ANG II is required for optimal overload-induced skeletal muscle hypertrophy

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Gordon, Scott E., Bradley S. Davis, Christian J. Carlson, and Frank W. Booth. ANG II is required for optimal overload-induced skeletal muscle hypertrophy. Am J Physiol Endocrinol Metab 280: 150–159, 2001.—ANG II mediates the hypertrophic response of overloaded cardiac muscle, likely via the ANG II type 1 (AT1) receptor. To examine the potential role of ANG II in overload-induced skeletal muscle hypertrophy, plantaris and/or soleus muscle overload was produced in female Sprague-Dawley rats (225–250 g) by the bilateral surgical ablation of either the synergistic gastrocnemius muscle (experiment 1) or both the gastrocnemius and plantaris muscles (experiment 2). In experiment 1 (n = 10/group), inhibiting endogenous ANG II production by oral administration of an angiotensin-converting enzyme (ACE) inhibitor during a 28-day overloading protocol attenuated plantaris and soleus muscle hypertrophy by 57 and 96%, respectively (as measured by total muscle protein content). ACE inhibition had no effect on nonoverloaded (sham-operated) muscles. With the use of new animals (experiment 2; n = 8/group), locally perfusing overloaded soleus muscles with exogenous ANG II (via osmotic pump) rescued the lost hypertrophic response in ACE-inhibited animals by 71%. Furthermore, orally administering an AT1 receptor antagonist instead of an ACE inhibitor produced a 48% attenuation of overload-induced hypertrophy that could not be rescued by ANG II perfusion. Thus ANG II may be necessary for optimal overload-induced skeletal muscle hypertrophy, acting at least in part via an AT1 receptor-dependent pathway.

SKELETAL MUSCLE adapts to its pattern of usage in humans (17) and animal models (2, 7, 16). For instance, functionally overloading the plantaris muscle by ablation of the synergistic gastrocnemius muscle in rat hindlimbs results in a 40% increase in mass and a 15% increase in muscle protein content in just 5 days (16). The mechanisms leading to skeletal muscle hypertrophy in response to mechanical overload are still unclear, although it is well established that input from both mechanical and hormonal stimuli is necessary for optimal skeletal muscle cell hypertrophy and/or skeletal α-actin gene promoter activity (a marker of muscle cell hypertrophy) in vivo (29) and in vitro (6, 35, 36). Furthermore, skeletal myotubes in culture exhibit stretch-induced growth only in the presence of both cell-released (paracrine/autocrine) and serum-dependent (endocrine) factors (35). Overall, the hormonal component of overload-induced skeletal muscle hypertrophy likely acts via endocrine and paracrine/autocrine input from some combination of at least 20 different hormones (4, 17), yet the potential role of ANG II has never been examined in this respect.

ANG II is strongly involved in overload-induced hypertrophy of cardiac muscle cells. The renin-angiotensin system (RAS), in which the precursor peptide angiotensigen is converted to ANG I by renin and subsequently to ANG II by angiotensin-converting enzyme (ACE), has an important endocrine function in control of systemic blood pressure (10, 25, 34). However, a local RAS has been identified in over a dozen tissue types in the past 20 years (25). This component of overload-induced cardiac hypertrophy can be attributed to autocrine/paracrine fashion. For instance, stretch overload quickly elicits a pronounced ANG II secretion in cultured cardiac myocytes (30). Additionally, whole animal models of overload-induced cardiac hypertrophy such as aortic restriction and coronary ligation in rats induce an upregulated gene expression of RAS components in the heart within 1 wk, including renin, angiotensigen, and ACE mRNA abundances, ACE activity, and intracellular renin, ANG I, and ANG II peptide contents (1, 8, 42). ACE activity was not increased in the plasma, providing further evidence that ANG II acts in an autocrine/paracrine fashion during cardiac hypertrophy (8). Moreover, the administration of an ACE inhibitor during cardiac overload prevented both the increase in cardiac tissue ACE activity and cardiac hypertrophy independent of blood pressure changes (1, 8). Similarly, the 15–20% left ventricular hypertrophy induced by 1 wk of systemic ANG II infusion in rats also occurs in a blood-pressure-independent manner (10, 34). Thus ANG II apparently acts directly on the heart to promote cardiac hypertrophy. This effect is probably mediated through the ANG II type 1 (AT1) receptor, since...
treatment with AT1 receptor antagonists prevents overload- and ANG II-induced cardiac hypertrophy in whole animals (10, 22, 34). Smooth muscle growth is also induced by ANG II in a fashion similar to that seen in cardiac myocytes (28). Administration of ANG II to cultured smooth muscle cells results in increases in smooth muscle α-actin expression (14), protein synthesis (3), and cell hypertrophy (12). Furthermore, ACE expression is increased in smooth muscle cells during vascular repair in humans (23). Not surprisingly, the smooth muscle cell growth response to injury is reduced or blocked by ACE inhibitors or AT1 receptor blockade in rats (28).

Because ANG II positively mediates overload-induced cardiac hypertrophy and smooth muscle growth, it was therefore the purpose of this investigation to examine the role of ANG II in the skeletal muscle hypertrophic response to mechanical overload. Here we show for the first time that ANG II is necessary for optimal overload-induced skeletal muscle hypertrophy. Furthermore, this effect appears to be at least partly mediated by the AT1 receptor.

METHODS

This investigation consisted of two separate experiments. Experiment 1 examined the effect of inhibiting ANG II production with an ACE inhibitor on compensatory hypertrophy of the plantaris and soleus muscles during a 28-day functional overload period. Experiment 2 examined the effect of local ANG II perfusion on the soleus muscle during a 10-day functional overloading period in animals undergoing either ACE inhibition or an AT1 receptor blockade. Experiment 2 also examined the effect of local ANG II perfusion on nonoverloaded soleus muscle.

Animals (experiments 1 and 2). Adult female Sprague-Dawley rats (225–250 g; Harlan) were individually housed at the University of Texas-Houston Health Science Center (UTHHSC) animal care facility, kept on a 12:12 h light-dark cycle, and given ad libitum access to water and rodent chow. During the overloading periods, body weights and food intakes were measured weekly (experiment 1) or every 5 days (experiment 2), whereas water intakes were measured daily in both experiments. Body weight values in experiment 2 were corrected for weight of the pump implants when appropriate. All procedures were approved by the UTHHSC Animal Care and Use Committee.

Design of experiment 1. Experiment 1 consisted of the following four groups of rats (n = 10/group): sham surgery (Sham), sham surgery with ACE inhibitor treatment (Sham/A-CE-I), overload surgery (Ovld), and overload surgery with ACE inhibitor treatment (Ovld/A-CE-I). The experimental treatment period lasted 28 days starting with the initial sham or overload surgery. During this time, the plantaris and soleus muscles of the animals in the Ovld groups were subjected to functional overload via bilateral gastrocnemius ablation, whereas the ACE-I groups received an ACE inhibitor in their daily drinking water. In anticipation that ACE inhibition might possibly promote skeletal muscle atrophy due to inhibition of maturation-related muscle growth over the 28-day period, an additional group of body weight-matched rats (n = 10) was killed on the day of initial survival surgery to assess muscle mass at that time point.

Design of experiment 2. As described in detail later, all rats in experiment 2 were implanted with osmotic pumps to enable continuous local perfusion of the right soleus muscle with either saline or ANG II. The following seven groups of rats (n = 8/group) were used in this experiment: sham surgery with saline perfusion (Sham/Sal), sham surgery with ANG II perfusion (Sham/ANG II), overload surgery with saline perfusion (Ovld/Sal), overload surgery with ACE inhibitor treatment and saline perfusion (Ovld/A-CE-I/Sal), overload surgery with ACE inhibitor treatment and ANG II perfusion (Ovld/A-CE-I/ANG II), overload surgery with AT1 receptor blockade and saline perfusion (Ovld/AT1-B/Sal), and overload surgery with AT1 receptor blockade and ANG II perfusion (Ovld/AT1-B/ANG II). Pumps were implanted during the initial sham or overload surgery, after which the experimental treatment period lasted 10 days. During the 10-day period, the soleus muscles of the animals in the Ovld groups were subjected to functional overload from the bilateral ablation of the gastrocnemius and the plantaris muscles. Note that this second experiment differed from experiment 1 in that the gastrocnemius and the plantaris muscles were both bilaterally removed in experiment 2 to isolate and optimize the hypertrophic stimulus on the soleus muscle only. During this time, the ACE-I groups received an ACE inhibitor in their daily drinking water, whereas groups undergoing AT1 receptor blockade (AT1-B) received an AT1 receptor antagonist daily via gastric gavage.

Surgical procedures for experiment 1. Functional overload of the plantaris and soleus muscles of animals in the two Ovld groups was produced by the bilateral surgical ablation of the synergistic gastrocnemius muscle using a modified procedure of that described by Baldwin et al. (2). Briefly, rats were weighed and anesthetized with an intraperitoneal injection of a cocktail containing ketamine, xylazine, and acepromazine (75, 3, and 5 mg/kg body wt, respectively). Under sterile conditions, the gastrocnemius muscles were then exposed by a posterior longitudinal incision through the skin and biceps femoris muscle of each lower hindlimb. After the soleus and plantaris muscles were separated from the gastrocnemius muscle at the Achilles tendon, the distal two-thirds of both heads of each gastrocnemius muscle was then excised. Sham surgeries in the two Sham groups consisted of the exact same procedure with the exception of gastrocnemius excision. Incisions were closed using wound clips. We observed little or no postoperative complications using this procedure, and animals were active immediately after recovering from anesthesia.

Surgical procedures for experiment 2. Rats were weighed and lightly anesthetized with an intraperitoneal injection of a ketamine (50 mg/kg body wt), after which they were prepared for surgery and then completely anesthetized with 3–5% isoflurane for the duration of the surgical procedure. Under sterile conditions, bilateral ablation of the distal halves of the gastrocnemius and plantaris muscles of both legs was accomplished to create a functional overload of soleus muscles of both hindlimbs in the five Ovld groups. To accomplish this, longitudinal incisions were made through the skin and biceps femoris muscle on the lateral side of both lower hindlimbs, and the soleus, plantaris, and gastrocnemius muscles were then separated at the Achilles tendon. After excision of the gastrocnemius and plantaris muscles of both legs, an osmotic pump (model 2ML2; Alza, Palo Alto, CA) was implanted subcutaneously on the back of the animal, and a Silastic catheter attached to the pump was fed subcutaneously to the right leg only (Fig. 1). The distal end of the catheter tubing was sutured to the remaining distal gastrocnemius tendon, enabling the constant local delivery of either ANG II or vehicle (saline) directly to the length of the right soleus muscle through two fenestrations in the catheter. The
biceps femoris and skin incisions were sutured shut over the catheter so that the catheter was underlying the biceps femoris distal to the right knee. These sutures also served to further secure the catheter in position. All sutures were accomplished with 5-0 Ethilon nylon suture (Ethicon, Somerville, NJ). With the exception of gastrocnemius and plantaris excisions, animals in the two Sham groups underwent the exact same surgical procedures as those animals undergoing an overload operation. The positioning of the catheter adjacent to the lateral head of the gastrocnemius muscle enabled full exposure of the fenestrations to the soleus muscle in the Sham animals. All animals were ambulatory immediately upon recovery from anesthesia, and the pump and catheter placement had no visible effect on animal mobility or right hindlimb use throughout the overloading period. Furthermore, soleus muscle wet weights and protein contents were not different between the catheterized and noncatheterized legs of any Sham or Ovld groups receiving saline perfusion (see RESULTS).

ACE inhibitor treatment (experiments 1 and 2). For animals scheduled to receive enalapril (ACE-I groups), the ACE inhibitor enalapril maleate was dissolved in their drinking water at a concentration of 0.2 mg/ml according to the methods of Baker et al. (1). Water intake data showed that this concentration delivered a dosage greater than that delivered by Chiba et al. (8) per unit body weight. Both prior investigations prevented loading-induced cardiac hypertrophy in rats with their respective dosages (1, 8). Furthermore, Chiba et al. (8) showed that their dosage lowered ACE activity in the heart, lung, kidney, and plasma. The drug and water were replaced fresh daily in the current experiments. ACE inhibitor treatment was started on the day of initial surgical treatment in experiment 1; however, it was started on the day before surgery in experiment 2 to initiate ACE inhibition before any ANG II delivery from the osmotic pump implant. Serum ACE activity was assessed in venous serum samples obtained at the time of death in experiment 1 (Sigma Diagnostics Kit 305-UV; Sigma Chemical, St. Louis, MO). As expected, serum ACE activity was significantly lower in both ACE-I groups compared with both non-ACE-I groups (means ± SE): Sham: 83.4 ± 6.2 U/l; Sham/ACE-I: 25.2 ± 5.2 U/l; Ovld: 85.4 ± 4.1 U/l; and Ovld/ACE-I: 37.6 ± 7.2 U/l.

AT1 receptor blockade (experiment 2). The AT1 receptor antagonist losartan potassium was administered one time daily by gastric gavage to animals undergoing AT1 receptor blockade (AT1-B groups). The dosage used (60 mg/kg body wt−1·day−1) was the same as that used by Susic et al. (34) to block ANG II-induced left ventricular hypertrophy. Gavage treatments were started on the day before surgery to initiate the AT1 receptor blockade before any ANG II delivery from the osmotic pump implant.

ANG II administration (experiment 2). For the duration of the 10-day overloading period, the osmotic pump implants continuously delivered either saline or synthetic human ANG II (Sigma catalog no. A-9525, 0.35 mg/ml in saline) at a flow rate of 5 μl/h to the right soleus of all animals. This dosage of ANG II (~0.175 mg·kg body wt−1·day−1) has been previously shown in rats to elicte left ventricular hypertrophy without causing hypertension when delivered subcutaneously by osmotic pump for 7 days (34). The human and rat ANG II molecules are octapeptides with 100% homology in amino acid sequence (40).

Tissue mass determination and preservation (experiments 1 and 2). Twenty-eight days after the initial surgery (experiment 1) or 10 days after the initial surgery (experiment 2), animals were weighed and anesthetized with an intraperitoneal injection of a cocktail containing ketamine, xylazine, and acepromazine (75, 3, and 5 mg/kg body wt, respectively). In experiment 1, the soleus and plantaris muscles of both hindlimbs were removed from all animals. In experiment 2, the soleus muscles were removed from both hindlimbs of the overloaded groups, whereas the soleus, plantaris, and gastrocnemius muscles were removed from both hindlimbs of the sham-operated animals. The gastrocnemius muscles in experiment 2 were then further separated into the medial and lateral heads. In experiment 1, blood samples (~3 ml) were obtained from the abdominal vena cava after the skeletal muscles were removed. The blood samples were allowed to clot on ice and centrifuged at 1,500 g for 20 min at 4°C, and the serum was stored at −80°C until analysis for ACE activity. The hearts were also removed from all animals in both experiments. After excision, the skeletal muscle and heart samples were immediately trimmed of excess fat and connec-
tive tissue, weighed on an analytical balance, frozen in liquid nitrogen-chilled isopentane, and stored at -80°C until further processing. Both tibias were removed from all animals, and their length was measured. Skeletal muscle and heart measurements were normalized to mean length of the tibia pair within each animal to assess if any potential effect of surgery or drug treatment was due to generalized animal growth.

Muscle protein determination (experiments 1 and 2). A representative section of frozen tissue was sliced under liquid nitrogen from one plantaris and one soleus muscle from each animal. Tissues sections were then weighed and homogenized as a 5% (wt/vol) solution in a buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na$_2$P$_2$O$_7$, 10H$_2$O, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg/ml leupeptin, 50 μg/ml pepstatin, and 33 μg/ml aprotinin. All homogenizations were accomplished on ice in a ground glass homogenizer using a variable speed motor. Before protein determinations, all sample homogenates and protein assay standards (BSA) were diluted 1:10 in a 0.5 M KOH solution (final concentration) and subjected to alkaline hydrolysis for 24 h at room temperature. Protein concentration of the sample homogenates was then assessed in triplicate using a modified Lowry procedure (DC Protein Assay; Bio-Rad, Hercules, CA). Protein assay results were then used to calculate total protein per whole muscle to complement muscle wet weight as an index of muscle hypertrophy.

Statistical analyses (experiments 1 and 2). Multivariate analyses of variance (MANOVAs) were employed for all statistical analyses in this investigation (Statistica; StatSoft, Tulsa, OK). Repeated measures were used for all MANOVAs requiring a within-subjects analysis. Post hoc comparisons were accomplished via a Fisher’s least significant difference test. Statistical significance in this study was chosen as $P < 0.05$.

RESULTS

Body weight, food intake, and water intake (experiments 1 and 2). The combination of overload surgery and ACE inhibition resulted in a slightly but significantly lower body weight for the Ovld/ACE-I group at all postsurgery time points during the 28-day overloading period in experiment 1, primarily from an initial body weight loss within the 1st wk of the experiment (Fig. 2). During the 10-day overloading period in experiment 2, the body weights of all overloaded groups were significantly lower than the Sham/Sal group at all postsurgery time points, with the exception of the Ovld/ACE/ANG II group at the 10-day time point (Table 1). Furthermore, the mean body weight of the Ovld/AT$_1$-B/Sal group was lower than that of the Ovld/Sal group at day 10. Previous studies have also observed a similar slight loss of body weight in response to cardiac overload surgery (alone or in combination with ACE inhibition) and in response to AT$_1$ receptor blockade (1, 8, 10). Although food and water intake data were not reported for the previous investigations, the body weight differences between groups in both of the two present experiments apparently did not result from food or water intake differences. In experiment 1, the overall food intake of the Ovld group was significantly higher than all other groups (Fig. 2). Additionally, while the water intake in experiment 1 tended to be higher in the two groups receiving an ACE inhibitor, the Sham/ACE-I group had by far the highest water intake. In contrast to experiment 1, there were no differences between groups in total (10-day) food or water intake during the overloading period in experiment 2 (Tables 2 and 3).

Skeletal muscle wet weights and total protein contents (experiments 1 and 2). Figure 3 presents the wet weights and total protein contents of the plantaris and soleus muscles from experiment 1. The protein content results reflected the wet weights for both muscles. Overloading resulted in significantly higher plantaris and soleus muscle total protein contents (by 37 and 20%, respectively) in the Ovld group compared with the Sham group. This hypertrophy was significantly attenuated by ACE inhibition in both muscles (by 57% in the plantaris and by 96% in the soleus). In fact, the wet weight and total protein content of soleus muscles in Ovld/ACE-I animals were statistically equal to those of Sham animals. There was no effect of ACE inhibition on nonoverloaded plantaris or soleus muscles (Sham/ACE-I group), indicating that the ACE inhibitor did not act by generally inhibiting maturation-related muscle growth or basal maintenance of muscle size over the 28-day overloading period. Further supporting this observation is the fact that the plantaris and soleus muscle wet weights and protein contents of all four experimental groups were significantly higher than those of the rats that were body weight matched on the day of initial survival surgery and killed before the overloading period (data not shown).

Because ACE inhibition attenuated hypertrophy to a much greater extent in the soleus muscle than in the plantaris muscle in experiment 1, we chose to isolate and optimize the hypertrophic stimulus on the soleus muscle in experiment 2. This was accomplished by the bilateral ablation of both the gastrocnemius and the plantaris muscles in the Ovld groups. Focus on just one muscle also allowed for the direct perfusion of saline or ANG II on the soleus. The wet weights and total protein contents of the soleus muscles in experiment 2 can be seen in Fig. 4. The total protein content differences generally reflected the wet weight differences of the soleus muscles of both legs in all groups except for the Sham/ANG II group, where wet weight increased in the perfused soleus but total protein content did not (as described below). Ten days of overload resulted in a significantly higher total protein content in the soleus muscles of the Ovld/SAI group vs. those of the Sham/Sal group (33% increase using the average value of both legs in each group). This hypertrophy was attenuated by 55% with ACE inhibition (Ovld/ACE-I/Sal) and by 48% with the AT$_1$ receptor blockade (Ovld/AT$_1$-B/Sal). However, as seen in the right leg soleus protein content values of the Ovld/ACE-I/ANG II group, local ANG II administration rescued 71% of the lost soleus hypertrophy in ACE-inhibited animals. This rescued protein content in the right soleus of the Ovld/ACE-I/ANG II group was restored to a level statistically equal to that of the Ovld/Sal group. Local ANG II administration did not affect soleus wet weight or protein content in AT$_1$
receptor-blocked animals (Ovld/AT1-B/ANG II). In non-overloaded soleus muscles, local ANG II perfusion resulted in a significant 12% increase in wet weight but no corresponding increase in protein content (Sham/ANG II group). Thus the increased wet weight in that condition was likely due to increased water content. Last, the wet weights of the plantaris, medial gastrocnemius, and lateral gastrocnemius muscles in the Sham/ANG II group were not affected by ANG II administration when compared either with the contralateral (nonperfused) leg in the Sham/ANG II group or with the perfused leg in the Sham/Sal group (data not shown).

The 55% attenuation of soleus hypertrophy by ACE inhibition in experiment 2 was less than the 96% attenuation seen in experiment 1. However, we attribute the difference to the fact that both the gastrocnemius and the plantaris muscles were removed in experiment 2, placing the increased loadbearing entirely on the soleus. In contrast, only the gastrocnemius muscle was removed in experiment 1, leaving the plantaris muscle with the perfused leg in the Sham/Sal group (data not shown).

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Table 1. Animal body weights during the 10-day soleus overloading protocol in experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Presurgery</td>
</tr>
<tr>
<td>Sham/Sal</td>
<td>241.8 ± 3.0</td>
</tr>
<tr>
<td>Sham/ANG II</td>
<td>242.7 ± 1.1</td>
</tr>
<tr>
<td>Ovld/Sal</td>
<td>242.0 ± 2.3</td>
</tr>
<tr>
<td>Ovld/ACE-I/Sal</td>
<td>242.3 ± 3.0</td>
</tr>
<tr>
<td>Ovld/ACE-I/ANG II</td>
<td>242.5 ± 3.8</td>
</tr>
<tr>
<td>Ovld/AT1-B/Sal</td>
<td>241.6 ± 3.1</td>
</tr>
<tr>
<td>Ovld/AT1-B/ANG II</td>
<td>241.9 ± 3.4</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 8/group). Functional overload surgery occurred at the beginning of day 1 and consisted of the bilateral ablation of the gastrocnemius and plantaris muscles to overload the soleus muscle in both hindlimbs. During the 10-day overloading period, the right hindlimb soleus muscle of all animals was continually perfused with either ANG II or vehicle (saline) via osmotic pump. Some animals (specified) daily received an angiotensin-converting enzyme (ACE) inhibitor in drinking water or an ANG II type 1 (AT1) receptor antagonist by gastric gavage beginning 1 day before the overloading period and continuing throughout the experiment. Sham/Sal, sham surgery with saline perfusion; Sham/ANG II, sham surgery with ANG II perfusion; Ovld/Sal, overload surgery with saline perfusion; Ovld/ACE-I/Sal, overload surgery with ACE inhibitor treatment and saline perfusion; Ovld/ACE-I/ANG II, overload surgery with ACE inhibitor treatment and ANG II perfusion; Ovld/AT1-B/Sal, overload surgery with AT1 receptor blockade and saline perfusion; Ovld/AT1-B/ANG II, overload surgery with AT1 receptor blockade and ANG II perfusion. *Significantly different (P < 0.05) from Sham/Sal at specified time point; †significantly different from Ovld/Sal at specified time point; ‡significantly different from Ovld/ACE-I/Sal at specified time point.

Fig. 2. Body weight (BW, A), food intake (B), and water intake (C) of animals during the plantaris and soleus overloading protocol in experiment 1, with and without the administration of an angiotensin-converting enzyme (ACE) inhibitor during the 28-day overloading period (means ± SE; n = 10/group). Functional overload of the soleus and plantaris muscles was achieved by the bilateral surgical ablation of the gastrocnemius muscle. Body weights and food intakes were measured weekly, while water intakes were measured daily. Average daily food intake for each animal within each 7-day time period was calculated using the 7-day total. Individual food and water intake values within each consecutive 7-day time period were normalized to the average of the individual body weight values taken at the beginning and the end of that time period. For each animal, the four individual food and water intake values for the consecutive 7-day time periods were used to calculate average individual intake values for the entire 28-day experiment (All Days). Sham, sham surgery; Sham/ACE-I, sham surgery with ACE inhibitor treatment (in daily drinking water); Ovld, overload surgery; Ovld/ACE-I, overload surgery with ACE inhibitor treatment. Significant differences (P ≤ 0.05) at each time point: *Sham vs. Sham/ACE-I; ¤Sham vs. Ovld; ¥Sham vs. Ovld/ACE-I; ¥Sham/ACE-I vs. Ovld; ¥Sham/ACE-I vs. Ovld/ACE-I; ¥Ovld vs. Ovld/ACE-I.
In the present investigation, we found that using ACE inhibition to partially block endogenous ANG II production significantly attenuated overload-induced skeletal muscle hypertrophy, with this effect being much greater in the slow-twitch soleus muscle than in the fast-twitch plantaris muscle. Moreover, we were able to rescue 71% of the lost hypertrophy in the right soleus muscles of ACE-inhibited animals by locally perfusing the muscles with exogenous ANG II in a continuous fashion throughout the loading period. The effect of ANG II in overload-induced skeletal muscle hypertrophy may be at least partly mediated via the AT1 receptor, as overload-induced soleus hypertrophy was attenuated by AT1 receptor blockade regardless of ANG II administration. The demonstration that ANG II is necessary for optimal overload-induced skeletal muscle hypertrophy is a completely novel finding.

Because ACE inhibition did not cause atrophy of nonoverloaded (sham-operated) soleus or plantaris muscles in experiment 1, the effect of ANG II on skeletal muscle appears to be primarily overload dependent. It is possible that the RAS is of low importance for normal maintenance of skeletal muscle mass but is upregulated by mechanical overloading and thus more easily affected by ACE inhibition under conditions of overload. Such a loading-induced phenomenon has already been identified in the heart, which exhibits increased renin, angiotensinogen, and ACE mRNA abundances; increased ACE activity; increased intracellular renin, ANG I, and ANG II peptide contents; and increased ANG II secretion in response to overloading (1, 8, 30, 42). Nevertheless, in contrast to the heart, solely increasing ANG II input (via exogenous ANG II administration) did not significantly increase the protein content of the nonoverloaded soleus muscle in experiment 2. Thus it is likely that other factors, such as AT1 receptor density, input from other hormones, or mechanical signaling, are also altered by the loading stimulus on the soleus.

**DISCUSSION**

ANG II is strongly involved with cardiac hypertrophy and smooth muscle growth, particularly under conditions of mechanical overload (1, 8, 10, 22, 30, 34, 42). We therefore hypothesized that ANG II also mediates overload-induced skeletal muscle hypertrophy. In the present investigation, we found that using ACE inhibition to partially block endogenous ANG II production significantly attenuated overload-induced skeletal muscle hypertrophy, with this effect being much greater in the slow-twitch soleus muscle than in the fast-twitch plantaris muscle. Moreover, we were able to rescue 71% of the lost hypertrophy in the right soleus muscles of ACE-inhibited animals by locally perfusing the muscles with exogenous ANG II in a continuous fashion throughout the loading period. The effect of ANG II in overload-induced skeletal muscle hypertrophy may be at least partly mediated via the AT1 receptor, as overload-induced soleus hypertrophy was attenuated by AT1 receptor blockade regardless of ANG II administration. The demonstration that ANG II is necessary for optimal overload-induced skeletal muscle hypertrophy is a completely novel finding.

Because ACE inhibition did not cause atrophy of nonoverloaded (sham-operated) soleus or plantaris muscles in experiment 1, the effect of ANG II on skeletal muscle appears to be primarily overload dependent. It is possible that the RAS is of low importance for normal maintenance of skeletal muscle mass but is upregulated by mechanical overloading and thus more easily affected by ACE inhibition under conditions of overload. Such a loading-induced phenomenon has already been identified in the heart, which exhibits increased renin, angiotensinogen, and ACE mRNA abundances; increased ACE activity; increased intracellular renin, ANG I, and ANG II peptide contents; and increased ANG II secretion in response to overloading (1, 8, 30, 42). Nevertheless, in contrast to the heart, solely increasing ANG II input (via exogenous ANG II administration) did not significantly increase the protein content of the nonoverloaded soleus muscle in experiment 2. Thus it is likely that other factors, such as AT1 receptor density, input from other hormones, or mechanical signaling, are also altered by the loading stimulus and interact with ANG II signaling to elicit over-

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### Table 1. Animal food intakes during the 10-day soleus overloading protocol in experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Intake, g·kg body wt⁻¹·day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 1–5</td>
</tr>
<tr>
<td>Sham/Sal</td>
<td>87.7±3.1</td>
</tr>
<tr>
<td>Sham/ANG II</td>
<td>90.0±2.6</td>
</tr>
<tr>
<td>Ovl/Sal</td>
<td>89.7±7.2</td>
</tr>
<tr>
<td>Ovl/ACE-I/Sal</td>
<td>81.1±5.1</td>
</tr>
<tr>
<td>Ovl/ACE-I/ANG II</td>
<td>85.3±5.2</td>
</tr>
<tr>
<td>Ovl/AT₁-B/Sal</td>
<td>73.3±3.8††</td>
</tr>
<tr>
<td>Ovl/AT₁-B/ANG II</td>
<td>84.1±5.1</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 8/group). Food intakes were measured every 5 days and were used to calculate average daily food intake for each animal within each 5-day time period. Individual intake values within each consecutive 5-day time period were normalized to the average of the individual body weight values taken at the beginning and the end of that time period. For each animal, the two individual intake values for the consecutive 5-day time periods were used to calculate average individual intake values for the entire 10-day experiment (All days). Further experimental details and description of experimental groups can be found in the legend for Table 1.

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### Table 2. Animal food intakes during the 10-day soleus overloading protocol in experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Days 1–5</th>
<th>Days 6–10</th>
<th>All days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham/Sal</td>
<td>208.7±6.9</td>
<td>212.7±11.3</td>
<td>210.7±7.9</td>
</tr>
<tr>
<td>Sham/ANG II</td>
<td>195.9±10.0</td>
<td>203.7±5.4</td>
<td>199.8±6.9</td>
</tr>
<tr>
<td>Ovl/Sal</td>
<td>196.7±13.7</td>
<td>207.1±15.3</td>
<td>201.9±13.4</td>
</tr>
<tr>
<td>Ovl/ACE-I/Sal</td>
<td>185.0±10.7†</td>
<td>208.9±5.3</td>
<td>196.9±7.0</td>
</tr>
<tr>
<td>Ovl/ACE-I/ANG II</td>
<td>194.0±11.6</td>
<td>204.0±15.6</td>
<td>199.0±13.3</td>
</tr>
<tr>
<td>Ovl/AT₁-B/Sal</td>
<td>169.0±10.9††</td>
<td>198.8±13.7</td>
<td>183.9±11.1</td>
</tr>
<tr>
<td>Ovl/AT₁-B/ANG II</td>
<td>186.2±12.5*</td>
<td>232.6±9.9††</td>
<td>209.4±10.6</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 8/group). Individual intake values within each consecutive 5-day time period were normalized to the average of the individual body weight values taken at the beginning and the end of that time period. For each animal, the two individual intake values for the consecutive 5-day time periods were used to calculate average individual intake values for the entire 10-day experiment (All days). Further experimental details and description of experimental groups can be found in the legend for Table 1.
load-induced skeletal muscle hypertrophy. In fact, loading-induced growth of most cell types, including skeletal muscle, is now widely believed to be a complex integration of intracellular signaling pathways activated by mechanical and diverse hormonal input in a complementary fashion (5, 6, 26, 35, 36). Furthermore, in light of the finding that AT1 receptor mRNA abundance is increased in overloaded hypertrophying cardiac myocytes (42), it could be postulated that AT1 receptor gene expression and possibly receptor density is also upregulated in skeletal muscle under conditions of overload. However, the existence of the AT1 receptor in skeletal muscle cells is currently debatable (20, 33, 37).

The extent to which skeletal muscle is sensitive to local vs. systemic levels of ANG II cannot be conclusively determined from the results of the present investigation. It appears that the soleus was only sensitive to locally elevated levels of exogenous ANG II during overloading, because the nonperfused contralateral overloaded soleus was not affected in the same group of animals. Additionally, the fact that the heart wet weight responded to ANG II administration in a manner almost identical to the perfused soleus wet weight argues for the probability that exogenous ANG II did indeed enter the systemic circulation. In a previous study by Susic et al. (34) using the same rate of ANG II delivery per unit of body weight, cardiac hypertrophy was elicited to a similar extent as the current investigation when ANG II was administered subcutaneously via osmotic pumps on the back. This occurred independent of blood pressure changes, indicating that the ANG II became systemic and acted directly on the heart (34). Therefore, it is likely that ANG II was elevated systemically by ANG II perfusion during the course of the unloading period in the present experiment. If systemic ANG II concentration was elevated by ANG II administration to the right soleus, the effect on the heart and not the contralateral soleus might be explained by the observation that binding and accumulation of systemic ANG II (and thus possibly ANG II sensitivity) are higher in cardiac muscle than in skeletal muscle (37). Still, regardless of systemic ANG II level, there is little doubt that the perfused soleus was exposed to a much higher ANG II concentration than was the contralateral soleus. Further research is warranted to determine the local concentration of ANG II necessary to contribute to skeletal muscle hypertrophy.

If systemic ANG II input does not alter skeletal muscle mass in untreated animals, then ANG II could function in an autocrine or paracrine fashion during overload-induced skeletal muscle hypertrophy in a manner similar to the local RAS upregulation seen in overload-induced cardiac hypertrophy (8, 30, 42). Although skeletal myocytes require paracrine/autocrine factors for stretch-induced growth (35) and can secrete growth factors such as insulin-like growth factor I in response to mechanical stress (24), the possibility that ANG II may be included as such a growth factor has not been examined. Although some have discounted skeletal muscle as a source of ANG II (32), others have
reported that 59% of the ANG II in the venous blood leaving the skeletal muscle vascular bed is attributable to local de novo ANG II synthesis from the conversion of both locally produced and circulating ANG I (9). It remains to be determined if ANG II is secreted by skeletal muscle cells themselves, since RAS activity of the endothelial and/or smooth muscle cells in the vascular wall (23, 25) might also account for much of the ANG II found in the venous plasma exiting the skeletal muscle vascular bed.

The mechanisms by which ANG II contributes to overload-induced skeletal muscle hypertrophy are unknown, especially since there are equivocal findings as to the presence of the AT1 receptor in skeletal muscle cells (20, 33, 37). However, the existence of nonskeletal...
muscle cells within all skeletal muscles also raises the distinct possibility that ANG II may also indirectly mediate skeletal muscle hypertrophy by acting on other cell types that are nevertheless essential to the hypertrophic response. For instance, the rate of skeletal muscle fiber hypertrophy in the synergist ablation model used in the present investigation has previously been shown to be closely related to the rate of increase in capillary density (27). It is therefore interesting that ANG II induces smooth muscle cell proliferation in the vessel wall and microvessel growth in skeletal muscle in a manner partly dependent on the AT1 receptor (13, 28) and that ACE inhibition decreases skeletal muscle microvascularity (39). It has been postulated that there is a paracrine secretion of ANG II in the microvasculature from endothelial cells and targeted for smooth muscle cells (25). On the other hand, ANG II-mediated skeletal muscle hypertrophy may also involve skeletal muscle fibroblasts; cardiac fibroblasts are extremely important for ANG II-stimulated cardiac hypertrophy under normal conditions and during mechanical stretch in the heart (18). Moreover, cardiac myocyte protein synthesis and hypertrophy are stimulated by supernatant from cultured fibroblasts treated with ANG II (18), possibly via paracrine factors such as endothelin-1, transforming growth factor-β, fibroblast-derived factor, and several other growth factors (18).

It is unclear why ACE inhibition attenuated overload-induced hypertrophy to a much greater extent in the slow-twitch soleus muscle than in the fast-twitch plantaris muscle in experiment 1. The difference could possibly be attributed to the respective amounts of increased loadbearing taken up by the soleus and plantaris muscles after elimination of the gastrocnemius. Because slow-twitch muscle is typically recruited a great deal more than fast-twitch muscle for normal daily loadbearing (15), the potential for increased loadbearing and subsequent hypertrophy may be greater in the plantaris than in the soleus (as supported by the almost 2-fold greater hypertrophy in the plantaris than in the soleus in the absence of ACE inhibition in experiment 1). The upregulation of other hypertrophic mechanisms responding to the overloading stimulus may therefore be greater in the plantaris and thus more able to override the effects of ACE inhibition. This would also explain why the soleus hypertrophy in experiment 2 (in which all of the overloading stimulus was placed on the soleus due to removal of both the plantaris and gastrocnemius muscles) was only attenuated by ACE inhibition to an extent similar to that of the plantaris in experiment 1 (55 vs. 57%, respectively). Nevertheless, despite potential loading differences between the two muscles, it is also possible that the slow-twitch soleus muscle is more sensitive to ACE inhibition than the fast-twitch plantaris muscle solely because it expresses components of the RAS at a higher level than the plantaris. Future investigations must attempt to separate the loading- vs. fiber type-dependent differences in skeletal muscle sensitivity to ACE inhibition during overload-induced hypertrophy.

Our finding that ANG II contributes to the hypertrophic response of skeletal muscle may at first appear to contradict studies undertaken in patients with congestive heart failure (CHF). In this population, long-term ACE inhibition or AT1 receptor blockade significantly reverses impairments in skeletal muscle strength, slow- and fast-twitch fiber area, oxidative capacity, and exercise capacity (11, 19, 31, 38). However, it is believed that the rescued skeletal muscle mass in CHF patients undergoing such interventions may be a product of their increased loading patterns resulting from greatly improved functional capacity and activity (31). Thus it is difficult to extrapolate findings observed in the impaired skeletal muscle of this clinical population to those observed under conditions of overload-induced hypertrophy of normal healthy skeletal muscle.

In summary, this investigation is the first to show that ANG II is necessary for optimal overload-induced skeletal muscle hypertrophy. This effect may be partly mediated by the AT1 receptor and is apparently more pronounced in the slow-twitch soleus muscle than in the fast-twitch plantaris muscle. The mechanisms by which ANG II mediates skeletal muscle hypertrophy under conditions of overload are currently unknown. However, ANG II may act directly on the skeletal muscle cells themselves, indirectly through the stimulation of neighboring fibroblasts, or via the promotion of capillary angiogenesis. Neither systemic nor local sources can currently be ruled out as the origin of ANG II input in this phenomenon. The present results, when taken in concert with the purported association between ACE genotype polymorphism and endurance exercise trainability in humans (21, 41), indicate the existence of a complex RAS involvement in the skeletal muscle adaptation to increased usage. This potential phenomenon is an exciting candidate for further investigation.

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