Enhancement of Rho/Rho-kinase system in regulation of vascular smooth muscle contraction in tachycardia-induced heart failure

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Abstract


Methods: Heart failure (HF) was produced by chronic rapid RV pacing (250 bpm, 28 days, six dogs). Indo1-AM was loaded into endothelium-denuded femoral artery segments for measuring intracellular [Ca²⁺]. Tension and changes in intracellular [Ca²⁺] [the change in the ratio (418 nm / 468 nm) of Indo1 fluorescence (F)] were simultaneously measured in Krebs–Ringer solution.

Results: In HF: (i) norepinephrine (10 µM) produced greater tension (784 ± 652 g/cm) than in control (502 ± 64 g/cm) despite a similar increase in F, indicating increased Ca sensitivity in vascular smooth muscle; (ii) nifedipine attenuated this enhanced response by only a maximum of 27% at 1 µmol/l with a 56% reduction in F; (iii) Y-27632 attenuated it by a maximum of 80% at 100 µmol/l without a significant change in F; (iv) RhoA protein and mRNA expression levels in the femoral artery were up-regulated by 110% and 56%, respectively, while those of Rho-kinase were unchanged.

Conclusions: The Ca-sensitizing mechanism involving the Rho/Rho-kinase system may be deeply involved in the enhanced arterial vasoconstriction seen in HF. Since Y-27632 attenuated this response in small arteries, it shows potential as a novel, potent vasodilator for the treatment of HF. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Adrenergic (ant)agonists; Calcium (cellular); Heart failure; Vasoconstriction/dilation

1. Introduction

In vascular smooth muscle, an increase in intracellular [Ca²⁺] following stimulation to various receptors plays an important role in regulating vasomotor tone [1]. However, because the cytosolic concentration of Ca²⁺ is not always proportional to the extent of myosin light chain (MLC) phosphorylation and contraction, an additional mechanism — one that regulates the Ca²⁺ sensitivity of both processes — has been proposed [2]. In this regard, an inhibition of agonist-induced Ca²⁺-sensitization by GDPβS [3] and a Ca²⁺-sensitizing effect of GTPγS in permeabilized smooth muscle without a detectable change in [Ca²⁺] [4–6] established that an upstream, membrane-coupled G-protein was on a major pathway mediating Ca²⁺-sensitization [7,8].

Recently, RhoA — a small, monomeric G-protein, and a member of the Rho subfamily of the Ras family of G-proteins — has been identified as the upstream component.

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of a major pathway for physiological Ca²⁺-sensitization [9]. Many of the same receptors that activate the phosphatidylinositol cascade also activate RhoA, and with the help of GEFs, dissociate cytosolic RhoA-GDP from GDI. This allows the exchange of GTP for GDP on RhoA. Active RhoA-GTP activates Rho kinase, which then phosphorylates the regulatory subunit of myosin phosphatase and inhibits its catalytic activity [10]. Uehata et al. [11] demonstrated that Y-27632, an inhibitor of Rho kinase, reduced both Ca²⁺ sensitization and vascular contraction in response to a variety of G-protein-coupled receptor agonists.

Congestive heart failure (CHF) is a clinical condition associated with alterations in the normal balance of neurohumoral agents and factors that act on the vascular wall. In heart failure, the vasomotor tone increases in response to an increased activity in the sympathetic nervous system [12]. Several investigators have demonstrated that the sensitivity to the α₁-adrenoceptor agonists, phenylephrine and norepinephrine also increases during the development of heart failure [13]. However, since such changes were not seen with KCl, they may be unique to α-adrenoceptor function in CHF, and not due to general increases in contractile performance. It is also likely that the enhanced vascular contractile activity seen in CHF is due to events beyond receptor interaction, which hence may involve the G-protein-mediated pathway.

The goal of this study was to examine the involvement of the Rho/Rho-kinase system in the exaggerated vasoconstriction seen in heart failure.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Production of pacing-induced heart failure

Heart failure was induced in beagle dogs of either sex by 28 days of rapid right ventricular pacing at a rate of 250 bpm using an externally programmable miniature pacemaker (Medtronic inc., Minneapolis), as described previously [14–16]. In this model of heart failure, both LV end-diastolic and end-systolic diameter are significantly increased, in association with a decreased ejection fraction (<35%) [14–16].

The study group consisted of 12 beagle dogs weighing 10–15 kg, of which six were designated CHF and six were sham-operated controls. The care of the animals and the protocols used were in accord with guidelines laid down by the Animal Ethics Committee of Yamaguchi University School of Medicine.

2.2. General tissue bath experiments

Lengths of femoral arteries were withdrawn under anesthesia [2% isoflurane, 1.5 l/min, a mixture of nitrous oxide and oxygen (2:1)] and placed in Krebs bicarbonate solution containing (in mmol/l) 120 NaCl, 5.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.17 NaH₂PO₄, 10 d-glucose, and 25 NaHCO₃, which was continuously bubbled with 95% O₂–5% CO₂. Each length of artery was cleaned of adhering tissues, and a segment ~2–3 mm long was cut, mounted on wires, and suspended between two stainless-steel wires. One wire was in a fixed position and the other was connected to a force–displacement transducer (159901A Isometric Force Transducer; Radnoti, Monrovia, CA). The whole preparation was mounted in a two-hook 20-ml organ chamber and bathed in Krebs bicarbonate solution aerated with a mixture of 95% O₂ and 5% CO₂ at 37°C via a circulating water pump (RTE-100; NESLAB, NH). Segments were placed under a resting tension of 1.0 g and allowed to equilibrate for 1 h, during which frequent washing with Krebs bicarbonate solution was performed. In 4–5 preparations from normal dogs and 4–6 from dogs with heart failure, the endothelium was kept intact, while in all other preparations the endothelium was denuded as evidenced by a lack of relaxation to 1.0 μmol/l acetylcholine.

2.3. Experimental design

Concentration–effect curves for the response to norepinephrine (10⁻⁷–10⁻⁴ mol/l) were constructed cumulatively. Briefly, the lowest concentration of norepinephrine was administered, and the resulting contraction was allowed to develop until it reached a plateau, at which point another, higher concentration of norepinephrine was introduced. After control concentration–effect curves had been obtained, increasing concentrations of nifedipine (10⁻⁹–10⁻⁶ mol/l) or Y-27632 (10⁻⁷–10⁻⁴ mol/l) were added to the organ bath 15 min before the reconstruction of the concentration–effect curves. This equilibration period was chosen on the basis of preliminary data showing that an approximately 15-min exposure to nifedipine or Y-27632 was necessary to produce a peak inhibition of the norepinephrine-induced contraction. Consecutive concentration–effect curves were separated by a washout period of 1 h.

To assess whether the endothelium influences receptor-operated or membrane voltage-dependent contraction, the contraction induced by norepinephrine (1 μmol/l) or KCl (60 mmol/l) was assessed with an intact endothelium. The vasorelaxant effects of nifedipine and Y-27632 were also evaluated during the angiotensin II (0.1 μmol/l)-induced contraction. Angiotensin II induces a peak contraction at this dose [17]. Such an experiment could be performed once only with angiotensin II because of tachyphylaxis, as previously observed [17].
2.4. Calcium measurements

Arterial segments were loaded with Indo1-acetoxy-methyl ester (Indo1-AM; Molecular Probes, Eugene, OR) for 1–2 h at 20°C by incubating with 2 ml Krebs bicarbonate solution containing 10 μmol/l Indo1-AM dissolved in 10% cremophore (Sigma Chemicals, St. Louis, MO) [18,19]. The loaded segments were mounted in the organ chamber, and the Indo1 fluorescence signal during the vasoconstrictor response to norepinephrine was measured using a spectrophotometer (RSP-6015; Unisoku, Osaka, Japan). Light from a 450-W xenon–mercury lamp, filtered through a 360-nm interference filter, was used for excitation. Emitted fluorescent light was monitored by two photomultiplier tubes preceded by 418- and 468-nm interference filters with bandwidths of ±5 nm. The ratio of the 418- and 468-nm signals ($F_{ratio}$) provides a measure of intracellular $[Ca^{2+}]$ [19]. In an attempt to calibrate the signals, in some experiments we obtained minimum ($R_{min}$) and maximum ($R_{max}$) values for the fluorescence ratio. For this purpose, the tissues were incubated in the calcium ionophore ionomycin (50 μmol/l) for 40–60 min to obtain stable fluorescence signals [20]. The $R_{min}$ signal was obtained using nominally a $Ca^{2+}$-free solution containing 2 mmol/l EGTA, while the $R_{max}$ signal was obtained using a solution containing 10 mmol/l $Ca^{2+}$. However, ionomycin may be less effective in tissues than in single cells, and consistent $R_{min}$ and $R_{max}$ signals were obtained in less than 50% of all preparations, a value consistent with one in a previous report (25%) [18]. For that reason, the results are expressed as a fluorescence ratio rather than as absolute $[Ca^{2+}]$.

2.5. Immunoblot analysis for determination of Rho A and Rho kinase protein levels

Femoral arteries were immersed in iced Tris-buffer (5 mmol/l, pH 7.4) containing the protease inhibitors leupeptin (5 mg/l) and PMSF (0.1 mmol/l), then homogenized for 30 s using a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 100 g for 10 min to remove particulate matter and unbroken cells. The pellet was resuspended in Tris-buffer, and this fraction was rapidly frozen in liquid nitrogen and stored at −80°C. An aliquot was retained for protein assay using the method of Lowry et al. [21].

Immunoblot analysis was performed as previously described [22], with some modifications. The protein was electrophoresed on SDS–polyacrylamide gels using a Laemmli buffer system, and the proteins in the gel were transferred to a protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were then treated with 5% nonfat dry milk in phosphate-buffer, incubated with a solution containing commercially available monoclonal antibodies (anti-Rho kinase from Transduction Laboratories, Lexington, Sandiego, and anti-Rho A protein from Santa Cruz Biotechnology, CA), and incubated further with peroxidase-conjugated secondary antibody. The amount of protein recognized by the antibodies was quantified by means of an ECL immunoblotting detection system (Amersham, Bucks, UK), the membrane being exposed to X-ray film. Quantitative densitometry of immunoblots was performed using a microcomputer imaging device (AE-6900M; ATTO, Tokyo). The relative activity associated with the Rho A or Rho kinase in each sample was calculated by dividing the activity associated with the Rho A or Rho kinase protein products by the activity associated with the positive control (rat cerebellum for Rho A [23] and RSV-3T3 cell lysate (Rouse Sarcoma-infected 3T3 mouse fibroblast cell line) for Rho kinase).

2.6. RT-PCR

Total cellular RNA was isolated from each frozen tissue sample by the method of acid guanidinium thiocyanate/phenol/chloroform extraction [24], then stored at −80°C. cDNA was prepared using a Takara RNA PCR Kit (Takara, Tokyo, Japan), as previously described [25], in a buffer containing 10 mmol/l Tris–HCl, pH 8.3, 50 mmol/l KCl, 5 mmol/l MgCl$_2$, and 1 mmol/l each of dCTP, dGTP, dTTP, and dATP, with 20 units of recombinant ribonuclease inhibitor, 2.5 M random 9 mers, 1.3 μg of total RNA, 5 units of avian myeloblastosis virus reverse transcriptase, all in a volume of 20 μl. This reaction mixture was incubated for 10 min at 30°C followed by 30 min at 42°C to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at 99°C for 5 min, and this mixture was then used for the amplification of specific cDNAs by PCR. PCR was performed as follows: to 20 μl of the RT reaction mixture was added 2 μl of 0.1 mol/l forward primer, 2 μl of 0.1 mol/l reverse primer, 8 μl of 10× amplification buffer (100 mmol/l Tris–HCl, pH 8.3, 0.5 mol/l KCl), 12 μl of 25 mmol/l MgCl$_2$, 55 μl of H$_2$O, 0.5 μl of [α-32P]dCTP (Amersham), and 0.5 μl (2.5 U/100 μl) of Taq polymerase. The primers for the amplification of Rho A, Rho kinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed from published sequences. For Rho A, they were based on human sequences [26,27]: at positions 83–104 (sense primer, 5′-ACC AGT TCC CAG AGG TGT ATG T-3′) and 304–326 (antisense primer, 5′-TTG GGA CAG AAG TGC TTG ACT TC-3′) (predicted length of PCR product, 244 bp). For Rho kinase, they were based on human sequences [26,27]: at positions 495–521 (sense primer, 5′-GAG CAA CTA TGA TGT GCC TGA AAA AT-3′) and 985–1006 (antisense primer, 5′-GAT GTC GTT GAA CAG TGC TTG ACT TC-3′) (predicted length of PCR product, 512 bp). For GAPDH, they were based on human sequences [26,27]: at positions 102–125 (sense primer, 5′-CTT CAT TGA CCT CAA CTA CAT GGT-3′) and
2.7. Quantitation of PCR products

The optimal number of amplification cycles needed to allow quantitation of Rho A, Rho kinase, and GAPDH gene PCR products was determined. The PCR products for each cycle were subjected to 5% polyacrylamide gel electrophoresis (PAGE) and autoradiography, and the associated radioactivity was measured using an imaging analyzer (model BAS-2000; Fuji Photo Film Co., Tokyo, Japan). The optimal number of cycles was found to be 25 for Rho A, Rho kinase, and GAPDH.

2.8. Assessment of expression of Rho A and Rho kinase mRNA

The relative radioactivity associated with Rho A and Rho kinase PCR products in each sample was calculated by dividing the radioactivity associated with the Rho A and Rho kinase PCR products by the radioactivity associated with the GAPDH gene product (internal control; amplified simultaneously). Each level of RT-PCR product was obtained as the average of duplicate data. We used GAPDH as an internal control because the densitometric scores for the mRNAs did not differ between the groups of dogs. Furthermore, this enzyme of the glycolytic pathway is constitutively expressed in most tissues and is the most widely accepted internal control in the molecular biology literature [28].

2.9. Plasma neurohumoral measurements

Plasma norepinephrine, angiotensin II, and plasma atrial neuro-peptide (ANP) were measured by means of high-performance liquid chromatography [29], angiotensin II radioimmunooassay kit (SRL Co., Ltd., Tokyo, Japan) [30], and Siono-RIA ANP kit (Shionogi Co., Ltd., Osaka, Japan) [31], respectively.

2.10. Statistics

An unpaired t-test was used to compare data between normal and heart failure. Changes within the same group were analyzed by one-way analysis of variance (ANOVA) for repeated measures and subsequent Scheffe’s post-hoc test. Differences between two groups were analyzed by two-way ANOVA and subsequent Scheffe’s post-hoc test. Statistical significance was defined by $P<0.05$.

3. Results

3.1. Neurohormonal activity in congestive heart failure

As summarized in Table 1, plasma norepinephrine, angiotensin II, and ANP were all higher with rapid chronic pacing than in sham-operated dogs.

3.2. Contractile responses to norepinephrine and K$^+$

Fig. 1 shows norepinephrine- and K$^+$-induced contractions of arterial segments in the presence or absence of endothelium, and Table 2 summarizes the maximum contractile responses to norepinephrine (1 $\mu$mol/l) and K$^+$ (60 mmol/l). In heart failure, the contractile response to norepinephrine was greater than normal, whether or not the endothelium was intact. However, in accord with previous reports [13,32] there was no difference in the contractile response to K$^+$ between normal and heart failure groups, whether or not the endothelium was intact.

As shown in Fig. 2, the cumulative addition of norepinephrine in concentrations ranging from $10^{-7}$ to $10^{-4}$ mol/l produced concentration-dependent increases in tension in normal and in heart failure dogs. The maximum response to norepinephrine was significantly greater in the heart failure than in the normal group at all concentrations of norepinephrine. However, there was no significant difference in EC50 between normal (1.21 ± 0.10 $\mu$mol/l) and heart failure (1.04 ± 0.2 $\mu$mol/l) groups.

3.3. Effects of nifedipine and Y-27632 on contractile responses to norepinephrine

Fig. 3 shows concentration–effect curves for the effects of increasing concentrations of nifedipine or Y-27632 on the responses to two concentrations of norepinephrine. Nifedipine and Y-27632 each produced a concentration-dependent decrease in the norepinephrine-induced tension at each concentration of norepinephrine. However, the inhibition was much larger with Y-27632 than with nifedipine. In fact, in heart failure the enhanced contractile response to norepinephrine was almost completely in-

<table>
<thead>
<tr>
<th>Hormonal data$^*$</th>
<th>Norepinephrine (pg/ml)</th>
<th>Angiotensin II (pg/ml)</th>
<th>ANP (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ($n=5$)</td>
<td>186±104</td>
<td>25±10</td>
<td>31±7</td>
</tr>
<tr>
<td>Heart failure ($n=5$)</td>
<td>612±165*</td>
<td>145±27*</td>
<td>205±59*</td>
</tr>
</tbody>
</table>

$^*$ Data represent mean±S.D. The number of experiments is indicated in parenthesis.

$^{*}P<0.01$ vs. normal.
Fig. 1. Time course of vasoconstriction in response to 1 μmol/l norepinephrine (A, B) or 60 mmol/l K⁺ (C, D) in the presence or absence of endothelium (ET). Normal (—), Heart failure (—). Note that the contractile response to norepinephrine was greater in heart failure than in the normal condition whether or not the endothelium was intact.

Inhibited by Y-27632, whereas nifedipine only partially inhibited it.

3.4. Effects of nifedipine and Y-27632 on tension–[Ca²⁺] relationship for norepinephrine-induced contraction

Fig. 4 shows simultaneous time courses for tension and the Indo-1 fluorescence ratio. In normal and heart failure groups, both tension and \( F_{\text{ratio}} \) increased after the addition of norepinephrine. In the presence of nifedipine (1 μmol/l), tension and \( F_{\text{ratio}} \) both showed a smaller increase in response to norepinephrine, the \( F_{\text{ratio}} \) response being more strongly affected than the tension response. In the presence of Y-27632, however, the tension response was decreased with no change in the \( F_{\text{ratio}} \) response. Fig. 5 summarizes the relation between tension and \( F_{\text{ratio}} \) after the addition of norepinephrine in the absence or presence of nifedipine and Y-27632. In heart failure group, norepinephrine (1 μmol/l) produced a higher tension than in the normal group.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Tension (g·cm⁻²)</th>
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<th>KCl (60 mmol/l)</th>
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<tbody>
<tr>
<td></td>
<td>Norepinephrine (1 μmol/l)</td>
<td></td>
<td>KCl (60 mmol/l)</td>
<td></td>
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<tr>
<td>ET (−)</td>
<td>ET (+)</td>
<td></td>
<td>ET (−)</td>
<td>ET (+)</td>
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<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>297±41 (5)</td>
<td>289±38 (4)</td>
<td>308±17 (5)</td>
<td>300±10 (6)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>486±38* (5)</td>
<td>481±48* (4)</td>
<td>311±24 (5)</td>
<td>289±22 (5)</td>
</tr>
</tbody>
</table>

*Data represent mean±S.D. The number of experiments is indicated in parenthesis.

\*P<0.01 vs. normal.
group despite a similar increase in $F_{\text{ratio}}$, indicating an increase in the Ca$^{2+}$ sensitivity of the vascular smooth muscle in heart failure. In the heart failure group (i) nifedipine inhibited this norepinephrine-induced enhanced vasoconstriction by only a maximum of 27% at 1 μmol/l, although this was associated with a 56% reduction in $F_{\text{ratio}}$; (ii) in contrast, Y-27632 inhibited it by a maximum of 80% at 100 μmol/l without a significant change in $F_{\text{ratio}}$.

3.5. Effects of nifedipine and Y-27632 on angiotensin II-induced contraction

Fig. 6 shows the time course of the tension response to angiotensin II (0.1 μmol/l). In both normal and heart failure groups, the tension curve showed a spontaneous decline after reaching a peak in response to angiotensin II. As shown in Fig. 7, angiotensin II induced a higher peak tension in heart failure than in the normal group, as in the case of norepinephrine. Once again, Y-27632 caused a larger inhibition of the contraction (this time, induced by angiotensin II) than nifedipine.

3.6. mRNA and protein expression levels for RhoA and Rho-kinase

Fig. 8 shows the expression levels of Rho A protein (A) and Rho A mRNA (B) in femoral arteries isolated from heart failure dogs and from normal dogs. The level of Rho A protein was significantly higher in heart failure (0.82±0.09) than in the normal animals (0.40±0.20), as was the expression level of the mRNA encoding Rho A (0.28±0.08 vs. 0.18±0.04). However, there was no significant difference in the Rho-kinase protein or mRNA expression levels between the heart failure and normal groups.

4. Discussion

It is commonly accepted that contraction of the smooth muscle cell mainly depends upon the concentration of Ca$^{2+}$ within the cytoplasm. Two major pathways exist at the level of the sarcolemma for the entry of Ca$^{2+}$ in response to appropriate stimuli: voltage-operated Ca$^{2+}$ channels and receptor-operated Ca$^{2+}$ channels [33]. Membrane depolarization and ligand binding to the receptor, respectively, result in the opening of these channels, allowing Ca$^{2+}$ to diffuse into the cell. Studies in which force and Ca$^{2+}$ have been simultaneously measured in intact smooth muscle tissue have suggested that the force/Ca$^{2+}$ ratio is greater in agonist-stimulated contractions than in [K$^+$]-stimulated contractions [34]. Indeed, there is evidence showing that stimulation of receptors significantly increases the myofilament Ca$^{2+}$ sensitivity of
smooth muscle [34]. Recently, the key element in the modulation of Ca\textsuperscript{2+} sensitivity was identified as a small, monomeric G-protein, RhoA [10].

Changes in peripheral vascular sensitivity to \(\alpha\)-adrenoceptor agonists have been demonstrated in certain models of hypertension and diabetes [35,36]. Further, in pacing-induced canine heart failure an enhanced reactivity of blood vessels to \(\alpha\)-adrenoceptor agonists has been noted [37], and this enhanced response occurred early in the development of the heart failure [38]. We confirmed here that the vascular reactivity to norepinephrine was significantly enhanced in heart failure, by comparison with the normal condition. Moreover, the present study — by assessing the Ca\textsuperscript{2+}-desensitizing effect of Y-27632 upon norepinephrine-induced vasoconstriction in parallel with the effect of a Ca\textsuperscript{2+}-antagonist, nifedipine — permitted a
greater insight into the mechanism underlying this enhanced vasoconstriction.

The major findings of this study are as follows. First, the enhancement of the norepinephrine-induced vasoconstriction in heart failure was largely ascribable to an increase in Ca\(^{2+}\) sensitivity rather than to an increase in cytoplasmic free Ca\(^{2+}\). Second, nifedipine inhibited this enhanced contraction by only a maximum of 27% in association with a decrease of 56% in free [Ca\(^{2+}\)]. In contrast, Y-27632 inhibited it by a maximum of 80% with no change in free [Ca\(^{2+}\)], indicating that the enhancement of the vasoconstriction was indeed induced by a marked increase in Ca\(^{2+}\) sensitivity mediated through activation of the Rho/Rho-kinase system. Third, immunoblot and RT-PCR analyses showed that the expressions of RhoA protein and its mRNA were increased in heart failure, whereas the expressions of Rho kinase protein and mRNA were unchanged (Fig. 9). Possibly, the mode of expression of these two proteins may be differently regulated in heart failure.

Another subject of this study was the role of the renin–angiotensin system (RAS) in the evolution of heart failure. This system has been considered a compensatory mechanism that initially acts to increase blood pressure and renal perfusion, although it appears to have an important role in elevating systemic vascular resistance in advanced heart failure.
failure [39]. Recently, a local type of RAS has been identified in a variety of tissues, including vascular smooth muscle, and several lines of evidence suggest that the angiotensin II produced by local RAS is heavily involved in the increase in systemic vascular resistance caused by the enhanced vasoconstriction seen in heart failure [40]. Interestingly, Y-27632 exhibited a counteracting (vasodilating) action against both agonists (norepinephrine and angiotensin II), suggesting that an exaggerated activation of the Rho/Rho-kinase system is common to the mechanisms underlying the enhanced vasoconstrictor response to these two agonists.

Since endothelial dysfunction has been said to contribute to the increased vascular resistance seen in heart failure [41,42], we examined the vasoconstrictor responses to norepinephrine and K⁺ under both endothelium-intact and endothelium-denuded conditions. In fact, the contractile response of vascular segments to norepinephrine was augmented in heart failure to a degree that did not depend on the existence of the endothelium (Table 2). This finding is consistent with the observation that the acetylcholine-induced relaxation response in the dorsal pedal artery remains unaltered in experimental heart failure [13].

Before drawing firm conclusions, several issues remain to be resolved. First, a Ca²⁺ channel blocker, nifedipine, also significantly inhibited the enhanced vasoconstriction induced by norepinephrine in heart failure, although the inhibition was smaller than that produced by the Rho-kinase inhibitor, Y-27632. In accord with this, Forster et al. [43] showed a significant inhibition by nifedipine of the enhanced vasoconstriction induced by norepinephrine in the same model of heart failure. A relevant finding may be that of Han et al. [44], who demonstrated that two distinct

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**Fig. 8.** Expression level of Rho A protein (A) and mRNA (B). Normal (n=6), Heart failure (n=6). Data represent mean±S.D.

**Fig. 9.** Expression level of the Rho kinase protein (A) and mRNA (B). Normal (n=6), Heart failure (n=6). Data represent mean±S.D.
subtypes of α1-adrenoceptor cause contractile responses, one of which causes contractions that require an influx of extracellular Ca\(^{2+}\) through dihydropyridine-sensitive channels.

Second, a causal link needs to be established between the observed up-regulation of RhoA in heart failure and the increased Ca\(^{2+}\) sensitivity of vascular smooth muscle (and the resulting enhanced vasoconstriction). With regard to this point, Kimura et al. [10] demonstrated a few years ago that an over-expression of Rho A increased the phosphorylation of the myosin-binding subunit of myosin phosphatase, an event that leads to the inactivation of myosin phosphatase.

Third, in heart failure structural and functional alterations in the components of vascular beds (such as smooth muscle, endothelium, and nerve endings) occur under the control of various neurohumoral factors [1]. Therefore, more work is clearly needed to elucidate whether, and to what extent, the Rho/Rho-kinase system contributes to enhanced vasoconstriction in the intact circulation in heart failure.

Fourth, enhanced vascular contractile activity seen in heart failure may be due to the quantitative or qualitative alteration at receptor level in tachycardia-induced heart failure. Luchner et al. [45] demonstrated that in tachycardia-induced heart failure the tissue angiotensin II was increased in heart and aorta, suggesting that local renin–angiotensin-system is activated in this model. Also, Foster et al. [13] showed that vaso-contractile response to selective α1-agonist, phenylephrine was enhanced in tachycardia-induced heart failure, suggesting that α1-adrenoceptor activity in arterial smooth muscle increases in congestive heart failure. To our knowledge, there is no direct evidence that the density of angiotensin II receptor or α receptor in vascular wall changes in a model of tachycardia-induced heart failure. However, since the receptor density has been shown to change dramatically in other cardiac hypertrophy and/or failure (up-regulation of AT1 receptor in experimental infarct myocardium [46], down-regulation of AT1 receptor in human failing myocardium [47,48], unchanged α1 receptor density in human failing heart [49]), it is likely that these changes in the receptor density also occur in vascular wall, contributing to the hyper-vasoconstriction seen in this model of heart failure.

Fifth, although this model of tachycardia-induced heart failure causes well-defined, predictable, and progressive LV dilatation, contractile dysfunction, and neurohumoral activation [50], it does not resemble chronic heart failure inasmuch as the pacing-induced failure lacks a hypertrophic compensatory phase [51], and disappears if pacing is discontinued after 3 weeks [52]. Clearly, research using other models of heart failure is needed if we are fully to elucidate the alterations in vascular contractile function seen in chronic heart failure.

In conclusion, a G-protein-coupled increase in myofilament Ca\(^{2+}\) sensitivity mediated through the Rho/Rho-kinase system is heavily involved in the mechanism underlying the enhanced vasoconstriction observed in heart failure. This finding may point the way to a new vasodilator therapy, a form of therapy now recognized as the most important in severe congestive heart failure.

Acknowledgements

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