Urotensin II induces hypertrophic responses in cultured cardiomyocytes from neonatal rats

Yunzeng Zou[a], Ryozo Nagai[a], Tsutomu Yamazaki[b,*]

[a] *Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b] *Department of Pharmacoepidemiology, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

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Abstract Urotensin II (UII), a cyclic neuropeptide, functions not only in the central nervous system but also in non-neural systems including cardiovascular systems. In the present study we examined whether UII regulates hypertrophy in cardiomyocytes. The exposure of cultured cardiomyocytes from neonatal rats to UII dose-dependently activated extracellular signal-regulated kinases (ERKs), important molecules in the development of cardiac hypertrophy. ERK activation by UII at 100 nM peaked at 8 min after stimulation. UII markedly induced expression of specific genes encoding atrial natriuretic peptide and brain natriuretic peptide, and significantly increased amino acid incorporation into proteins. Incubation of cardiomyocytes with UII increased cell size and myofibril organisation. UII, then, might participate in cardiomyocyte hypertrophy.

Key words: Urotensin II; Cardiomyocyte; Hypertrophy

1. Introduction

Cardiac hypertrophy is not only an adaptational state preceding cardiac failure but also an independent risk factor for cardiac morbidity and mortality [1]. Therefore, it is even more important to determine the molecular mechanism of the development of cardiac hypertrophy. Mechanical stretch is an initiating factor for cardiac hypertrophy in response to haemodynamic overload [2,3]. Additionally, a number of neurohumoral factors such as angiotensin II (AngII), endothelin 1 (ET-1), and catecholamines are reported to be associated with development of hypertrophy [4–8].

Urotensin II (UII), a cyclic neuropeptide originally isolated from the fish spinal cord [9], recently has been detected and found to be functionally implicated in mammalian central nervous and cardiovascular systems [10–12]. In the cardiovascular system, many reports indicated that UII might regulate arterial blood pressure as a potent vasoactive 'somatostatin-like' peptide [9–13]. Low concentrations of UII produced relaxations, while higher concentrations induced contraction of vascular smooth muscle [9]. UII also can increase or decrease myocardial contractility, depending on dose [11]. Moreover, UII administration to human subjects induced ST segment changes in the electrocardiogram observed in myocardial ischemia [11]. Actions of UII in mammals are proposed to be mediated through interactions with certain somatostatin receptor subtypes [9]. Recently an orphan human G protein-coupled receptor (GPR) homologous to rat GPR14 has been found to be expressed predominantly in cardiovascular tissues and to function as a receptor for UII [11]. Taken together, such evidence strongly suggests that UII may influence cardiovascular homeostasis as well as pathologic processes such as ischemic heart disease and heart failure. However, whether UII is involved in the regulation of cell enlargement has not been reported. We therefore examined the role of UII in the development of cardiomyocyte hypertrophy, and found that this peptide induces hypertrophic responses in cardiomyocytes of neonatal rats.

2. Materials and methods

2.1. Cell culture

Primary cultures of cardiac myocytes were prepared from ventricles of 1-day-old Wistar rats as described previously [4,7] according to the method of Simpson [14]. In brief, cardiomyocytes were plated at a field density of 1×10^5 cells/cm^2 on 35-mm culture dishes with 2 ml of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) with 10% foetal bovine serum (FBS). 24 h after seeding, the culture medium was changed to DMEM with 0.1% FBS for another 48 h before treatment with UII (human) (Peptide Institute, Osaka, Japan).

2.2. Assay for kinase activation

The activities of extracellular signal-regulated kinases (ERKs) were measured using myelin basic protein (MBP)-containing gels as described previously [4,7]. In brief, after stimulation with UII, cells were lysed with buffer A (25 mM Tris–HCl at pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were electrophoresed on a sodium dodecyl sulphate (SDS)–polyacrylamide gel containing 0.5 mg/ml MBP. ERK in the gels was denatured in 6 M guanidine HCl and renatured in 50 mM Tris–HCl at pH 8.0 containing 0.04% Triton X-100 and 5 mM 2-mercaptoethanol. Phosphorylation activities of ERK were assayed by incubating the gels with [γ-^{32}P]ATP (Du Point-New England Nuclear). After incubation the gels were washed extensively, dried and processed for autoradiography.

2.3. [^{3}H]Phenylalanine incorporation

Protein synthesis was determined by assessing the incorporation of labelled phenylalanine from the extracellular medium into total trichloroacetic acid-precipitable cell proteins [4,7]. Cardiac myocytes cultured for 24 h in DMEM with 10% FBS were starved in 0.1% FBS–DMEM for 24 h. Then, UII or saline was added into the medium and the cells were further cultured for 24 h. [^{3}H]phenylalanine (1 μCi/ml) (Du Point-New England Nuclear Co.) was added 2 h before harvest. At the end of the labeling incubation, plates were placed on ice, quickly washed twice with ice-cold phosphate-buffered saline, incubated for 30 min with 10% trichloroacetic acid, and washed again. Precipitates were solubilised for 30 min in 1 M NaOH and neutral-

*Corresponding author. Fax: (81)-3-5800 8751.
E-mail address: yama-tky@umin.ac.jp (T. Yamazaki).

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ised. Total radioactivity was measured by a liquid scintillation counter.

2.4. Northern blot analysis

Total RNA of cultured cardiomyocytes was extracted using zol B (Cinna Biotecx Laboratories). Total RNA (10 μg) was size-fractionated electrophoretically in 1.2% formaldehyde agarose gels and transferred to nylon membranes (Millipore). Blots were hybridised with [α-^{32}P]dCTP (Du Pont-New England Nuclear)-labelled cDNA fragments of sequences encoding atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Hybridised bands were quantitated by a densitometer.

2.5. Immunocytochemistry

Cardiomyocytes cultured on glass cover slides were fixed for 20 min in 4% paraformaldehyde and permeabilised for 10 min in 0.1% Triton X-100. The cells were incubated with phalloidin-tetramethylrhodamine isothiocyanate (TRITC) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) for 1 h at 37°C [15].

2.6. Statistical analysis

Statistical comparison was carried out within three independent experiments using one-way ANOVA and Dunnett's *t*-test. Values of $P < 0.05$ were considered to indicate statistical significance.

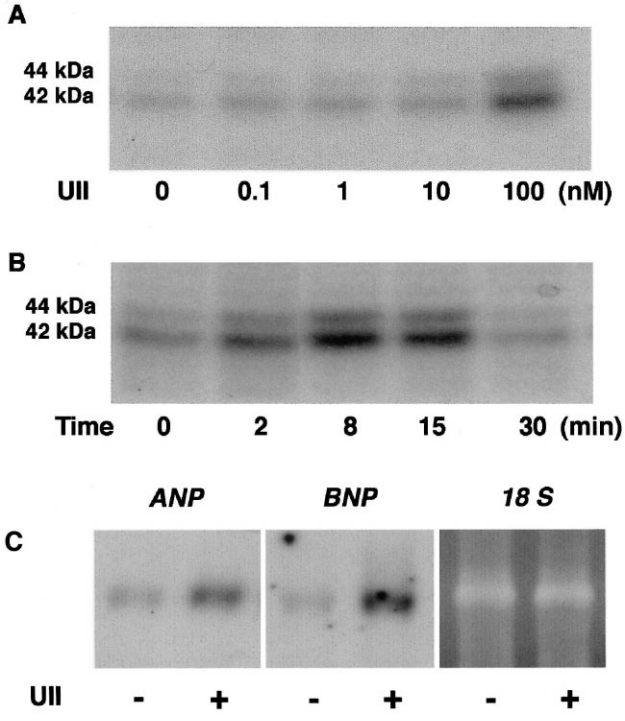

Fig. 1. Activation of ERKs by UII in cultured cardiomyocytes. Cardiomyocytes were incubated with UII at the indicated concentration for 8 min (A) or at 100 nM for the indicated period of time (B). Aliquots of the cell lysates were electrophoresed on an SDS–polyacrylamide gel containing 0.5 mg/ml MBP. After denaturation and renaturation we assayed phosphorylation activity of ERKs by incubating the gel with [γ-^{32}P]ATP. Then the gel was washed, dried and processed for autoradiography. Representative autoradiograms are shown. C: Quantitative analysis of ERK activity. The intensities of the 42-kDa ERK band were measured by densitometric scanning of the autoradiograms. The activity was expressed relative to that of the 42-kDa ERK obtained from unstimulated cardiomyocytes. The data are indicated as a mean of two experiments. ERK, extracellular signal-regulated kinase; UII, urotensin II; SDS, sodium dodecyl sulphate; MBP, myelin basic protein.

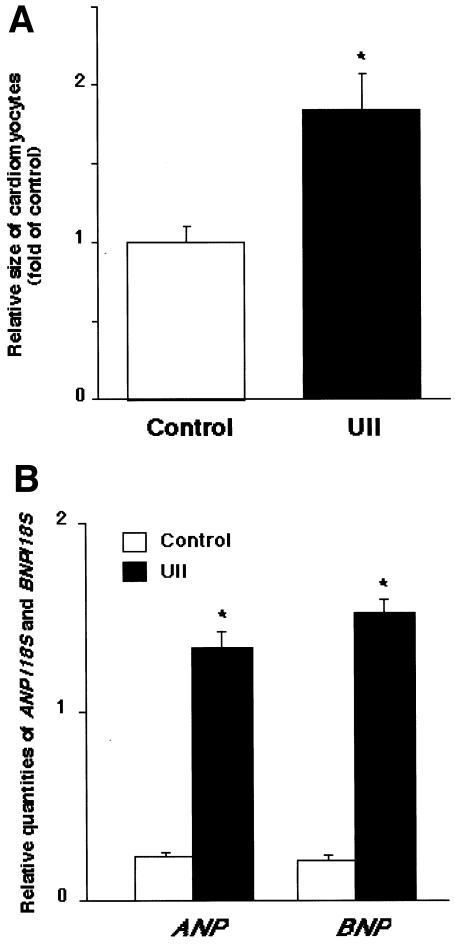

Fig. 2. Induction of expression of *ANP* and *BNP* genes by UII in cardiac myocytes. Cardiomyocytes were incubated with 100 nM UII or saline (control) for 2 h. Total RNA (10 μg) was size-fractionated in 1.2% formaldehyde agarose gels. Expression of *ANP* and *BNP* genes was examined by Northern analysis. Ethidium bromide staining of *18S* ribosomal RNA is presented to confirm integrity of RNA. A: Representative autoradiograms from three independent experiments are shown. B: The intensities of *ANP*, *BNP* or *18S* bands were measured by densitometric scanning of the autoradiograms. The ratio of relative amounts of *ANP* or *BNP* to that of *18S* are shown. Data are presented as mean ± S.E.M. from three independent experiments. $^*P < 0.05$ versus control. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; UII, urotensin II.

3. Results and discussion

3.1. Activation of kinases by UII in cardiac myocytes

A growing body of evidence has shown that stimuli that induce hypertrophy activate multiple intracellular protein kinases and phosphatases leading to reprogramming of gene expression and a consequent increase in protein synthesis in cardiac myocytes [3,16]. Mitogen-activated protein kinases (MAPK) have been reported to play important roles in the development of cardiac hypertrophy [3,16]. ERKs, which are members of the MAPK family, have been extensively investigated [3,17–21]. To elucidate the role of UII in cardiac hypertrophy, we first examined whether UII induces activation of ERKs in cultured cardiac myocytes. ERKs in cardiac myocytes were slightly activated by UII at 1 nM and strongly activated by UII at 100 nM (Fig. 1A and C), representing dose-dependent activation of ERKs. At 100 nM the increase

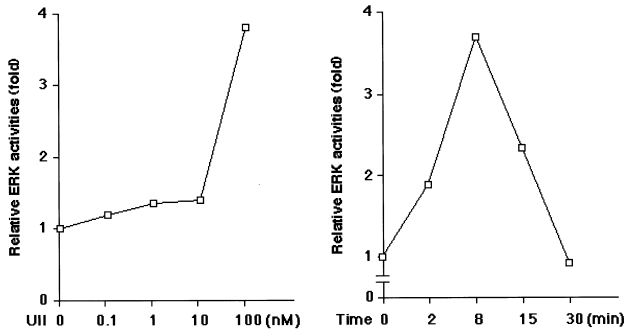

Fig. 3. Protein synthesis stimulated by UII in cardiac myocytes. Cardiac myocytes starved in DMEM with 0.1% FBS for 24 h were treated with 100 nM UII or saline (control) for 24 h, and then were pulse-labelled with [^{3}H]phenylalanine (1 μCi/ml) for the final 2 h. Incorporation of [^{3}H]phenylalanine into the acid-precipitable cellular fraction was determined by liquid scintillation counting. Data are presented as means ± S.E.M. from three independent experiments (control = 100%). *$P < 0.05$ versus control. UII, urotensin II.

in activation of ERKs was rapid with a slight increase at 2 min and a peak at 8 min followed by a decrease (Fig. 1B and C). The results suggest that UII can rapidly induce significant activation of ERKs in cardiac myocytes.

3.2. Expression of specific genes by cardiac myocytes in response to UII

Induction of specific gene expression is one of the responses of cardiomyocytes that cause hypertrophy [16]. Notably these genes include foetal genes such as *ANP* and *BNP* genes and they have been reported to be increased in the development of cardiac hypertrophy [16]. Activation of ERKs also has been reported to be involved in the expression of *ANP* and *BNP* genes [18,22]. We therefore examined whether UII induces increases in the expression of these two foetal type genes. Exposure of cardiac myocytes to UII for 2 h significantly increased expression of *ANP* and *BNP* genes beyond expression in control cells (Fig. 2A and B). The results suggest that UII stimulation results in induction of foetal type gene expression in cardiac myocytes.

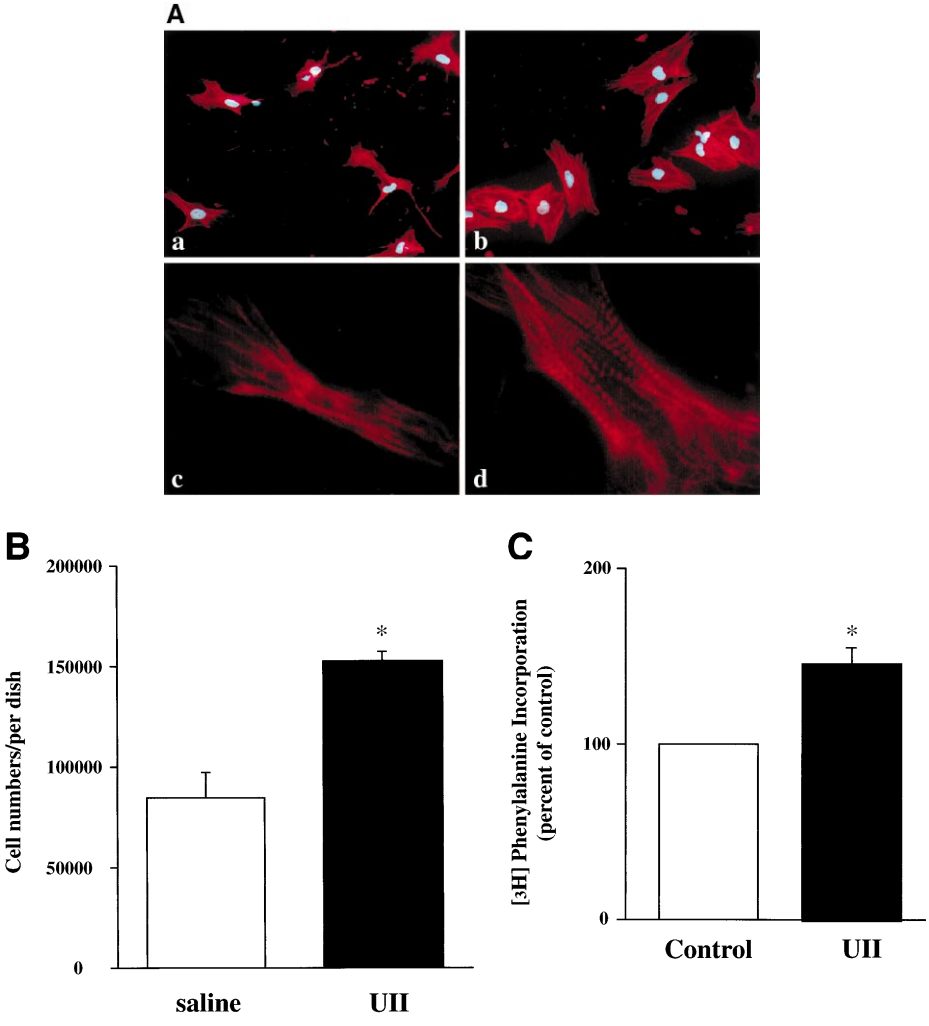

Fig. 4. UII-induced morphologic changes of cardiac myocytes. A: Cultured cardiac myocytes were exposed to saline (a and c) or to UII (b and d) at 100 nM for 24 h and then incubated with phalloidin-TRITC (red) and DAPI (blue). Representative stained preparation from three independent experiments are shown. a and b, ×200; c and d, ×400. B: Relative cardiomyocyte size. Surface area of cardiomyocytes was measured by using NIH Image software in 60 randomly chosen cells from three different dishes. Data are presented as mean ± S.E.M. *$P < 0.05$ versus saline. C: Numbers of cardiomyocytes with well-organised myofilaments were counted in whole dish. Data are presented as mean ± S.E.M. from three different dishes. *$P < 0.05$ versus saline. UII, urotensin II; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

3.3. UII-induced protein synthesis in cardiac myocytes

Activation of protein kinases and induction of specific gene expression can lead to increases in protein synthesis in cardiac myocytes [3,16]. [^{3}H]Phenylalanine incorporation from the extracellular medium into the total trichloroacetic acid-precipitable cell protein has been one method used to determine the protein synthesis rate [7]. When we examined UII-induced protein synthesis by assessing UII-induced incorporation of phenylalanine into total protein of cardiac myocytes, addition of UII for 24 h significantly accelerated protein synthesis compared to control cells incubated with saline ($P < 0.05$) (Fig. 3).

3.4. UII-induced morphologic changes in cardiac myocytes

Enhanced sarcomere organisation is a feature of cardiomyocyte hypertrophy [16] that we were able to observe in response to UII (Fig. 4A–C). Control cells incubated with saline for 24 h were small and irregularly shaped (Fig. 4Aa), and myofilaments were poorly organised (Fig. 4Ac). When the cells were incubated with UII for 24 h, they became larger (Fig. 4Ab), and myofilaments were well organised (Fig. 4Ad). These results suggest that increased cell size and myofibrillar organisation induced by UII accompanies the synthesis of new proteins.

The cyclic neuropeptide UII not only influences neuromuscular function but also participates in cardiovascular regulation [9–13]. We showed in the present study that UII is able to induce hypertrophic responses in cardiac myocytes including activation of ERKs, induction of foetal type gene expression, increases in protein synthesis and morphologic changes. The mechanism by which UII induces cardiomyocyte hypertrophy are unclear at present. Recently, UII has been reported to exert its effects on cardiovascular function through GPR14 [11], and an increase in inositol phosphates [23]. A quite recent report demonstrated that UII induced contraction, actin stress fibre formation and proliferation in arterial smooth muscle cells through the small GTPase RhoA and Rho kinase [24]. Since the Rho family is involved in the activation of ERKs and development of hypertrophy in cardiac myocytes [25,26], UII may induce ERK activation and cardiac hypertrophy through Rho family proteins. Otherwise, UII elevates intracellular Ca^{2+} levels [11], and Ca^{2+} and Ca^{2+}-binding proteins, such as Ca^{2+}/calmodulin-dependent protein kinases and calcineurin, have been reported to regulate the development of cardiac hypertrophy induced by AngII, ET-1 and catecholamines [8,15,27], UII may, therefore, also need participation of Ca^{2+}/Ca^{2+}-binding proteins to induce cellular events in cardiac myocytes. Further studies are necessary to issue these hypotheses.

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