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Transient receptor potential (TRP) protein 7 acts as a G protein-activated Ca^{2+} channel mediating angiotensin II-induced myocardial apoptosis

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Abstract

Transient receptor potential (TRP) proteins have been identified as cation channels that are activated by agonist–receptor coupling and mediate various cellular functions. TRPC7, a homologue of TRP channels, has been shown to act as a Ca^{2+} channel activated by G protein-coupled stimulation and to be abundantly expressed in the heart with an as-yet-unknown function. We studied the role of TRPC7 in G protein-activated signaling in HEK293 cells and cultured cardiomyocytes *in vitro* transfected with FLAG-tagged TRPC7 cDNA and in Dahl salt-sensitive rats with heart failure *in vivo*. TRPC7-transfected HEK293 cells showed an augmentation of carbachol-induced intracellular Ca^{2+} transient, which was attenuated under a Ca^{2+}-free condition or in the presence of SK&F96365 (a Ca^{2+}-permeable channel blocker). Upon stimulation with angiotensin II (Ang II), cultured neonatal rat cardiomyocytes transfected with TRPC7 exhibited a significant increase in apoptosis detected by TUNEL staining, accompanied with a decrease in the expression of atrial natriuretic factor and destruction of actin fibers, as compared with non-transfected cardiomyocytes. Ang II-induced apoptosis was inhibited by CV-11974 (Candesartan; Ang II type 1 [AT1] receptor blocker), SK&F96365, and FK506 (calcineurin inhibitor). In Dahl salt-sensitive rats, apoptosis and TRPC7 expression were increased in the failing myocardium, and a long-term treatment with temocapril, an angiotensin-converting enzyme inhibitor, suppressed both. Our findings suggest that TRPC7 could act as a Ca^{2+} channel activated by AT1 receptors, leading to myocardial apoptosis possibly via a calcineurin-dependent pathway. TRPC7 might be a key initiator linking AT1-activation to myocardial apoptosis, and thereby contributing to the process of heart failure.

Key words: angiotensin II, apoptosis, Ca^{2+} channels, G proteins, heart failure, transient receptor potential protein

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Introduction

Myocardial apoptosis has been suggested to be an important process contributing to the progression of heart failure in both animal models and humans caused by various etiologies [1]. The apoptotic process is initiated by many stimuli, such as anoxia and neurohormonal factors including catecholamines, angiotensin II (Ang II), and proinflammatory cytokines [2].

Ang II is an important agonist inducing myocardial apoptosis via Ang II type1 (AT1) receptor-mediated signaling [3]. Ang II binding to AT1 receptors evokes an elevation of intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) to activate calcium-dependent endogenous endonuclease, thus initiating the process of apoptosis. This [Ca^{2+}]$_i$ elevation has been thought to be a key initiator of the intracellular signaling of Ang II-induced apoptosis [4]. AT1 receptors couple to G proteins ($G_{11/12}$ and/or G_q) that activate phosphatidylinositol metabolism via phospholipase C activation, yielding inositol-1,4,5-trisphosphate and 1,2-diacylglycerol (DAG) [5]. Activation of AT1-G protein coupling mediates an increase in Ca^{2+} influx, possibly through L-type Ca^{2+} channels driven by DAG-activated protein kinase C [6]. Gq activation alone is sufficient to induce myocardial apoptosis [7, 8]. However, it is still largely unclear how [Ca^{2+}]$_i$ rises when AT1 receptors are activated.

The transient receptor potential (TRP) protein was first identified as a key component required for phototransduction in *Drosophila melanogaster* [9]. In *Drosophila*, rhodopsin couples to Gq protein, leading to activation of TRP protein as a receptor-activated cation channel that is relatively Ca^{2+}-selective. Since then, numerous homologues belonging to the TRP protein superfamily have been identified [10, 11]. One subfamily, named the ''canonical'' TRP channel (TRPC) family, consists of the 7 homologues TRPC1–7. TRPC7 is reported to be a Ca^{2+}-selective cation channel activated by G protein-coupled receptor (GPCR) and DAG [12], and is highly expressed in the brain and heart [10–12]. However, the role of TRPC in the heart remains unknown.

We hypothesized that TRPC7 acts as a Ca^{2+} channel mediating Ang II-induced myocardial apoptosis, and that this pathway is involved in the process of heart failure. To test this, we conducted two series of experiments: TRPC7-overexpressing experiments using HEK293 cells and cultured cardiomyocytes, and animal experiments using Dahl salt-sensitive rats with heart failure. Our results suggest that TRPC7 functions as a Ca^{2+} channel mediating AT1-induced myocardial apoptosis via a calcineurin-dependent pathway.

Materials and methods

Cell culture and measurement of changes in [Ca^{2+}]$_i$

Transfection efficiency of TRPC7 using FuGene6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) in HEK293 cells was high enough (about 50%), whereas in cultured cardiomyocytes was quite low (about 10%). Due to data errors it was difficult to compare the difference of [Ca^{2+}]$_i$ between transfected and non-transfected cardiomyocytes using Ca^{2+}-transient experiment. Therefore, we conducted the experiments measuring [Ca^{2+}]$_i$ change using HEK293 cells instead of cultured cardiomyocytes.

HEK293 cells were purchased from RIKEN Cell Bank, Tsukuba, Japan. Neonatal rat ventricular myocytes were prepared from hearts in 2–3-day-old Sprague–Dawley rats. Ventricular tissue was digested with collagenase, and a cardiomyocyte-enriched cell suspension was obtained using Percoll step gradients. HEK293 and myocardial cells were plated onto either gelatin-coated glass coverslips or 20 × 20 mm chamber slides and were cultured for 24 h in Dulbecco's modified Eagle medium containing 10% horse serum and antibiotics. Cells were further cultured under serum-free conditions with 4:1 Dulbecco's modified Eagle medium: medium 199 for 54 h. Transient transfection was performed 6 h after the culture medium was changed to serum-free conditions. Ang II was added to the medium 24 h after transfection was started, and cells were further cultured for 24 h, resulting in 24 h-stimulation with Ang II.

HEK293 cells on glass coverslips were loaded with fura-2 by incubation in HEPES-buffered physiological salt solution (PSS) containing 4 μmol/l fura-2/AM (Molecular Probes, Inc., Eugene, OR, USA) for 50 min at 30 °C. The coverslips were then placed in a 30 °C perfusion temperature-controlled chamber with 200 μl volume mounted on the stage of an inverted microscope (Leica DM IRB; Leica, Mannheim, Germany) equipped with a × 40 objective lens and a cooled charge-coupled device camera, Cascade:512F (Roper Scientific Inc., Tucson, AZ, USA). The fura-2 fluorescence at an emission wavelength of 510 nm was observed by exciting fura-2 alternately at 340 and 380 nm. The 340:380 nm fluorescence ratio images of the cells were captured and analyzed with a ratio image analysis system (Slidebook; Intelligent Imaging Innovations Inc., Denver, CO, USA). GFP fluorescence excited at a wavelength of 488 nm was observed at an emission wavelength of 510 nm.

Construction of the TRPC7 expression vector

Mammalian expression plasmids pCI-neo (Promega, Madison, WI, USA) encoding mouse TRPC7 (pCI-neo-TRPC7) were kindly provided by Prof. Y. Mori (Kyoto University). TRPC7 cDNA was excised from these plasmids and reconstructed into a C-terminal FLAG-tagged mammalian expression vector (pFLAG-CMV-5a; SIGMA, St. Louis, MO, USA) as follows:

Using pCI-neo-TRPC7 as a template, the C-terminal region 2333–2633 of pCI-neo-TRPC7 was amplified with the specific primers 5′-CAGGCTGGCATGAGGAATTCTG-3′ (sense) and 5′-ATAGGTACCAATGTCCTTGCCCTGGTTCACC-3′ (antisense), and the resulting fragments were digested with *Eco*RI/*Kpn*I and subcloned into the pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA). The N-terminal region 8143–2333 of pCI-neo-TRPC7 was excised by digestion with *Xba*I/*Eco*RI, and the resulting fragments were inserted into the pBluescript II SK (+) vector (pBluescript II SK (+)-TRPC7). TRPC7 cDNA (1–2633 without a stop codon) was excised from pBluescript II SK (+)-TRPC7 by digestion with *Not*I/*Kpn*I and subcloned into pFLAG-CMV-5a (pFLAG-TRPC7).

Gene transfection

Transient transfection was performed 6 h after the culture media was changed to serum-free conditions as follows:

HEK293 cells were co-transfected with 3.2 μg of pFLAG-TRPC7 and 0.8 μg of pEGFP-F vector (CLONTECH Laboratories, Inc., Palo Alto, CA, USA) using 8 μl of FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) in 500 μl of culture medium. After incubation for 48 h, cells were subjected to Ca^{2+} transient experiments.

Cultured cardiomyocytes were transfected with 4 μg of pFLAG-TRPC7 using 8 μl of FuGENE 6 Transfection Reagent in 1 ml of culture medium for 48 h. Ang II was added to the medium 24 h after transfection was started. Blockers for intracellular signaling were added to the medium 30 min prior to the addition of Ang II. CV-11974 was kindly provided by Takeda Chemical Industries, Ltd. (Osaka, Japan).

RT-PCR and Northern blot

RT-PCR was performed using a B*ca*BEST™ RNA PCR Kit Ver.1.1 (TaKaRa Biomedicals, Tokyo, Japan). RNA blot hybridization was performed using total RNA (20 μg) from cultured cardiomyocytes and myocardial tissues of Dahl rats. The probe used to detect TRPC7 mRNA was the $\sim$ 0.44-kb *Sac*I/*Pvu*II fragment excised from the C-terminal $\sim$ untranslated lesion (site 2558–2995) of mouse TRPC7 (accession number AF139923). Using rat myocardium mRNA as a template, the above fragment was amplified by RT-PCR with the specific primers 5′-CGGAATTCGAGCTCCTGGAGGAGAAGTCTC-3′ (sense) and 5′-CGGGTACCACAGCTGTCGTCCTGAGTCAAG-3′ (antisense), and the resulting fragments were digested with *Eco*RI/*Kpn*I and subcloned into the pFLAG-CMV-5a vector. The target probe was excised from this subcloned plasmid by digestion with *Sac*I/*Pvu*II. Hybridization was performed using a DIG-High Prime random labeling kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's specifications. The amount of TRPC7 mRNA was normalized to that of GAPDH.

Animal model of heart failure

Dahl salt-sensitive (DS, strain SS/Jr) and salt-resistant (DR, strain SR/Jr) rats were used as reported previously [13, 14]. All rats were fed an 8% NaCl diet for 9 weeks until 17 weeks of age. The rats were divided into 3 groups: DR rats used as a normotensive control (DR), DS rats receiving no treatment (DS/T−), and DS rats treated with temocapril (DS/T+), an angiotensin-converting enzyme inhibitor (ACE-I). Temocapril, kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan), was dissolved in drinking water (10 mg/kg/day) and administered orally to DS rats from 10 to 17 weeks of age. At 17 weeks of age, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Left ventricular function was assessed by transthoracic echocardiographic study using an echocardiographic system equipped with an 11-MHz phased-array transducer (LOGIC 400; GE Yokogawa Medical Systems, Tokyo, Japan). After echocardiographic study, hearts were excised and were either stored in liquid nitrogen until used for the biochemical examinations or fixed by retrograde perfusion with 4% paraformaldehyde for histological examinations. Study protocols were approved by the Committee on the Ethics of Animal Experiments, Kyushu University, and were in accordance with the *Guidelines for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised in 1996).

Histological examinations

Samples prepared as follows were observed under a confocal laser scanning microscope (LCM510; Zeiss, Jena, Germany) using optimal filter sets according to the manufacturer's instructions.

Detection of apoptosis
Apoptosis was detected by *in situ* terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH) according to the manufacturer's instructions.

Cultured cardiomyocytes. Cardiomyocytes were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 containing 0.1% sodium citrate, and blocked in PSS containing 10% normal goat serum. Cells were then incubated with mouse anti-FLAG M2 monoclonal antibody (SIGMA) for 1 h at room temperature. After being washed with PSS, cells were further subjected to the TUNEL reaction for 1 h at 37 °C under co-incubation with rhodamine-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Non-transfected cardiomyocytes were counted up to 4000–5000 cells under several high-power fields, and transfected cells, identified by positive staining for FLAG, were counted in the whole section and found to number 500–600 cells. Apoptosis was quantified as the percentage of TUNEL-positive cardiomyocytes to total cardiomyocytes counted (as 100%) in each group.

Myocardial section of Dahl rats. Transverse-sectioned tissue blocks of 4 mm thickness were excised at the level of the papillary muscle of the hearts, fixed with 4% paraformaldehyde, and embedded in paraffin. Paraffin-embedded tissues were sliced on silane-coated slides at 2 μm-thickness, dewaxed and rehydrated, treated with proteinase K, and incubated with mouse anti-rabbit troponin T monoclonal antibody (SIGMA) for 1 h at room temperature to identify cardiomyocytes. After being washed with PSS, cells were further subjected to the TUNEL reaction for 1 h at 37 °C under co-incubation with rhodamine-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) followed by 2-min incubation with Hoechst 33342 (Molecular Probes) to identify nuclei. Apoptotic cells identified by TUNEL-positive stained nuclei were counted in the whole section. Apoptosis was quantified as the percentage of TUNEL-positive nuclei of cardiomyocytes to total nuclei of cardiomyocytes counted in the whole section.

Atrial natriuretic factor (ANF) and actin staining
Cultured cardiomyocytes were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked in PSS containing 10% normal goat serum. Cells were then incubated with rabbit anti-rat ANF polyclonal antibody (CHEMICON International, Temecula, CA, USA) and mouse anti-FLAG M2 monoclonal antibody (SIGMA) for 1 h at room temperature. After being washed with PSS, cells were further incubated with Oregon green-conjugated goat anti-rabbit IgG antibody and rhodamine-conjugated goat anti-mouse IgG antibody for 1 h at room temperature. Non-transfected cardiomyocytes were counted up to 4000–5000 cells in several high-power fields, and transfected cells were counted in the whole section and found to number 500–600 cells. ANF expression was quantified as the percentage of ANF-positive cardiomyocytes to total cardiomyocytes counted in each group.

Another aliquot of cells was incubated with Oregon green-conjugated phalloidin (Molecular Probes) and mouse anti-FLAG M2 monoclonal antibody for 1 h at room temperature. After being washed with PSS, the cells were further incubated with rhodamine-conjugated goat anti-mouse IgG antibody for 1 h at room temperature.

Statistical analysis

The measured values were expressed as the mean ± S.E. Comparison of a single parameter among groups was performed by one-way ANOVA followed by Bonferroni/Dunn's *post-hoc* test. Dose-dependency of Ca^{2+} transients on carbachol was assessed by repeated measures one-way ANOVA. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

TRPC7 as a Ca^{2+}-permeable channel (Fig. 1)

Carbachol, a muscarinic agonist, provoked a transient monophasic rise in $[Ca^{2+}]_i$ in HEK293 cells. In TRPC7-transfected HEK293 cells, as identified by a co-transfected marker EGFP, 10 μmol/l carbachol-induced transient rise in $[Ca^{2+}]_i$ was augmented as compared with those in the non-transfected control and EGFP-transfected cells (Fig. 1A). The peak Ca^{2+} transient level was increased in response to the dose of carbachol (Fig. 1B). The whole Ca^{2+} transient was attenuated in Ca^{2+}-free solution containing 2 mM EGTA (Fig. 1C) or in the presence of 25 μmol/l SK&F96365, a blocker for Ca^{2+}-permeable channels (Fig. 1D).

TRPC7 mediates Ang II-induced apoptosis in cardiomyocytes (Figs. 2–5)

As a positive control for apoptosis, cultured cardiomyocytes were treated with 5 μmol/l A23187 for 24 h. This treatment induced a high rate of apoptosis, as shown in Fig. 2A (80.2 ± 2.6% of total cardiomyocytes, $n = 6$). As shown in Fig. 2D, apoptosis was scarcely induced in non-stimulated cells, both in non-transfected cells (0.05 ± 0.02%, column A)

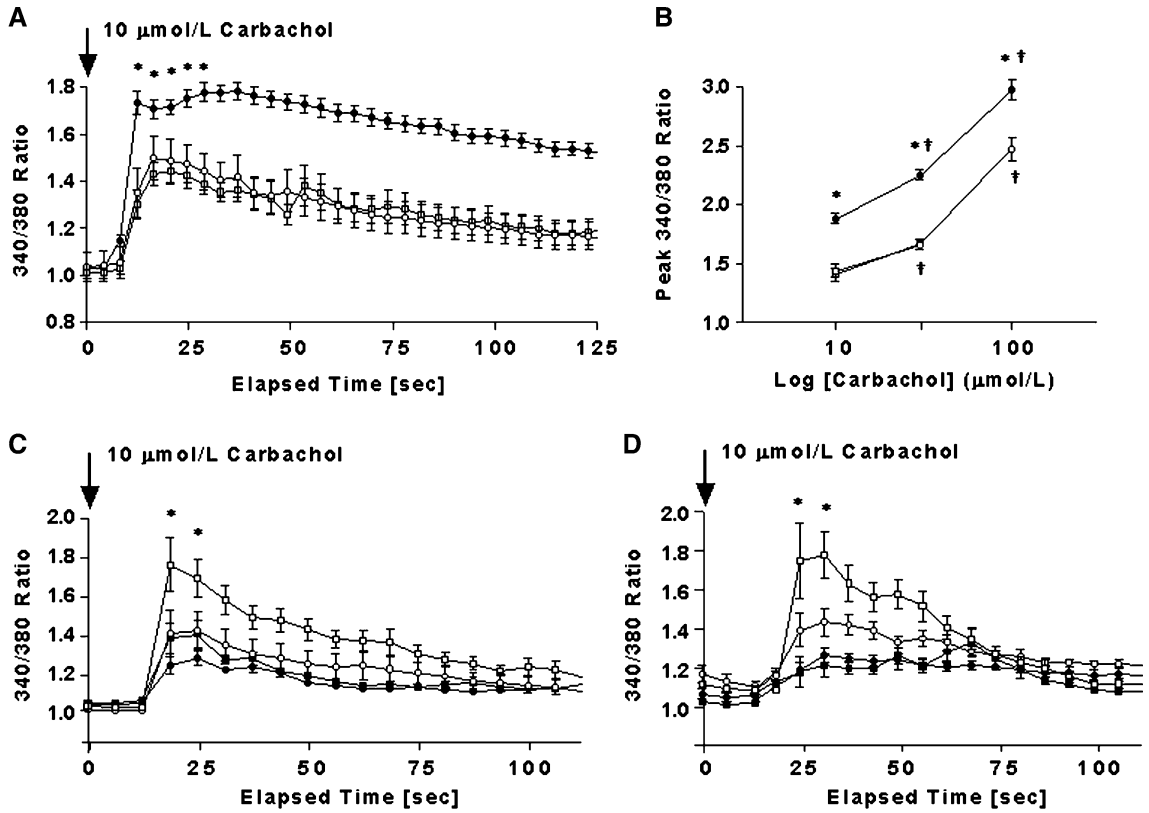

Fig. 1. Time course of changes in $[Ca^{2+}]_i$ induced by carbachol in HEK293 cells. The 340:380 nm fluorescence ratios of fura-2 are plotted against the elapsed time after the application of carbachol. Plots are shown as means ± S.E. (n = 16 each). (A) Ca^{2+} transients induced by 10 μmol/l carbachol. ○ Non-transfected, □ Transfected with control vector and EGFP, ● Transfected with TRPC7 and EGFP. * $p < 0.01$ vs. ○ and □ at the same elapsed time. (B) Dose-dependency of the peak 340:380 nm fluorescence ratios on carbachol. ○ Non-transfected, □ Transfected with control vector and EGFP, ● Transfected with TRPC7 and EGFP. *$p < 0.01$ vs. ○ and □ at the same dose of carbachol; †$p < 0.01$ vs. ● at 10 μmol/l carbachol. (C) Effects of extracellular Ca^{2+} on carbachol-induced Ca^{2+} transients. ○ Non-transfected in the presence of 1.6 mmol/l $[Ca^{2+}]_o$, ● Non-transfected in the absence of $[Ca^{2+}]_o$ (2 mmol/l EGTA), □ Transfected with TRPC7 and EGFP in the presence of 1.6 mmol/l $[Ca^{2+}]_o$, ■ Transfected with TRPC7 and EGFP in the absence of $[Ca^{2+}]_o$ (2 mmol/l EGTA). *$p < 0.05$ vs. ■ at the same elapsed time. (D) Effects of SK&F96365 on carbachol-induced Ca^{2+} transients. ○ Non-transfected, ● Non-transfected in the presence of 25 μmol/l SK&F96365, □ Transfected with TRPC7 and EGFP, ■ Transfected with TRPC7 and EGFP in the presence of 25 μmol/l SK&F96365. *$p < 0.01$ vs. ■ at the same elapsed time.

and cells transfected with control vectors (0.09 ± 0.09%, column D). Ang II stimulation slightly increased apoptosis in non-transfected cells (Fig. 2B), but without statistical significance (0.10 ± 0.02% by 0.1 μmol/l Ang II [column B] and 0.13 ± 0.03% by 1 μmol/l Ang II [column C], respectively). In TRPC7-transfected cells, as identified by positive staining for FLAG (Fig. 2C), apoptosis was significantly increased even without Ang II stimulation (4.09 ± 1.37%, column F). Upon stimulation with Ang II, TRPC7-transfected cells exhibited a further increase in apoptosis. Ang II-induced apoptosis was almost maximal at 0.1 μmol/l Ang II (9.48 ± 1.62% by 0.1 μmol/l Ang II [column G] and 9.65 ± 2.30% by 1 μmol/l Ang II [column H], respectively). As shown in Fig. 2C, an apoptotic cell exhibited nuclear condensation and fragmentation, which are typical features of apoptosis.

Figure 3 shows the effects of blockers for Ang II-mediated signaling on apoptosis. Ang II (0.1 μmol/l)-induced apoptosis that appeared in TRPC7-transfected cardiomyocytes (11.65 ± 0.53%) was significantly inhibited by 0.1 μmol/l CV-11974 (Candesartan; AT1 receptor blocker, 5.43 ± 0.92%), 12 μmol/l SK&F96365 (5.81 ± 0.48%), and 125 nmol/l FK506 (calcineurin inhibitor, 5.47 ± 0.85%). The degree of inhibitory effect was comparable among these three blockers. There was no significant additive inhibitory effect on myocardial apoptosis in the presence of three blockers (5.12 ± 0.83%).

We further observed the expression of ANF as a marker gene for myocardial differentiation [15] in relation to apoptosis. Consistent with many previous reports, Ang II induced a significant increase in the expression of ANF (Fig. 4A vs. B), which was almost maximal at 0.1 μmol/l

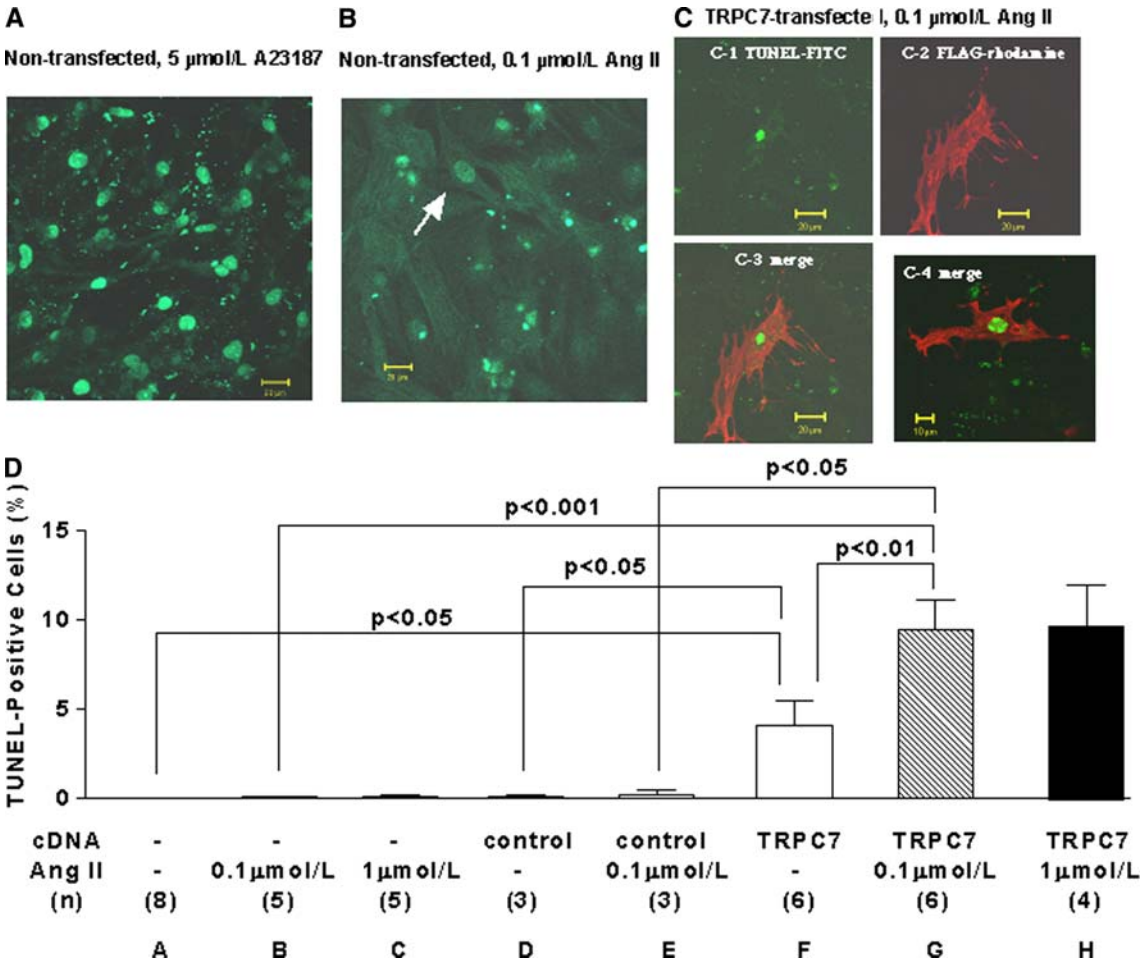

Fig. 2. Ang II-induced myocardial apoptosis detected by TUNEL staining. (A) Non-transfected cardiomyocytes stimulated by 5 μmol/l A23187. (B) Non-transfected cardiomyocytes stimulated by 0.1 μmol/l Ang II. A nucleus of positive TUNEL stain is shown (arrow). (C) TRPC7-transfected cardiomyocytes stimulated by 0.1 μmol/l Ang II. C-1, Positive stain for TUNEL with a condensed nucleus; C-2, TRPC7-transfected cardiomyocytes identified by anti-FLAG-tag staining; C-3, Merge; C-4, Another typical apoptotic cardiomyocyte showing a fragmented nucleus. Bars indicate 20 μm except C-4 (10 μm). (D) Quantified data of myocardial apoptosis. Bars indicate means ± S.E. The numbers of experiments are indicated in the parentheses.

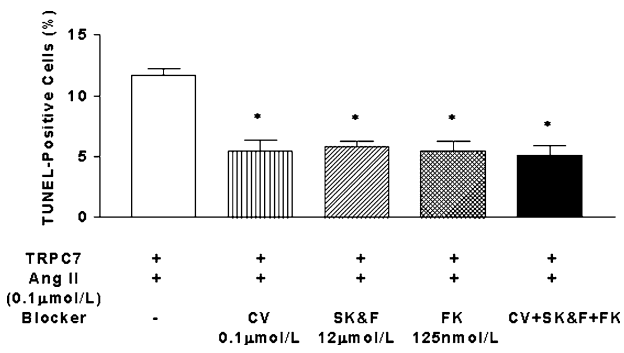

Fig. 3. Effects of AT1-signaling blockers on 0.1 μmol/l Ang II-induced myocardial apoptosis. Bars indicate means ± S.E. (n = 5 each) *$p < 0.05$ vs. TRPC7-transfected, Ang II-activated cells without blockers (open bar).

Ang II (48.5 ± 7.8% vs. 23.6 ± 4.9% in the control, column B vs. A in Fig. 4D). TRPC7-transfected cardiomyocytes showed a decrease in the ANF expression (8.5 ± 0.8%, column D in Fig. 4D) as compared with the non-transfected control (23.6 ± 4.9%, column A in Fig. 4D), but without statistical significance. Ang II stimulation induced a decrease in the ANF expression of TRPC7-transfected cardiomyocytes (Fig. 4C). This effect was significant as compared with that in non-transfected cells with Ang II-stimulation (48.5 ± 7.8% in column B vs. 9.1 ± 1.6% in column E, and 47.4 ± 6.5% in column C vs. 9.3 ± 2.0% in column F, respectively).

Figure 5 shows the morphological changes in apoptotic cardiomyocytes. Ang II (0.1 μmol/l) promoted sarcomere organization in non-transfected cells, as detected by staining

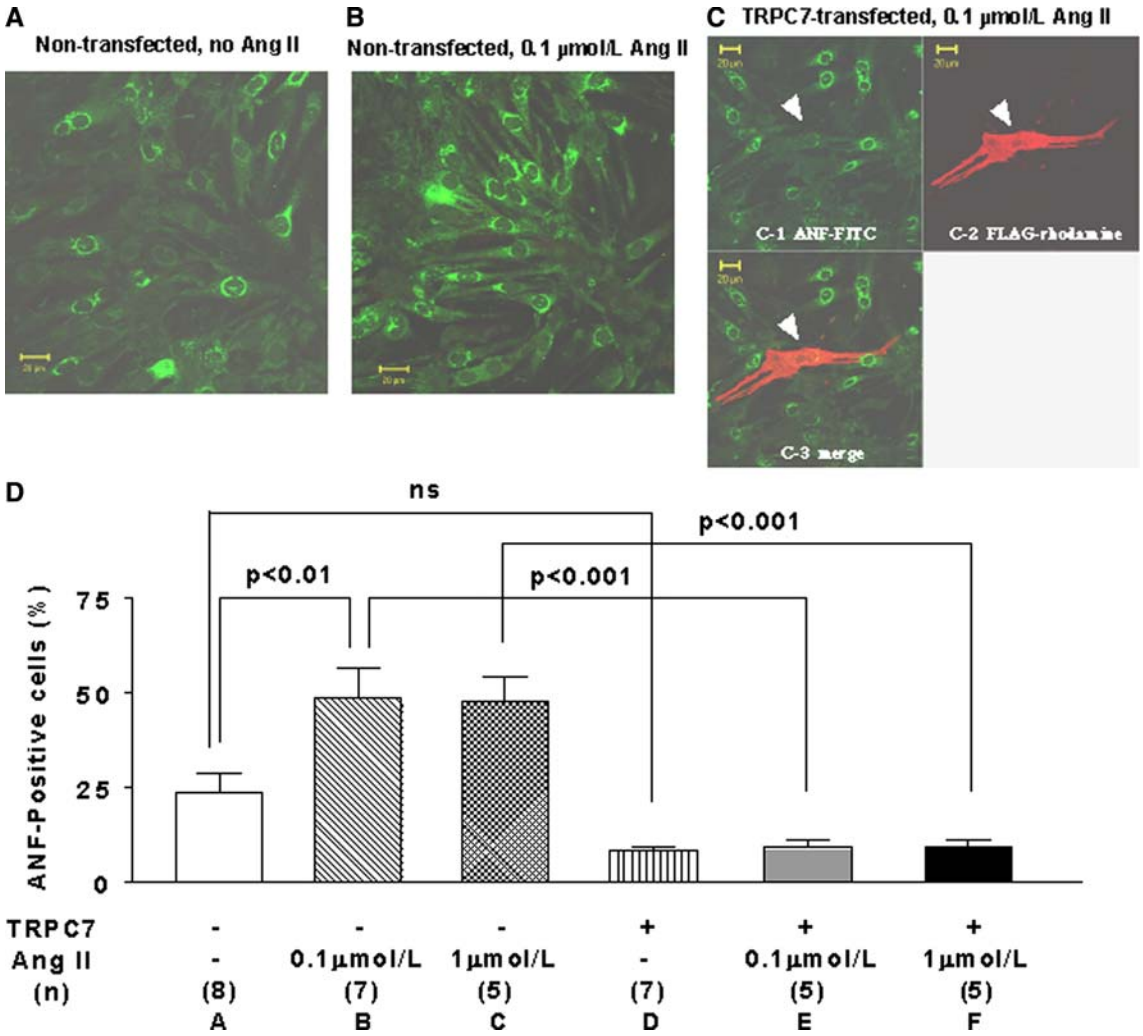

Fig. 4. Changes in the expression of ANF by Ang II stimulation. (A) Non-transfected cardiomyocytes. (B) Non-transfected cardiomyocytes stimulated by 0.1 μmol/l Ang II. (C) TRPC7-transfected cardiomyocytes stimulated by 0.1 μmol/l Ang II. C-1, Positive stain for ANF; C-2, TRPC7-transfected cardiomyocytes identified by anti-FLAG-tag staining; C-3, Merge. The TRPC7-transfected cell exhibited a decreased expression of ANF (arrowheads). Bars indicate 20 μm. (D) Quantified data of the ANF expression. Bars indicate means ± S.E. The numbers of experiments are indicated in the parentheses.

of actin fibers with thick sarcomeres (Fig. 5A vs. B). On the other hand, in TRPC7-transfected cells, Ang II induced the destruction of actin fibers in apoptotic cells, as shown by fiber fragmentation (Fig. 5C).

Apoptosis and TRPC7 expression in animals with heart failure (Table 1 and Figs. 6, 7)

It has been suggested that apoptosis is an important process contributing to the progression of heart failure in Dahl salt-sensitive rats [16–18]. Therefore, we observed changes in myocardial apoptosis and TRPC7 expression in DS rats with heart failure. As shown in Table 1, there was no significant difference in systolic blood pressure among the groups before the treatment, whereas the systolic blood pressure was elevated to a similar extent in both DS/T− and DS/T+ rats at 17 weeks of age. LV mass corrected for body weight was increased in DS/T− rats compared to that in DR rats, and it was significantly decreased in DS/T+ rats. LV ejection fraction and fractional shortening, which were decreased in DS/T− rats at 17 weeks of age, were almost normalized in DS/T+ rats after the treatment.

Consistent with previous reports, myocardial apoptosis (Fig. 6A) was increased in DS rats with heart failure (0.41 ± 0.08%) as compared with that in DR rats (0.08 ± 0.01%), and it was suppressed in DS rats treated with temocapril (10 mg/kg/day) for 7 weeks (0.15 ± 0.01%, Fig. 6B). Similarly, the expression of TRPC7 (Fig. 7) mRNA was up-regulated in DS rats with heart failure (1.65 ± 0.2

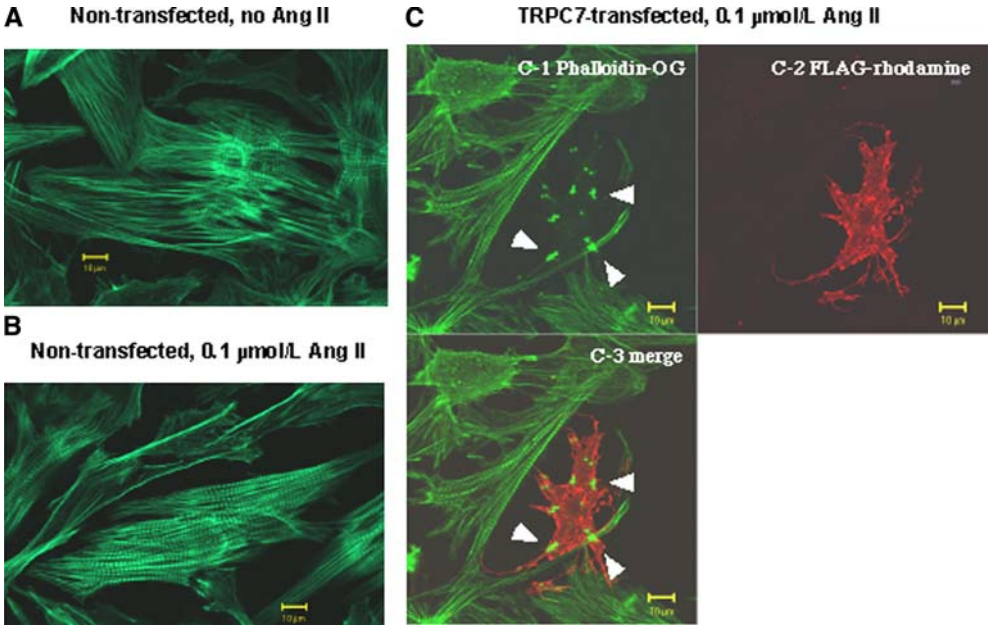

Fig. 5. Changes in sarcomere organization in apoptotic cardiomyocytes detected by actin fiber staining. (A) Non-transfected cardiomyocytes. (B) Non-transfected cardiomyocytes stimulated by 0.1 μmol/l Ang II. (C) TRPC7-transfected cardiomyocytes stimulated by 0.1 μmol/l Ang II. C-1, Actin staining with phalloidin-oregon green (OG); C-2, TRPC7-transfected cardiomyocytes identified by anti-FLAG-tag staining; C-3, Merge. TRPC7-transfected cell shows destructed and shrunk actin fibers (arrowheads). Bars indicate 10 μm.

arbitrary unit [AU]) as compared with that in DR rats (1.13 ± 0.04 AU). This up-regulated expression was suppressed by the long-term treatment with temocapril (1.01 ± 0.13 AU).

Discussion

TRPC7 is abundantly expressed in the heart as much as in the eye and lung [12], and its function in the heart has been remained unknown. The present study showed that TRPC7 could act as a Ca^{2+} channel activated by GPCR AT1, thereby inducing myocardial apoptosis. This process might be mediated via a calcineurin-dependent signaling pathway and be involved in the process of heart failure induced by hypertension, for example.

Table 1. Data of Dahl rat LV function

		DR	DS/T−	DS/T+
SBP (mmHg)	Pre	124 ± 5	123 ± 4	125 ± 4
	Post	134 ± 6	206 ± 5†	201 ± 10†
LV mass (mg/g BW)		2.45 ± 0.12	5.43 ± 0.24†	4.05 ± 0.17†,§
LVEF (%)	Pre	80.0 ± 2.8	80.2 ± 1.9	79.3 ± 2.0
	Post	86.5 ± 1.2	66.5 ± 3.9†	79.9 ± 2.1§
FS (%)	Pre	43.6 ± 2.9	42.6 ± 2.0	42.1 ± 2.1
	Post	49.4 ± 1.6	32.9 ± 2.8†	43.6 ± 2.2§

DS/T−, DS rats without treatment; DS/T+, DS rats treated with temocapril; SBP, systolic blood pressure; Pre, at 8 weeks of age before the treatment; Post, at 17 weeks of age after the treatment; LVEF, LV ejection fraction; FS, % fractional shortening.
*$p < 0.05$ vs. DR, †$p < 0.01$ vs. DR, §$p < 0.01$ vs. DS/T− (n = 6 each).

TRPC7 works as a GPCR-activated Ca^{2+} channel

Although rat TRPC7 cDNA or mRNA has not been identified as yet, we think that rat TRPC7 is almost identical to mouse TRPC7 based on the following findings. (1) When RT-PCR was performed using specific primers for mouse TRPC7, a specific band was detected in rat mRNA from both cultured cardiomyocytes and myocardial tissue as a template (data not shown). (2) When Northern blot was performed using a specific probe for mouse TRPC7, a specific band was detected in rat mRNA from myocardial tissues as shown in Fig. 7.

HEK293 cells express muscarinic receptors that couple to the G protein Gq/11, which has been linked to phospholipase C activation [19], based on the finding that carbachol-induced Ca^{2+} current in TRPC6-overexpressing HEK293 cells is suppressed in the presence of U73122, a phospholipase C inhibitor [20]. We thus think that Ang II is similar to carbachol, of course not identical, in terms of TRPC7

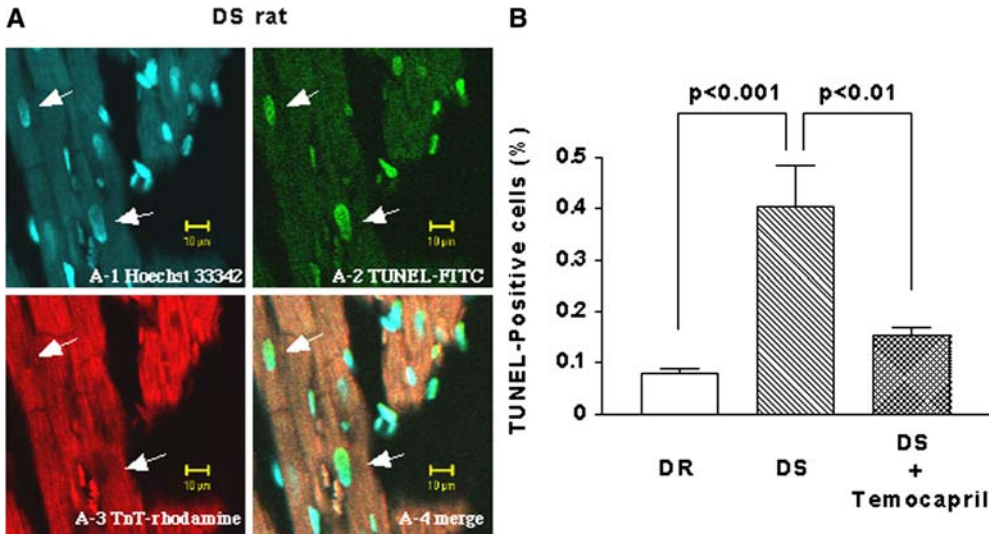

Fig. 6. Myocardial apoptosis in Dahl rats. (A) Representative histology showing apoptotic cardiomyocytes in DS rats with heart failure (arrows). A-1, Hoechst 33342 staining to identify all nuclei; A-2, TUNEL staining; A-3, Anti-troponin T (TnT) staining to identify cardiac cells; A-4, Merge. Bars indicate 10 μm. (B) Quantified data of myocardial apoptosis in Dahl rats. DR, Dahl salt-resistant rat; DS, Dahl salt-sensitive rat fed with high-salt diet. DS + temocapril, DS rats treated with temocapril. Bars indicate means ± S.E. (n = 8 each).

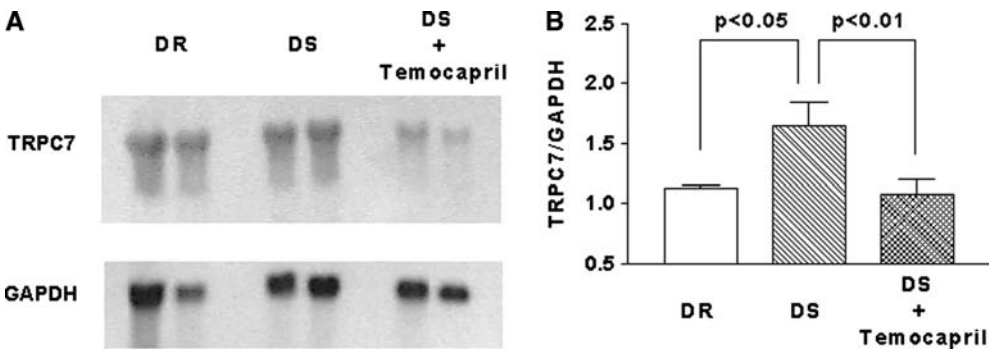

Fig. 7. TRPC7 mRNA expression in the myocardium of Dahl rats. (A) Typical Northern blot. DR, Dahl salt-resistant rat; DS, Dahl salt-sensitive rat fed with high-salt diet. DS + temocapril, DS rats treated with temocapril. (B) Quantified data of the TRPC7 mRNA expression normalized to that of GAPDH. Bars indicate means ± S.E. (n = 6 each).

activation. TRPC7-overexpressimg HEK293 cells showed an augmentation of carbachol-induced Ca^{2+} transient in a dose-dependent manner, as compared with non-transfected cells. The increase in $[Ca^{2+}]_i$ was attenuated under a Ca^{2+}-free condition or in the presence of SK&F96365, a blocker for Ca^{2+}-permeable channels, including TRPC7. Using patch clamp recording, Okada *et al*. demonstrated that TRPC7-expressed HEK293 cells show increased inward cation currents in response to ATP and 1-oleoyl-2-acetyl-*sn*-glycerol, a DAG analogue [12]. Taken together, the present results suggest that TRPC7 serves as a Ca^{2+} permeable channel activated by GPCR.

TRPC7 mediates myocardial apoptosis

When TRPC7 was overexpressed in cardiomyocytes, the incidence of apoptosis was increased even without Ang II stimulation as compared with non-transfected cells or cells transfected with control vector. This could be because the TRPC7 prepared in this study has a small degree of basal activity, as observed by Okada *et al*. [12] Ang II promoted a further increase in apoptosis in TRPC7-overexpressing cardiomyocytes. In association with apoptosis, TRPC7-overexpressing cardiomyocytes exhibited decreased expression of ANF, destruction of actin fibers, and shrinkage of the cell

body. This Ang II-induced apoptosis might be dependent on AT1-receptor activation, transmembrane Ca^{2+} influx, and calcineurin activation, based on the findings that AT1 blocker CV-11974, Ca^{2+} channel blocker SK&F96365, and calcineurin inhibitor FK506 inhibited Ang II-induced myocardial apoptosis with no additive effects.

Shibasaki *et al*. demonstrated a pivotal role of calcineurin in Ca^{2+}-induced apoptosis in BHK-21 cells [21]. Furthermore, recent reports have demonstrated calcineurin-dependent myocardial apoptosis *in vitro* [22, 23]. This calcineurin-dependent apoptosis is reported to be mediated via BAD dephosphorylation catalyzed by activated calcineurin [24]. Previous reports showed that Ang II induces myocardial apoptosis via an AT1-mediated signaling pathway *in vitro* [3, 4]. There has also been a report showing the link between AT1 receptor and calcineurin *in vivo* [25]. Based on these previous reports, there seems to be a close link between AT1 activation and myocardial apoptosis in connection with calcineurin-dependent signaling. Whereas this AT1-mediated apoptosis is dependent on Ca^{2+} influx, how Ca^{2+} enters into cells is still largely unknown. Since SK&F96365 is not a specific blocker to TRPC7, Ca^{2+} influx induced by AT1-activation is not solely via TRPC7 channels. However, our results may present one possible underlying link between AT1 activation and Ca^{2+} influx partly arising from TRPC7-activation, which might lead to the activation of calcineurin-dependent signaling. Another recent report also demonstrated that TRPM7, a member of the TRP channels superfamily, plays a key role in anoxic neuronal death [26].

Role of TRPC7 in heart failure

Recent studies have shown that Ang II induces up-regulation of T-type Ca^{2+} channels in cardiomyocytes. This mechanism is proposed to be involved in the Ca^{2+} overload and arrhythmias seen in myocardial hypertrophy [27].

The present study showed another new possibility that TRPC7 contribute to the process of heart failure observed in DS rats induced by hypertension, because the up-regulated Ang II signaling plays a pivotal role in the development of heart failure in this animal model as discussed below.

Apoptosis has been suggested to be one of the pathological mechanisms involved in the process of heart failure in DS rats [16–18]. The present study also confirmed that apoptosis is increased in the failing hearts of DS rats. Apoptosis was suppressed by a long-term treatment with temocapril, an ACE-I. It has been established that the blockade of Ang II signaling exerts a protective effect against heart failure. Several mechanisms have been proposed to explain the beneficial effects of ACE-I on the failing heart, which include anti-hypertrophic, anti-oxidative, anti-inflammatory, and anti-fibrotic effects [5, 28]. All these effects lead to the inhibition of cardiac remodeling. In a canine model of heart failure, ACE-I has been shown to prevent myocardial apoptosis and thereby preserve cardiac function by unclarified intracellular mechanisms [29]. In Dahl rats as well, blockade of the Ang II-mediated pathway has a beneficial effect on myocardial hypertrophy and remodeling [25, 30, 31]. The present study may demonstrate a novel mechanism of the beneficial effects of ACE-I. The expression of TRPC7 mRNA in the myocardium was up-regulated in DS rats with heart failure, as compared with that in DR rats. This TRPC7 up-regulation was suppressed by the long-term treatment with temocapril. These results suggest that TRPC7-mediated myocardial apoptosis contributes to the process of heart failure in DS rats, and that ACE-I exerts a beneficial effect through the blockade of Ang II-TRPC7-induced apoptosis, although how TRPC7 is up-regulated in the failing heart remains to be elucidated.

In conclusion, TRPC7 could act as a Ca^{2+} channel activated by AT1–G protein interaction, thereby leading to myocardial apoptosis. This process might depend on a calcineurin-mediated pathway. TRPC7 might be a novel target for the treatment of heart failure, to which the apoptotic process may partially contribute.

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