Angiotensin II Receptor Subtypes in the Skeletal Muscle Vasculature of Patients With Severe Congestive Heart Failure

Slawomir L. Malendowicz, MD; Pierre V. Ennezat, MD; Marco Testa, MD, PhD; Laura Murray, BSc; Edmund H. Sonnenblick, MD; Todd Evans, PhD; Thierry H. LeJemtel, MD

**Background**—Vascular remodeling occurs in the skeletal muscle of patients with severe congestive heart failure (CHF); this remodeling is mediated in part by increased activity of the renin-angiotensin system. Animal models suggest that in the vasculature, angiotensin II receptor type 2 (AT2-R) expression may be upregulated in pathological states associated with vascular remodeling. The therapeutic effects of an AT1-R antagonist may, therefore, be in part due to increased plasma angiotensin II levels, which stimulate AT2-R. However, whether AT2-R is expressed in the skeletal muscle vasculature of patients with severe CHF is unknown.

**Methods and Results**—The steady-state transcript levels of the AT1-R and AT2-R genes were analyzed by reverse transcription–polymerase chain reaction in RNA samples prepared from the skeletal muscle of 12 patients with severe CHF (V\text{O}_2 <10 \text{ mL \cdot kg}^{-1} \cdot \text{min}^{-1}) and 5 age-matched healthy subjects who underwent vastus lateralis biopsies. Human fetal skeletal muscle RNA served as a positive control for the expression of AT1-R and AT2-R gene transcripts. Transcripts from the AT1-R gene were detected readily in all samples. In contrast, transcripts from the AT2-R gene were only detected in fetal skeletal muscle samples and could not be detected in the skeletal muscle vasculature of healthy subjects or that of CHF patients, who were treated with either angiotensin-converting enzyme inhibitors or AT1-R antagonists.

**Conclusions**—The AT2-R gene is not expressed in the skeletal muscle of patients with CHF. In the absence of detectable AT2-R gene transcripts, the AT2-R pathway is unlikely to contribute to the effects of AT1-R antagonists on the skeletal muscle vasculature in patients with severe CHF. (*Circulation*. 2000;102:2210-2213.)

**Key Words:** angiotensins • receptors • heart failure • muscle, skeletal

Increased activity of the renin-angiotensin system is an important contributor to cardiac and vascular remodeling in patients with congestive heart failure (CHF). This increased activity and the resulting elevated plasma and tissue levels of angiotensin II exert remodeling effects by activating transduction pathways modulated by angiotensin II receptors type 1 (AT1-R) and 2 (AT2-R). Vascular remodeling in patients with CHF reduces skeletal muscle perfusion and peak aerobic capacity during exercise. However, angiotensin-converting enzyme (ACE) inhibitors induce improvement in skeletal muscle perfusion and enhance peak aerobic capacity in patients with CHF.

The importance of the renin-angiotensin system in vascular remodeling has been recently underscored by the results of the Heart Outcomes Prevention Evaluation (HOPE) trial. In the absence of left ventricular dysfunction, the ACE inhibitor ramipril reduced mortality in patients with coronary vascular disease, stroke, and peripheral arterial disease. Most of the known effects of angiotensin II, including vasoconstriction, hypertrophy, cellular growth, catecholamine release, and aldosterone secretion, are mediated by the AT1-R. The AT2-R pathway counters the effects of the AT1-R pathway and has been associated with growth inhibition, apoptosis induction, and bradykinin-mediated vasodilatation. Treatment with AT1-R antagonists further increases angiotensin II levels, thereby stimulating AT2-R activity in patients with CHF who are not conjointly treated with ACE inhibitors. Thus, the stimulation of AT2-R induced by a further increase in angiotensin II levels may contribute to the therapeutic benefits of AT1-R antagonists in patients with CHF.

During human development, the AT2-R gene is expressed in the fetal but not the adult skeletal muscle vasculature. The purpose of the present study was to determine whether the vascular remodeling that occurs in the skeletal muscle beds of patients with severe CHF is associated with the re-expression of the AT2-R gene.
Methods

Study Population

Twelve patients with New York Heart Association class IV CHF and 5 age-matched healthy subjects participated in the study. The patient group included 4 women and 8 men whose age, left ventricular ejection fractions, and peak VO$_2$, averaged 62±6 years, 24±6%, and <10 mL·kg$^{-1}$·min$^{-1}$, respectively. The cause of CHF was ischemic cardiomyopathy in 10 patients and nonischemic cardiomyopathy in 2. Medical therapy consisted of ACE inhibitors (lisinopril, n=4; fosinopril, n=3; 40 mg/d) or AT1-R antagonists (160 mg/d valsartan, 5), furosemide (mean dose, 114±40 mg/d), and digoxin in all patients, long-acting oral nitrate preparations in 10 patients, and β-adrenergic blockers in 2 patients. The control group consisted of 5 sex- and age-matched healthy subjects who had a peak VO$_2$ of >30 mL·kg$^{-1}$·min$^{-1}$. The Ethical Review Board of the Albert Einstein College of Medicine approved the study. All patients gave written informed consent before the study.

Muscle Biopsies

Biopsies of the vastus lateralis muscle 15 cm above the patella were performed using a needle biotome (Bard). A sample of the vastus lateralis muscle taken from a 23-week-old fetus was provided by the Albert Einstein College of Medicine Tissue Bank. All samples were immediately plunged in liquid nitrogen and transferred to a freezer, where they were kept at −80°C until analysis.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction Analysis of Skeletal Muscle Samples

Each tissue sample was transferred to a 6-mL polypropylene tube containing 1 mL of Trizol (Gibco/BRL) and was homogenized with a hand-held tissue tearer (Biospec Products). Extracted RNA was isolated by isopropanol precipitation. After the nucleic acids were washed in 70% ethanol, they were resuspended in distilled water and the DNA was removed by digestion with 1 U of RQ1 RNase-free DNase (Promega Corp) for 10 minutes at 37°C. The concentration of purified RNA was determined by optical density and then diluted to 0.125 μg/μL. Each RNA sample (0.5 μg) was reverse-transcribed at 37°C for 90 minutes in a 20-μL reaction containing 100 pmol of the random hexamer pdN6 (Pharmacia) and 40 U of murine leukemia virus reverse transcriptase (Gibco/BRL).

For polymerase chain reaction (PCR), 2 μL of each reverse transcription (RT) product was used in a 20-μL reaction containing 1.5 mmol/L MgCl$_2$, 1.0 mmol/L each primer, 200 μmol/L deoxyribonucleoside triphosphate, and 0.1 μL of α$^{32}$P-dCTP as a tracer, when applicable. Fetal skeletal muscle RNA served as a positive control for expression of AT1-R and AT2-R gene transcripts. The integrity of all RNA samples was analyzed by amplifying transcripts for smooth muscle α-actin, β-actin, von Willebrand factor, and GAPDH. Mock reactions that lacked only the RT enzyme were conducted for all samples to exclude the presence of products derived from genomic DNA. Primer sequences are shown in the Table.$^{11–15}$

<table>
<thead>
<tr>
<th>Primer Sequences</th>
<th>AT1-R forward</th>
<th>AT1-R reverse</th>
<th>AT2-R forward</th>
<th>AT2-R reverse</th>
<th>Smooth muscle α-actin forward</th>
<th>Smooth muscle α-actin reverse</th>
<th>β-Actin forward</th>
<th>β-Actin reverse</th>
<th>GAPDH forward</th>
<th>GAPDH reverse</th>
<th>Von Willebrand factor forward</th>
<th>Von Willebrand factor reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5’-GAT GAT TGG CCC AAA AGT G 6</td>
<td>5’-TAG GTA ATT GCC AAA AGG C 11</td>
<td>5’-TTT CCT TCC ATG TCC TGA C 6</td>
<td>5’-AAA CAC ACT GGC GAG CTT CT 12</td>
<td>5’-CCA GCT ATG TGA AGA AGA AGA G 5</td>
<td>5’-GTG ATC TTC TCC TGC ATT CGG T 13</td>
<td>5’-AAG GAT TCC TAT GTG GGC 5</td>
<td>5’-CAT TTC CTC GCA GAA GTC 11</td>
<td>5’-CAT GGC AAA TCC CAT GGC ACC GT 5</td>
<td>5’-TGG GGA CAC GGA AGG CCA TGC 14</td>
<td>5’-TCC TGC AGT TCC CCA GCT TC 5</td>
<td>5’-CTT CTC ATT CCC ATC TCT C 15</td>
</tr>
</tbody>
</table>

Results

Total RNA was purified from the skeletal muscle of healthy individuals or CHF patients and used in PCR reactions to determine the presence of specific transcripts. As a positive control for AT1-R and AT2-R, RNA was also derived from a fetal sample of skeletal muscle because both genes are expressed at this stage of development.$^{2}$ The PCR conditions used were not designed necessarily to be quantitative but rather to ensure very high sensitivity for detecting relatively low transcript levels. Transcripts encoding AT1-R, von Willebrand factor, and GAPDH were detected readily, as expected, in all samples; some examples are shown in Figure 1. The GAPDH product is derived from a highly expressed housekeeping gene and serves as a positive control for general RNA integrity; products were also detected similarly in all samples using primers specific for smooth muscle α-actin or β-actin (data not shown). The von Willebrand factor gene is a specific marker for endothelial cells, and it was analyzed here to demonstrate the presence of vascular tissue in each of the biopsied samples.

Figure 1. Representative examples of RT-PCR products detecting expression of AT1-R, von Willebrand factor (VWF), and GAPDH in skeletal muscle of patients with severe CHF and healthy controls. FT indicates fetal skeletal muscle; NL, skeletal muscle from normal control; ARB, skeletal muscle sample from patient treated with AT1-R antagonist; and ACEI, skeletal muscle sample from patient treated with ACE inhibitor.
microcirculation of the skeletal muscle. In the adult rat throughout the aortic wall, with higher levels reported in the aorta and can be detected by autoradiography. In the rat vasculature, the AT2-R gene is expressed at low levels in the aorta and can be detected by autoradiography. To increase the level of sensitivity, PCR reactions were amplified in the presence of trace $^{32}$P-dCTP, and products were analyzed by phosphorimage analysis after polyacrylamide electrophoresis. As shown in Figure 3, AT2-R gene transcripts were measurable in fetal skeletal muscle samples diluted at least 100-fold, but they could not be detected in undiluted skeletal muscle RNA from normal subjects or CHF patients. Therefore, we confirm that AT2-R gene expression is restricted to fetal-stage skeletal muscle and conclude that the gene is not re-expressed in CHF patients, even at 1/100 this level.

**Discussion**

In the rat vasculature, the AT2-R gene is expressed at low levels in the aorta and can be detected by autoradiography throughout the aortic wall, with higher levels reported in the microcirculation of the skeletal muscle. In the adult rat skeletal muscle microcirculation, AT2-Rs were localized using anti–AT2-R antibodies to the endothelial cells, vascular smooth muscle cells, fibroblasts, mast cells, macrophages, and pericytes. The AT2-R gene is expressed in endothelial cells derived from the spontaneous hypertensive rat coronary arteries but not in endothelial cells derived from bovine aorta or porcine peripheral arteries.

The expression of AT2-R in the adult rat skeletal muscle vasculature is in apparent contrast to normal human adults. The AT2-R gene is highly expressed during fetal development, but its products decrease rapidly after birth and, in the adult, they are restricted to the adrenals, uterus, ovary, lung, heart, and specialized nuclei in the brain. In human primary cell cultures, AT2-R transcripts are found in coronary endothelial cells using RT-PCR, but the receptors are not detected by radioligand assay in vascular smooth muscle cells from the renal artery. In the human kidney, AT2-R expression was localized using in situ hybridization to the medial layer of the interlobular arteries. In the failing heart, AT2-R expression is unchanged or upregulated, whereas AT1-R expression levels are decreased.

Importantly, the similar level of von Willebrand factor and smooth muscle $\alpha$-actin transcripts measured in all samples (and detected easily in both fetal and adult skeletal muscle samples) indicates that the endothelial and smooth muscle cell compartment was not under-represented in the biopsy samples. Because endothelial cells are the primary site of AT2-R expression, this control provides confidence that the experiments are not biased by cell-type differences among samples.

Forced overexpression of the AT2-R gene in transgenic mice is sufficient to offset a AT1-R–mediated vasoconstrictive and pressor effect through an endothelium-dependent mechanism. AT2-R–mediated vasodilation is caused by paracrine effects of bradykinin liberated from vascular smooth muscle cells, which leads to the activation of the endothelial bradykinin type 2 receptor–mediated nitric oxide/cGMP system. However, AT2-R gene expression could not be detected in the skeletal muscle vasculature of adult controls or patients with severe CHF treated with ACE inhibitors or AT1-R antagonists. The RT-PCR assay was

**Figure 2.** AT2-R gene transcripts are not detected in skeletal muscle vasculature of healthy subjects (NL) or patients treated with either AT1-R antagonists (ARB) or ACE inhibitors (ACEI). In fetal skeletal muscle RNA samples, 191-bp AT2-R gene transcripts are detected in undiluted samples (F) and samples at 1:10 dilution. No product was detected by ethidium bromide staining using 1:100 or 1:1000 dilutions of fetal mRNA. M indicates DNA ladder mix (Gene Ruler), with 200-bp marker indicated.

**Figure 3.** Transcripts of AT2-R gene were detectable with RT-PCR using $^{32}$P-dCTP as tracer in fetal skeletal muscle RNA sample diluted up to 100-fold with RNA from peripheral blood. No expression of AT2-R gene is detectable in skeletal muscle from normal control subjects (NL) or from patients treated with either AT1-R antagonists (ARB) or ACE inhibitors (ACEI).
sensitive enough to detect AT2-R gene expression easily in a 1:100 dilution of fetal skeletal muscle mRNA. The lack of expression of the AT2-R gene in the skeletal muscle vasculature indicates that direct vasodilatation resulting from AT2-R activation by increasing levels of circulating angiotensin II is unlikely to contribute to the relaxing effects of AT1-R blockade on the skeletal muscle beds.

The purpose of this study was to evaluate the expression of AT2-R at the transcriptional level. It would be interesting to confirm the lack of AT2-R at the level of protein or signaling activity. Unfortunately, because of the limiting size of biopsy material in this clinical study, it was not feasible to investigate the presence of AT2-R using ligand binding or Western blotting assays. However, considering that the highly specific and sensitive RT-PCR assay was negative for AT2-R mRNA in skeletal muscle samples, the presence of protein without RNA is unlikely.

The absence of AT2-R in the skeletal muscle vasculature of patients with CHF is compatible with the recent observation that AT1-R antagonism with losartan does not seem to offer added benefits over ACE inhibition with captopril.26 In summary, the AT2-R gene is not re-expressed in the skeletal muscle vasculature of patients with CHF and severely decreased peak aerobic capacity. In the absence of detectable AT2-R gene transcripts, the AT2-R pathway is unlikely to contribute to the effects of AT1-R antagonists on the skeletal muscle vasculature of patients with severe CHF.

References
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