.66×10^{-5}	228±12	84±5	5.18±0.23	2715±165	1365±90
6.66×10^{-4}	232±16	85±6	6.6±0.3	2760±135	1493±128
6.66×10^{-3}	235±15	85±3	7.8±0.4	2850±173	1560±120
6.66×10^{-2}	238±16	88±7	5.8±0.4	2858±195	1388±120
0.67	240±20	88±4	5.2±0.5	2880±150	1365±113

HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure.

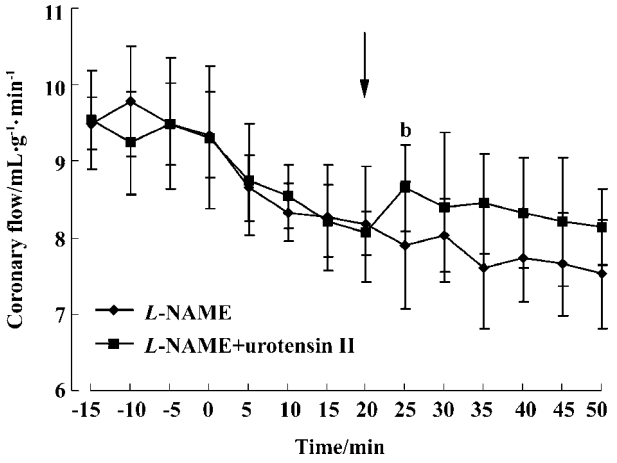

Fig 2. Effect of *L*-NAME (30.6 μg) on rat urotensin II (6.66×10^{-2} μg)-induced increase in coronary flow in isolated perfused rat heart. Arrow indicates urotensin II injection. *n*=9 in each group. Mean±SD. $^{b}P<0.05$ *vs* coronary flow at 20 min before urotensin II injection.

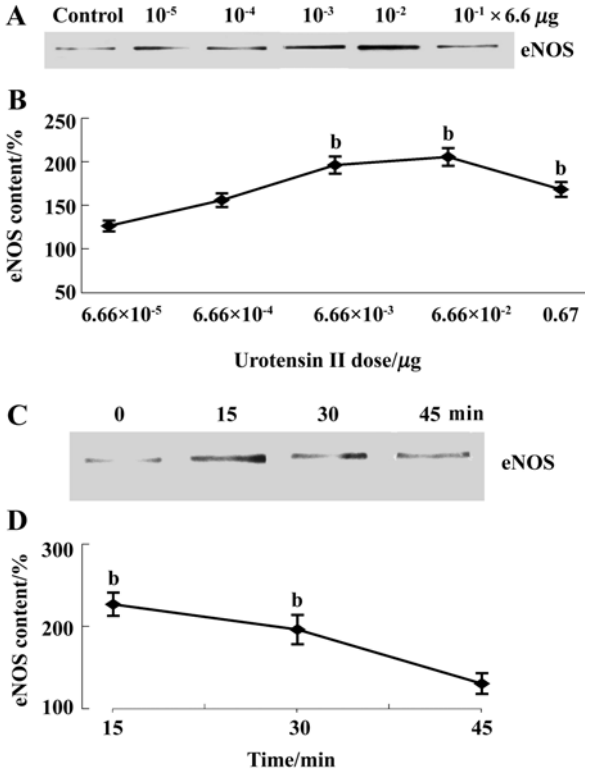

Fig 3. Effect of rat urotensin II on eNOS expression in myocardium. (A, B) Increase in eNOS protein expression in rat hearts treated with urotensin II for 30 min. (C, D) Time course (15, 30, and 45 min) of urotensin II-stimulated eNOS expression. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* control.

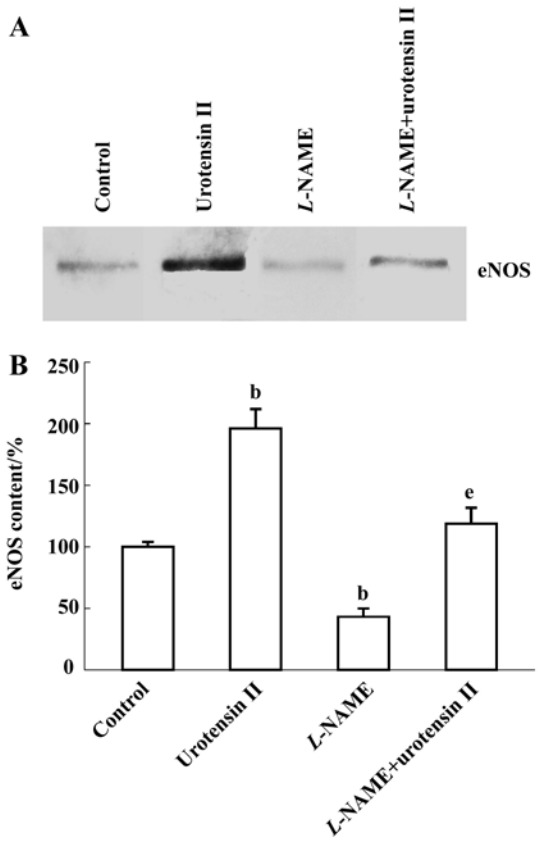

Fig 4. Effect of rat urotensin II (6.66×10^{-2} μg) on eNOS expression following treatment with *L*-NAME (30.6 μg) in isolated perfused rat heart. (A) eNOS expression was determined by immunoblotting. (B) Quantification of the protein content of eNOS by densitometry. $^{b}P<0.05$ compared with hearts left untreated. *n*=3. Mean±SD. $^{e}P<0.05$ compared with hearts treated with urotensin II alone.

Previous *in vivo* studies showed that systemic infusion of sub-lethal doses of human urotensin II into anesthetized cynomolgus monkeys caused an increase in vascular resistance, a moderate reduction in blood pressure, a fall in cardiac output and a severe depression in myocardial contractility[8]. Recently, it was shown that human urotensin II increased the contractile force in cardiomyocytes of human hearts as well as in endothelium-denuded human coronary arteries[13].

The present study showed that rat urotensin II increased the CF. This effect was similar to that of human urotensin II on the perfused rat heart[17]. *L*-NNA, an inhibitor of NOS, attenuated human urotensin II-induced increase of CF [17]. *L*-NAME increased the maximum coronary perfusion pressure and inhibited the later dilator response to human urotensin II, suggesting that human urotensin II modulated the coronary vascular tone by releasing NO[18]. However, our results did not indicate an involvement of NO since *L*-NAME did not affect the CF increase. Based the present results,

we can not explain the mechanisms underlying the urotensin II-induced CF increase. Katano *et al*[17] have shown that the cyclooxygenase inhibitor, diclofenac (10 mmol/L) significantly inhibited urotensin II-induced coronary vasodilation. Gray *et al*[18] have also reported that addition of cyclooxygenase inhibitor, indomethacin (1×10^{-5} mol/L) to the perfusion solution significantly increased the maximum constriction and inhibited the later dilator response to human urotensin II. These studies suggested that human urotensin II modulated coronary flow through factor such as cyclooxygenase products and nitric oxide to elicit coronary vasodilation. Further studies are therefore required to determine whether other endothelium-derived factors, such as PGI_2, endothelium-derived hyperpolarized factor or eicosanoid are involved in the vascular response to rat urotensin II.

Emerging evidence suggests a role of endogenous NO in the regulation of myocardial function. eNOS was the primary NOS isoform that was constitutively expressed not only in the vascular endothelial cells, but also in cardiomyocytes[19-22]. eNOS expression was detected in both atrial and ventricular myocytes from human hearts[24,25]. Our results showed that rat urotensin II upregulated eNOS protein expression in myocardium and this effect was not prevented by pretreatment with *L*-NAME in isolated rat hearts.

Recent studies suggest that NO in the cardiomyocyte may function as an important counterregulator of various biological processes such as regulation of Ca^{2+} homeostasics, inhibition of adrenoreceptor agonists and suppression of toxic effects of endothelin-1[26]. Upregulation of eNOS might balance the direct cardiac action of urotensin II. Rat urotensin II-induced the increase of eNOS activity and increased NO production may be important for maintaining normal myocardial contractility. Calcium-independent inducible NOS is also expressed in cardiomyocytes and is able to generate a large amount of NO. Further studies are needed to examine the effect of rat urotensin II on iNOS and the precise role of NO in rat urotensin II regulation of myocardial function.

In conclusion, the present work demonstrated that rat urotensin II did not alter the cardiac function but increased CF and myocardial eNOS protein levels in isolated rat heart. The increase in CF seems independent of activation of eNOS. There may exist differences in cardiovascular responses to rat urotensin II and human urotensin II.

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