Original Article

Histochemical Characteristics of Soleus Muscle in Angiotensin-Converting Enzyme Gene Knockout Mice

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We examined the histochemical characteristics of soleus muscle in the angiotensin-converting enzyme (ACE) gene (Ace in mice, ACE in humans) knockout mice. Serial sections of soleus muscle of wild-type (Ace+/+, *n*=20) and heterozygous mutant (Ace+/−, *n*=24) mice were stained for myosin adenosine triphosphatase activity to identify different muscle fiber types. Capillaries were visualized by amylase-periodic acid-Schiff staining. ACE activity in the serum and gastrocnemius muscle was higher in male mice than in female mice. Female and male Ace+/− mice had markedly lower ACE activity in the serum and the gastrocnemius muscle than did female and male Ace+/+ mice, respectively. In both male and female mice, the composition of fiber types (type I and IIa) did not differ significantly between Ace+/+ and Ace+/− mice. There was no significant gender difference in capillary density. Ace+/− mice had significantly more capillaries around type IIa fibers (5.44±0.18 *vs.* 5.01±0.13, $p<0.05$) than Ace+/+ mice. The differences in the number of capillaries around type I fibers and in the number of capillaries around per fiber (capillary:fiber ratio) between Ace+/− and Ace+/+ mice were not significant ($p<0.1$). There was no significant difference in the mean cross-sectional area occupied by one capillary and the number of capillaries per fiber area between Ace+/+ and Ace+/− mice. In conclusion, knockout of the Ace gene in mice increased capillary density, as expressed by the mean number of capillaries around type IIa fibers. This finding suggests a possible mechanism for the cardioprotective effects of ACE inhibitors. (*Hypertens Res* 2005; 28: 681–688)

***Key Words*: angiotensin-converting enzyme (ACE) knockout mice, soleus muscle, fiber types, capillary density, adenosine triphosphatase (ATPase)**

Introduction

The circulating human renin-angiotensin system (RAS) plays an important role in circulatory homeostasis (*1*). Angiotensin-converting enzyme (ACE) produces the vasoconstrictor angiotensin II and metabolizes vasodilatory kinins. A local RAS is known to exist in skeletal muscle (*2*), and may work

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with other metabolic tissue to influence the energy balance of the entire body (*3*). The human ACE gene shows a polymorphism based on the presence (insertion [I]) or absence (deletion [D]) of a 287-base-pair Alu-repeat element within intron 16. The I allele is associated with lower serum and tissue ACE activity (*4*, *5*). ACE I/D polymorphism has been shown to be associated with exercise performance: the I allele is found to be associated with superior exercise endurance (*6*–*8*), and the D allele is associated with power performance (*6*, *9*). Although the mechanism by which ACE I/D polymorphism is associated with endurance performance is not clear, the presence of the ACE-I allele has been indicated to confer enhanced mechanical efficiency (energy used per unit power output) in aerobically trained skeletal muscle (*10*). Human muscle is composed of various fibers characterized as type I (slow-twitch) fibers and type II fibers (fast-twitch; including type IIa and IIx fibers) based on the myosin adenosine triphosphatase (ATPase) reaction (*11*, *12*). We previously reported that the I allele of the ACE gene is associated with an increased percentage of slow-twitch type I fibers in human skeletal muscle in young untrained volunteers (*13*).

However, it is also possible that the metabolic and vascular properties of muscle may be related to ACE activity. In fact, inhibition of ACE activity by ACE inhibitors has been shown to increase glucose uptake in skeletal muscles in type 2 diabetic mouse KK-Ay (*14*) and increase muscle capillary density in animals (*15*–*18*). Muscle capillary density is increased in ischemic limbs of normal rabbits treated with quinaprilat (*18*), normal and diabetic mice treated with perindopril (*19*, *20*), spontaneously hypertensive rats (SHRs) treated with perindopril (*16*), and in heart of obese Zucker rats treated with a combination of perindopril and the diuretic indapamide (*15*). In contrast, the ACE inhibitor captopril failed to increase capillary density in the ischemic limbs of normal rabbits (*18*), and benazeprilat was found to have no effect on the capillary: fiber ratio in normal or ischemic soleus muscle in SHRs (*21*). In addtion, fosinopril has been shown to have no effects on capillary density in human muscle (*22*). Fabre *et al*. suggested that the different effects of the quinaprilat and captopril on angiogenesis in rabbit ischemic hindlimb may be due to differences in the effects on tissue ACE activity exerted by different ACE inhibitors (*18*).

The role played by ACE in terms of the histochemical characteristics of muscle may also be examined using transgenic mice that carry an insertional disruption of the murine Ace gene (ACE in humans, Ace in mice) (*23*). Mice that are heterozygous (Ace+/−) for this mutation have serum and tissue ACE activities that are approximately 30% and 40% lower, respectively, than those of wild-type (Ace+/+) mice (*23*, *24*). The current study using Ace-knockout mice was performed to examine whether or not ACE influences the composition of muscle fibers and capillary density within a defined genetic and environmental background. We examined the mouse soleus muscle because it normally contains approximately equal numbers of slow (type I) and fast (type IIa) fibers (*25*), and also because the type I fibers are evenly distributed throughout the length of the muscle (proximal to distal) (*26*).

Methods

Animals

Wild-type mice (Ace+/+, $n=20$) and heterozygous mutant mice (Ace+/−, $n=24$) were bred at the Animal Center of Fukuoka University from heterozygous F1 offspring of C57BL/6J mice, in which exon 14 of the Ace gene was disrupted (*27*). The founders (B6.129P2-Ace^{tm2Unc}) were originally obtained from the Jackson Laboratory (Maine, USA). Ace+/+ and Ace+/− mice were identified by measuring serum ACE activity, since there is no similarity in the serum ACE activity between wild-type mice and heterozygous mutant mice (*27*).

Mice were housed in individual cages in a sound-attenuated room, and were maintained under conditions of constant humidity, temperature, and light cycle (light 8 AM–8 PM). All mice were allowed *ad libitum* access to water and food. This project was approved by the Ethics Committee of Fukuoka University.

Determination of Serum ACE Activities

Blood was drawn from the tails of mice. Serum ACE activities were measured on a Hitachi autoanalyzer (Hitachi 7600-020S, Hitachi Inc., Tokyo, Japan) using a spectrophotometric method with a commercially available kit (Sigma Diagnostics, St. Louis, USA).

Histological Procedures

Animals were anesthetized by injecting pentobarbital sodium into the abdominal cavity (0.1 ml/10 g body weight). The soleus muscle was rapidly excised from the left hindlimbs, weighed, immediately mounted in an embedding medium (OCT compound, Tissue Tek, CA, USA), frozen in isopentane (2-methyl butane) cooled to near its freezing point with liquid nitrogen, and stored at −80°C until analysis (*12*). The gastrocnemius muscle was rapidly excised from the right hindlimbs, weighed, frozen in liquid nitrogen and stored at −80°C for the measurement of tissue ACE activity. Serial 10-μm cross-sections were cut from the middle of the left soleus muscle using a cryostat at −20°C, mounted on slides, and dried at room temperature for 1–2 h. Serial sections were then processed for acid-stable (pre-incubated at pH 4.5) and alkali-stable (pre-incubated at pH 10.3) staining for myofibrillar ATPase activity to identify different types of muscle fiber and for amylase-periodic acid-Schiff staining to visualize the capillaries according to methods as described previously (*12*). The sections were either used immediately or stored in an airtight container at 4°C (for ATPase staining) or −20°C (for amylase-periodic acid-Schiff staining). The density of the

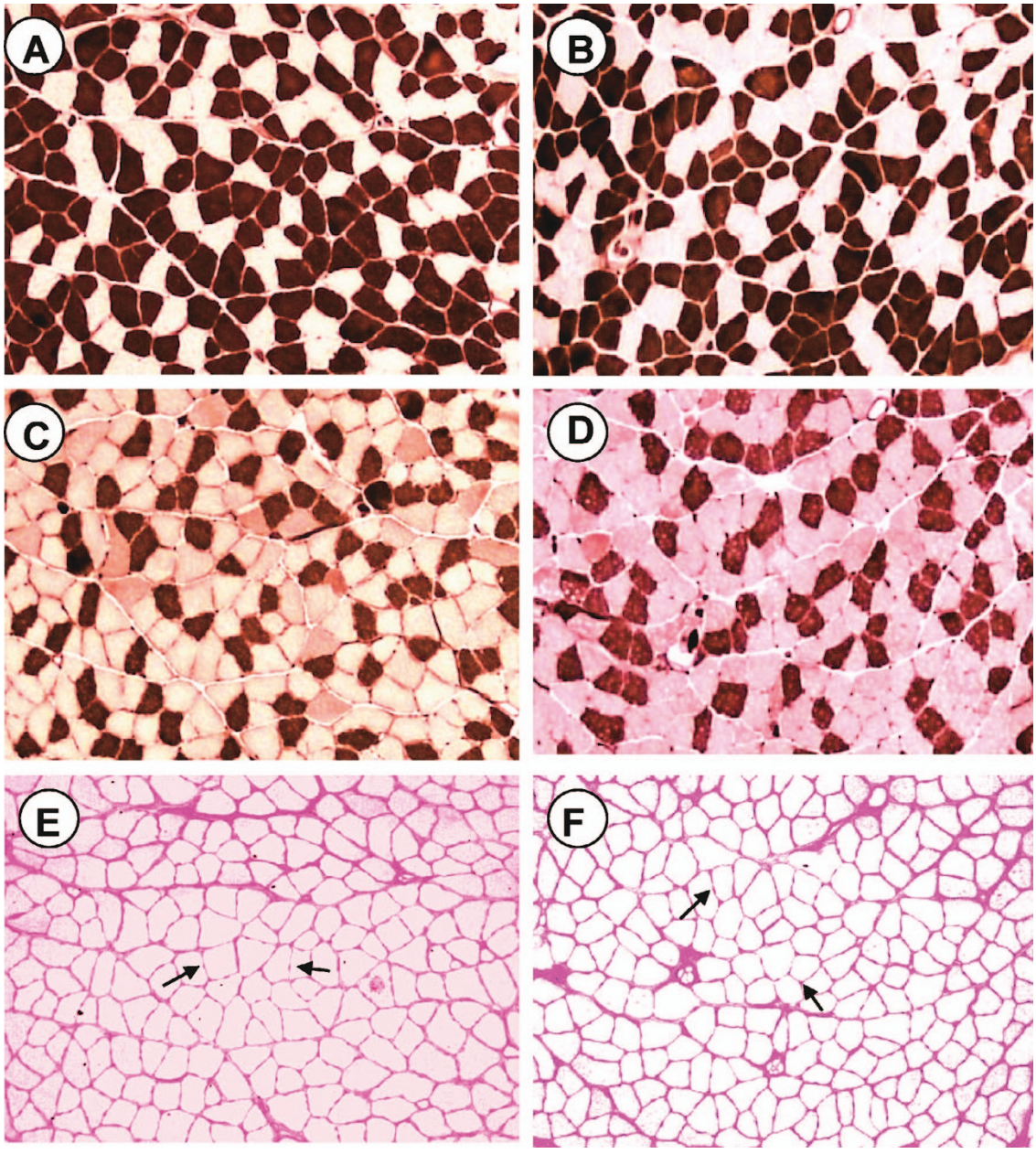

Fig. 1. *Serial sections of soleus muscle in wild-type (Ace+/+) mice (left: A, C, E) and heterozygous mutant (Ace+/–) mice (right: B, D, F). Myosin ATPase staining: preincubated at pH 10.3 (A, B), type I fibers stain light and type IIa fibers stain dark; preincubated at pH 4.5 (C, D), type I fibers stain dark and type IIa fibers stain light. Amylase-periodic acid-Schiff staining (E, F): arrows indicate capillaries. × 100.*

capillaries visualized by amylase-periodic acid-Schiff staining was expressed as 1) the number of capillaries per fiber (capillary:fiber ratio), 2) the number of capillaries per area (capillary/mm^2), 3) the number of capillaries around each fiber type, and 4) the cross-sectional area occupied by a single capillary (diffusion index). All of these values were calculated using COMFAS (Scan Beam, Hadsund, Denmark) as described previously (*12*).

Determination of ACE Activities in Gastrocnemius Muscle

Tissue ACE activity was measured in the gastrocnemius muscle from the right hindlimbs of mice using a synthetic peptide containing a fluorescent chromophore as the substrate, as described previously (*28*). Gastrocnemius muscle was homogenized in 8 volumes of homogenate buffer containing 50 mmol/l Tris-HCl, 0.25 mol/l sucrose, and 0.3 mol/l NaCl

Table 1. Serum ACE Activity and Histochemical Characteristics of Soleus Muscle Fibers in Wild-Type (Ace+/+) and Heterozygous Mutant (Ace+/–) Mice

	Ace+/+ (n=20)	Ace+/– (n=24)	p value
Age (months)	10.3±0.6	9.5±0.4	n.s.
Female/male	10/10	14/10	n.s.
Body weight (g)	27.5±1.2	26.6±0.9	n.s.
Serum ACE activity (U/l)	358±22	191±12	<0.01
ACE activity in gastrocnemius muscle (mU/mg protein)	1.62±0.61	0.70±0.21	<0.01
Soleus weight (mg/g body weight)	0.31±0.01	0.31±0.01	n.s.
Percent composition in No. (%)			
Type I	40.4±1.7	40.8±1.2	n.s.
Type IIa	59.4±1.8	59.3±1.5	n.s.
Percent composition in area (%)			
Type I	43.3±2.5	42.5±1.9	n.s.
Type IIa	56.6±2.5	57.8±1.9	n.s.
Mean fiber area (mm^2)			
Type I	1,828±72	1,813±49	n.s.
Type IIa	1,638±72	1,691±52	n.s.
No. of capillaries around the fiber			
Type I	5.17±0.12	5.57±0.16	<0.1
Type IIa	5.01±0.13	5.44±0.18	<0.05
Mean	5.11±0.12	5.51±0.17	<0.05
Capillary:fiber ratio	1.97±0.05	2.18±0.09	<0.1
Diffusion index (mm^2)			
Type I	354±16	333±14	n.s.
Type IIa	329±14	317±12	n.s.
Mean	341±14	325±13	n.s.
Capillary/mm^2	1,035±32	1,123±51	n.s.

Data are mean±SEM. ACE, angiotensin-converting enzyme; Ace, ACE gene in mice.

(pH 7.5) at 21,000 rpm for 3 × 30 s using a Handy Micro homogenizer NS-310E (Physcotron, Nichi-on Ltd., Tokyo, Japan) while the samples were cooled in an ice-water bath (*29*, *30*). After the homogenate was centrifuged at 4°C and 20,000 × *g* for 1 h in a high-speed micro refrigerated centrifuge (MTX-150, Tomy Seiko Co., Ltd., Fukuoka, Japan), the supernatant (S_1 fraction) was collected and stored at 4°C. The pellet was then homogenized again in 10 volumes of homogenate buffer that also contained 0.1% Triton X-100 at 21,000 rpm for 2 × 30 s. The suspension was shaken at 37°C for 1 h in a water bath for detergent extraction of the membrane-associated enzyme (*29*, *30*). The mixture was centrifuged at 4°C and 5,000 × *g* for 5 min and the supernatant (S_2 fraction) was collected. The ACE activities and protein concentrations of the S_1 and S_2 fractions were measured.

Stock substrate solution for ACE activity was prepared at 1 mmol/l by dissolving 7.04 mg of the substrate in 1 ml of solution containing 50 mmol/l Tris-HCl and 0.22 mol/l NaCl (pH 7.4). The stock substrate solution was diluted with a solution containing 50 mmol/l Tris-HCl and 0.3 mol/l NaCl (pH 7.5) to give a working substrate solution of 50 μmol/l. Thirty μl of the S_1 or S_2 fraction was mixed with 180 μl of substrate (50 μmol/l) in a white microplate (White Combiplate 8, Labsystems, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) using a plate shaker (Micro Mixer E-36, Taitec Corporation, Tokyo, Japan), and the increase in fluorescence was monitored (excitation: 355 nm, emission: 460 nm) at 37°C in intervals of 5 min for 30 min in a fluorescence plate reader (Labsystems Fluoroskan II, Labsystems, Dainippon Pharmaceutical Co., Ltd.). Samples that had been preincubated with 0.3 mmol/l captopril (Sigma Chemical Co., St. Louis, USA) for 30 min at room temperature were used as a tissue blank to define specific ACE activity (*31*). A standard curve was constructed by using a dilution series using a calibrator (ACE activity: 81 U/l, product No.: 30550, Sigma Diagnostics), and the relative fluorescence intensities were monitored at 15 min. ACE control-N and E (ACE activity: 33 U/l and 108 U/l, respectively, product Nos.: A6040 and A7040, Sigma Diagnostics) were used as quality controls. Specific ACE activity in the muscle was calculated using the standard curve. Protein concentrations were determined using the Micro BCA protein assay reagent (Pierce, Rockford, USA) with bovine serum albumin as a standard. ACE activities in S_1 and S_2 fractions were expressed as mU/mg protein, and muscle ACE activity

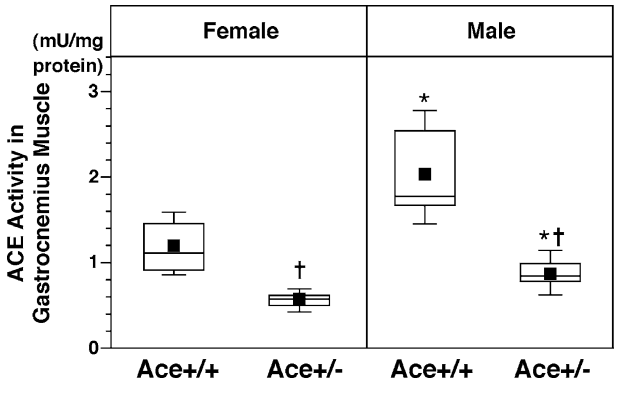

Fig. 2. *Box-and-whisker plots showing the mean (■), median (middle bar in the rectangle), and 10th (bottom bar), 25th (bottom of rectangle), 75th (top of rectangle), and 90th percentiles (top bar) of ACE activity in the gastrocnemius muscle in wild-type (Ace+/+) and heterozygous mutant (Ace+/–) mice according to gender.* *p < *0.01, male* vs. *female;* †p < *0.01, Ace+/– mice* vs. *Ace+/+ mice.*

was expressed as the mean ACE activity of the S_1 and S_2 fractions.

Statistical Analysis

All statistical analyses were performed using the SAS (Statistical Analysis System) Software Package (Version 8.2, SAS Institute, CA, USA). Data are presented as the mean±SEM, unless indicated otherwise. Variables were compared between groups by an analysis of variance and/or Wilcoxon rank-sum test. All *p* values were two-tailed. The significance level was considered to be 5% unless indicated otherwise.

Results

Figure 1 shows typical serial sections of soleus muscle in a wild-type (Ace+/+) and a heterozygous mutant (Ace+/–) mice. Myosin ATPase staining (Fig. 1A–D) was used to identify type I and II fibers, and amylase-periodic acid-Schiff staining was used to visualize capillaries (Fig. 1E and F). ATPase staining distinguishes between fiber types based on differences in myosin isoforms in response to acid *vs.* alkaline conditions. The soleus in normal mice contains two isomyosins (slow myosin and intermediate myosin) (*25*). Since slow myosin is more stable under acidic conditions, and intermediate myosin is acid-labile, type I fibers, which contain slow myosin, stain darkly due to high myofibrillar ATPase activity and type IIa fibers, which contain intermediate myosin, stain lightly due to a low myofibrillar ATPase activity after preincubation at pH 4.5. In contrast to acid-stable ATPase staining, alkali-stable ATPase staining exhibits the opposite pattern of fiber staining after preincubation at pH 10.3. As shown in Fig. 1, the type I fibers in the mouse soleus muscle were stained

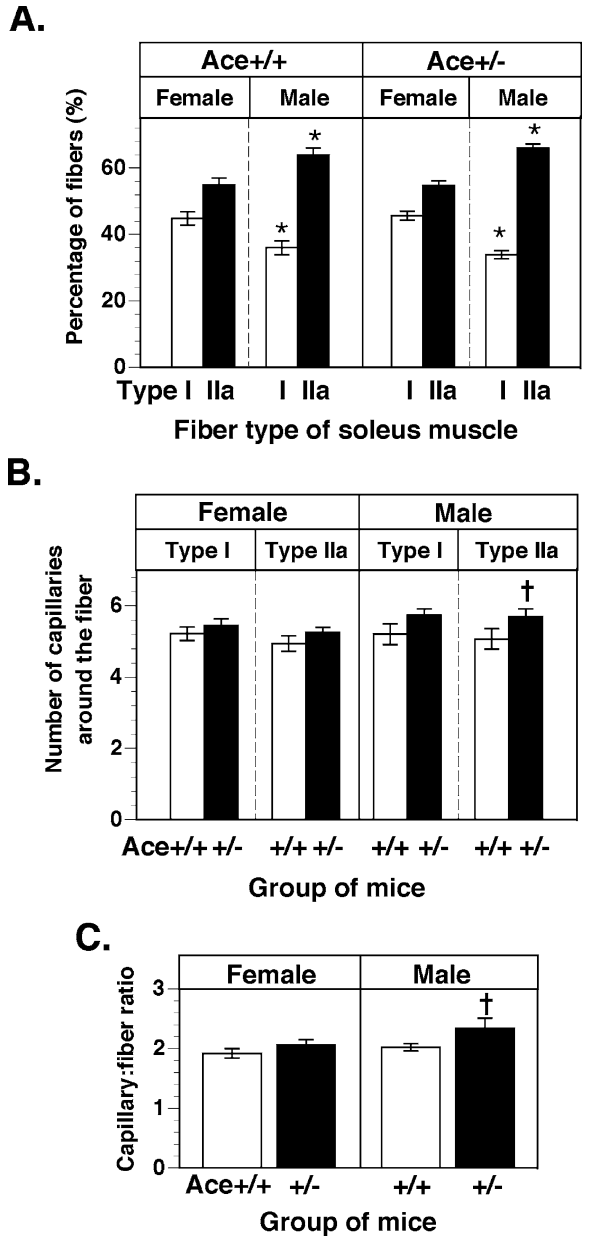

Fig. 3. *Percentage of fibers around type I and IIa fibers of soleus muscle in male and female wild-type (Ace+/+) and heterozygous mutant (Ace+/–) mice (A). The number of capillaries around type I and IIa fibers (B) and the number of capillaries per fiber (C) in Ace+/+ and Ace+/– mice according to gender.* *p < *0.05, male* vs. *female;* †p < *0.1, Ace+/– mice* vs. *Ace+/+ mice.*

light and dark after preincubation at pH 10.3 (Fig. 1A and B) and 4.5 (Fig. 1C and D), respectively, whereas the type IIa fibers were stained dark and light after preincubation at pH 10.3 (Fig. 1A and B) and 4.5 (Fig. 1C and D), respectively.

Table 1 shows ACE activity in the serum and gastrocnemius muscle, as well as the histochemical characteristics of soleus muscle fibers in Ace+/+ and Ace+/– mice. As shown

in the table, age, body weight, and the weight of the soleus muscle did not differ significantly between Ace+/+ and Ace+/− mice. As shown in Table 1, the mean ACE activities in the serum and gastrocnemius muscle in Ace+/− mice were approximately 47 and 57% lower than those in Ace+/+ mice ($p<0.01$). In both Ace+/+ and Ace+/− mice, males had significantly higher ACE activity in the serum (data not shown) and the gastrocnemius muscle (Fig. 2) than the females. Both female and male Ace+/− mice had much lower serum ACE activity than female and male Ace+/+ mice, respectively (144±3 *vs.* 271±8 U/l and 258±6 *vs.* 446±18 U/l, $p<0.01$). Moreover, in both female and male Ace+/− mice, lower ACE activity was observed in the gastrocnemius muscle than was detected in the gastrocnemius muscle of the female and male Ace+/+ mice, respectively (Fig. 2).

As shown in Table 1, there was no significant difference in terms of the percent composition (in number and area) of fiber types in the soleus muscle between Ace+/+ and Ace+/− mice. In addition, the mean areas of type I and type IIa fibers did not significantly differ between Ace+/+ and Ace+/− mice (Table 1). However, a significant gender difference in the fiber composition of the soleus muscle was found. As shown in Fig. 3A, male Ace+/+ and Ace+/− mice had a significantly lower percentage of type I soleus fibers than female Ace+/+ and Ace+/− mice, respectively. There were no significant differences in the distribution of type I and IIa fibers between Ace+/+ and Ace+/− mice in either male or female mice.

Table 1 also shows the capillary density in the soleus muscle in Ace+/+ and Ace+/− mice. As shown in the table, the number of capillaries around type IIa fibers in Ace+/− mice was significantly greater than that in Ace+/+ mice, but the differences between Ace+/− and Ace+/+ mice in terms of the number of capillaries around type I fibers were not significant ($p<0.1$). The mean number of capillaries around muscle fibers in Ace+/− mice was significantly greater than that in Ace+/+ mice (Table 1). The number of capillaries per fiber (capillary:fiber ratio) in Ace+/− mice also appeared to be greater than that in Ace+/+ mice, although the differences were not significant (Table 1). Figure 3B shows the number of capillaries around each type of fiber in Ace+/+ and Ace+/− mice according to gender. As shown in the figure, male Ace+/+ and Ace+/− mice were not significantly different from female Ace+/+ and Ace+/− mice, respectively, with regard to the number of capillaries around type I and IIa fibers. Male Ace+/− mice appeared to have more capillaries around type IIa fibers than male Ace+/+ mice, although the differences were not significant ($p<0.1$) (Fig. 3B). As shown in Fig. 3C, there was no significant gender difference in the capillary:fiber ratio between Ace+/+ and Ace+/− mice. Male Ace+/− mice appeared to have a higher capillary:fiber ratio than Ace+/+ mice, but the difference was not significant ($p<0.1$) (Fig. 3C).

As shown in Table 1, there was no significant difference in the cross-sectional area occupied by a single capillary (diffusion index) around type I and IIa fibers between Ace+/− and Ace+/+ mice. The mean cross-sectional area occupied by one capillary and the number of capillaries per area of fiber (capillary/mm^2) were also not significantly different between Ace+/− and Ace+/+ mice (Table 1).

Discussion

The present study examined the histochemical characteristics of the soleus muscle in Ace knockout mice. Mice that carry an insertional disruption of exon 14 in the murine Ace gene were originally produced by Krege *et al*. (*23*). Male homozygous mutant (Ace−/−) mice exhibit substantially reduced fertility (*23*, *32*). We confirmed that the proportion of Ace−/− mice that are produced by mating heterozygous mutant (Ace+/−) mice was extremely low (+/−:−/− = 24:2). Therefore, only heterozygous mutant mice (Ace+/−) were used in this study.

Our findings that 1) male mice had higher levels of serum ACE activity than did female mice, and 2) both female and male Ace+/− mice had much lower levels of serum ACE activity than did the respective Ace+/+ mice agree with those of Krege *et al*. (*27*). We also found that both female and male Ace+/− mice had lower levels of muscle ACE activity than did the respective Ace+/+ mice (Fig. 2). This finding agrees with the results reported by other authors who have demonstrated that Ace−/− mice had lower levels of ACE activity in the kidney, heart, and lung than did Ace+/+ mice in the corresponding tissues (*24*, *33*).

We previously reported an association between ACE I/D polymorphism and the distribution of different types of fiber in human skeletal muscle in young healthy subjects (*13*). In the present study, we did not observe any significant differences between Ace+/− and Ace+/+ mice with respect to percent composition (in terms of both number and area) of soleus muscle fibers (Table 1 and Fig. 3). This discrepancy is likely due to differences in the fiber composition of human skeletal muscle and the mouse soleus muscle. Human skeletal muscle contains type I (slow), IIa (intermediate), and IIb (fast) fibers, and we previously found that the ACE-I allele is associated with an increased percentage of type I fiber and a decreased percentage of type IIb fiber in human skeletal muscle (*13*). Our previous study showed no significant association between the ACE-I allele and the percentage of type IIa skeletal muscle. The mouse soleus muscle contains type I and IIa fibers, but no type IIb fiber (*25*). Therefore, our present finding does not contradict our previous finding. The mouse fast-twitch extensor digitorum longus muscle and gastrocnemius muscle contain large amounts of type IIb fibers, but very little type I fiber (*25*). The fast-twitch planaris muscle also contains very little type I fiber (*26*). Therefore, we did not examine these fast-twitch muscles.

We observed a significantly higher mean number of capillaries around fibers, and significantly more capillaries around type IIa fibers in Ace+/− mice, as compared with Ace+/+ mice (Table 1). These findings support those of other reports demonstrating that the inhibition of ACE activity by ACE

inhibitors (including quinaprilat and perindopril) increases capillary density in animals (*15–18*). However, other ACE inhibitors (including captopril, benazeprilat, and fosinopril) have been shown to have no significant effects on angiogenesis in humans and animals (*18*, *21*, *22*). Cilazapril has been shown to prevent capillary network remodeling and ameliorate myocardial hypertrophy in a rat model of human non-insulin-dependent diabetes that exhibits hypertension, obesity, hyperglycemia, and hyperlipidemia (*34*). In mice, ACE activity and mRNA levels are high in the kidney and heart, and low in the lung (*24*, *33*). Disruption of the Ace gene (*23*) caused about an approximately 40% reduction in ACE activity and an approximately 50% reduction in ACE mRNA in these organs in Ace+/– mice (*24*). Therefore, our findings support the hypothesis of Fabre *et al*. (*18*), namely, that the differences in the effects of ACE inhibitors on muscle capillary density are due to differences in their effects on tissue ACE activity. In fact, Reneland *et al*. (*22*), who reported that the ACE inhibitor fosinopril had no significant effects on capillary density in muscle biopsy specimens, showed that fosinopril substantially decreased serum ACE activity, but had no significant effect on muscle ACE activity. Moreover, the findings of Takai *et al*. indicated that in SHRs, trandolapril and peridopril, both of which inhibited ACE activity in the heart, significantly reduced cardiac hypertrophy; however, the administration of temocapril and enalapril, both of which failed to inhibit ACE activity in the heart, did not have this effect (*35*).

The mechanisms responsible for the association between the ACE gene and muscle capillary density remain to be elucidated. It seems unlikely that the effect of reduced serum and tissue ACE activity on the production of angiotensin II plays a role. First, angiotensin II is known to be a potent angiogenic factor (*36*). Second, Wei *et al*. (*33*) demonstrated that mice with homozygous or heterozygous deletion of the ACE gene did not differ from wild-type mice in terms of the angiotensin II concentrations in the kidney, heart, and lung, due to the compensatory production of angiotensin II by chymase. However, we found reduced muscle ACE activity in heterozygous Ace knockout mice (Fig. 2). Therefore, the association between disruption of the ACE gene and increased muscle capillary density may be mediated *via* bradykinin (BK) accumulation, since ACE catalyzes not only the conversion of angiotensin I to angiotensin II, but also the breakdown of BK into inactive peptides. In fact, an ACE inhibitor, perindopril, has been shown to increase capillary density in the ischemic hindlimbs of normal and diabetic mice *via* the BK B2 receptor pathway (*19*, *20*). It remains unclear whether or not an indirect effect of reduced activity of ACE, mediated by augmented endothelial levels of nitride oxide may contribute to this phenomenon (*18*).

In conclusion, heterozygous disruption of the ACE gene in mice increases the number of capillaries around fibers. This finding suggests a possible mechanism for the cardioprotective effects of ACE inhibitors.

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