form in myocytes, leading to a glycogen storage disease of the cardiac muscle (2).

The physiological relevance of the acute elevation of AMPK activity during exercise to muscle glycogen regulation has not been fully elucidated. Halse et al. (16) reported GS inactivation in accordance with AMPK activation after 2 h of glucose starvation in the medium in human myoblast cells in culture. They also showed that stimulation by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) or hydrogen peroxide caused AMPK activation and concurrent GS inactivation. Wojtaszewski et al. (45) demonstrated that the addition of AICAR to the circulation medium resulted in increased AMPK-α_2 activity and decreased GS activity in perfused rat skeletal muscles. AICAR is taken up into muscle cells and metabolized to form ZMP, a monophosphorylated derivative that mimics the stimulatory effects of AMP on AMPK without changing the concentration of AMP or ATP (17). In fact, GS Ser^7 can be phosphorylated and inactivated by AMPK in vitro (8) and in vivo (26). Wojtaszewski et al. also found significant activation of α_2-AMPK and inactivation of GS when muscle glycogen was depleted by prolonged exercise and restricted diet. In contrast, repeated activation of AMPK by the administration of AICAR for 5–28 days increased glycogen concentration in rat skeletal muscles (7, 21, 44). Aschenbach et al. (3) demonstrated acute activation of AMPK-α_2 in red and white gastrocnemius muscles in normal rats after a single intraperitoneal dose of AICAR. However, GS activity was reduced only in white gastrocnemius muscle, whereas GS activity conversely increased in red gastrocnemius muscle (3). They also made the contradictory observation that in vitro incubation with AICAR had no effect on GS activity in isolated rat muscles (epitrochlearis or flexor digitorum brevis) (3).

Therefore, we undertook the present study to elucidate the direct effects of acute AMPK activation, which is comparable to activation in contracting muscle on glycogenolysis and glycogen synthesis in mature skeletal muscle. For this purpose, we used an isolated rat muscle preparation and pharmacologically manipulated muscle AMPK with AICAR stimulation, which allowed us to activate AMPK-α_1 and -α_2 to a level similar to that observed after tetanic contraction in skeletal muscle. We demonstrate that activation of AMPK, in contrast to muscle contraction, does not cause GS or GP activation, nor does it cause a decrease in muscle glycogen. We propose that AMPK activation per se antagonizes glycogen synthesis in contracting muscle and facilitates the regeneration of ATP via glycolysis.

MATERIALS AND METHODS

Materials. All radioactive materials ([γ-^{32}P]ATP, 3-*O*-methyl-[^{3}H]glucose, [^{14}C]mannitol, [^{14}C]UDP-glucose, and [^{14}C]glucose 1-phosphate) were obtained from Perkin Elmer Japan (Yokohama, Japan), human insulin (Humulin R) from Eli Lilly (Indianapolis, IN), P81 filter paper from Whatman (Brentford, UK), and protein A-Sepharose from GE Healthcare Bioscience (Little Chalfont, UK). All other reagents were of analytic grade and were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Animals. Male Sprague-Dawley rats (120–130 g body wt; Japan SLC, Hamamatsu, Japan) were housed in an animal facility maintained at 20°C with a 12:12-h light-dark cycle and allowed free access to water and standard rodent chow. After an overnight fast, the rats were randomly assigned to experimental groups. The Kyoto University Graduate School of Medicine Committee on Animal Research approved all experimental procedures.

Muscle sample preparation. Animals were killed by cervical dislocation. The epitrochlearis muscles were rapidly isolated and incubated as previously described (19), with some modifications. Briefly, the muscles were preincubated for 40 min in 6 ml of Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, containing 2 mM sodium pyruvate (KRB-P). For the dose-response and time-course changes in AICAR-stimulated AMPK activity, muscles were incubated in the absence or presence of AICAR (0.03, 0.125, 0.5, 2, or 8 mM) for 40 min or in the presence of 2 mM AICAR for 10, 30, 40, or 60 min, respectively. On the basis of these experiments, we decided to stimulate muscles with 2 mM AICAR for 40 min in other experiments (see RESULTS). For insulin treatment, muscles were preincubated and then incubated in the presence of 1 μM insulin for 40 min. For epinephrine treatment, muscles were preincubated and then incubated in the presence of 3 μg/ml epinephrine for the last 15 min of the incubation period (total 40 min). Muscle contractions were induced by preincubation of muscles and then incubation in KRB-P for 40 min, followed by electrical stimulation during the last 10 min, as described previously (19) (1/min train rate, 10-s train duration, 100-Hz pulse rate, 0.1-ms pulse duration, 100 V). The buffers were continuously gassed with 95% O_2-5% CO_2 and maintained at 37°C. The muscles were then used for glucose uptake measurements (see *Glucose uptake*) or trimmed and immediately frozen in liquid nitrogen for other assays. For the measurement of lactate release, we used KRB containing 8 mM glucose during preincubation and stimulation. AICAR treatment and muscle contractions were performed as described above.

Isoform-specific AMPK activity. AMPK activity was determined as described previously, with slight modifications (40). Frozen muscles were homogenized in ice-cold lysis buffer (1:60, wt/vol) containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride and then centrifuged at 14,000 *g* for 20 min at 4°C. The supernatants (200 μg of protein) were immunoprecipitated with isoform-specific antibodies directed against the α_1- or α_2-subunit of AMPK (40) and protein A-Sepharose beads. The immune complex was washed extensively with 240 mM HEPES (pH 7.0) and 480 mM sodium chloride. Kinase activity was determined by the phosphorylation of the SAMS peptide (40). The kinase reaction was carried out in 40 mM HEPES (pH 7.0), 0.1 mM SAMS, 0.2 mM AMP, 80 mM sodium chloride, 0.8 mM dithiothreitol, 5 mM magnesium chloride, and 0.2 mM ATP (2 μCi of [γ-^{32}P]ATP) for 20 min at 30°C. The reaction products were then spotted onto P81 filter papers, which were extensively washed in 1% phosphoric acid, and the radioactivity on the dried papers was quantified with a liquid scintillation counter (Aloka, Tokyo, Japan).

Glucose uptake. We evaluated 3-*O*-methylglucose (3-MG) uptake as an index of glucose uptake activity. 3-MG uptake was determined as described previously (19), with modifications. After the incubation period, muscles were incubated in KRB containing 1 mM 3-MG, 1.5 μCi/ml [^{3}H]3-MG, 7 mM mannitol, and 0.3 μCi/ml [^{14}C]mannitol at 30°C for 10 min. The muscles were then trimmed and frozen in liquid nitrogen. The muscles were processed by incubation in 1 M NaOH at 85°C for 10 min, and the digestates were neutralized with HCl. The radioactivity in aliquots of the digestates was determined by liquid scintillation counting of dual labels, and the extracellular and intracellular spaces were calculated. The rate of 3-MG uptake was expressed as micromoles of 3-MG per milliliter of intracellular space per hour.

GS activity. GS activity was determined as described previously (19), with modifications. Frozen muscles were homogenized in lysis buffer (*buffer A*) containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 2 mM EGTA, 50 mM β-glycerophosphate, 4 mg/l leupeptin, 10 μg/ml aprotinin, 3 mM benzamidine, and 0.5 mM

phenylmethylsulfonyl fluoride and centrifuged at 14,000 *g* at 4°C for 30 min. The supernatants (40 μg of protein) were added to 80 μl of assay mixture containing 50 mM Tris (pH 7.8), 5 mM EDTA, 6.7 mM UDP-glucose, 10 mg/ml glycogen, 50 mM β-glycerophosphate, 50 mM sodium fluoride, and 0.1 mCi/mmol [^{14}C]UDP-glucose at 30°C, in the absence and presence of 10 mM glucose 6-phosphate (G-6-P), to measure G-6-P-independent (active form) and total GS activities, respectively. After 15 min the reaction solution was spotted onto a filter paper to terminate the reaction. The filter papers were washed extensively in 66% (vol/vol) ice-cold ethanol and then evaluated in a liquid scintillation counter for ^{14}C incorporation into glycogen. GS activity in the muscle was expressed as the active form ratio: G-6-P-independent activity ÷ total GS activity. The effect of ZMP on basal GS activity was evaluated by measurement of absolute GS activity in the presence of 6 mM ZMP, instead of 10 mM G-6-P, in the assay mixture. Absolute GS activity was expressed as ^{14}C incorporation activity (nmol·min^{-1}·mg protein^{-1}).

GP activity. Muscle GP activity was measured as described previously (15). Frozen muscles were homogenized in *buffer A*, and the supernatants were prepared as described for GS activity. Supernatants (40 μg of protein) were added to 80 μl of assay mixture containing 50 mM MES (pH 6.1), 100 mM glucose 1-phosphate, 200 mM potassium fluoride, 10 mg/ml glycogen, and 2.5 mCi/mol [^{14}C]glucose 1-phosphate, in the absence and presence of 6 mM 5′-AMP, to measure AMP-independent and total GP activities, respectively. After 15 min the reaction solution was spotted onto a filter paper to terminate the reaction. The papers were washed extensively in 66% (vol/vol) ice-cold ethanol and then evaluated in a liquid scintillation counter for ^{14}C incorporation into glycogen. GP activity in the muscle was expressed as the active form ratio: 5′-AMP-independent activity ÷ total GP activity. The effect of ZMP on basal GP activity was evaluated by measurement of absolute GP activity in the presence of 6 mM ZMP, instead of 6 mM 5′-AMP, in the assay mixture. Absolute GP activity was expressed as ^{14}C incorporation activity (μmol·min^{-1}·mg protein^{-1}).

Muscle glycogen. Frozen muscles were processed by incubation in 1 M NaOH at 85°C for 10 min. The digestates were neutralized with HCl. The glycogen in the digestates was hydrolyzed by incubation in 2 N HCl for 2 h at 85°C. The digestates were neutralized with NaOH, and the glucose released from the glycogen was measured enzymatically using a hexokinase glucose assay reagent (Sigma-Aldrich). Glycogen content was expressed as micromoles of glucosyl unit per gram wet muscle weight.

Lactate. Muscles were incubated and stimulated in KRB containing 8 mM glucose as described above. After muscle contraction for 10 min or AICAR stimulation for 40 min, aliquots of the incubation buffer were collected, and the lactate in the buffer was determined using the Determiner-LA kit (Kyowa Medex, Tokyo, Japan). The lactate released into the buffer was calculated and normalized to the wet muscle weight.

Statistical analysis. Values are means ± SE. Multiple means were compared by ANOVA. Two means were compared by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

AICAR acutely stimulates muscle AMPK in a dose- and time-dependent manner to a level comparable to that achieved by contraction. To evaluate the effects of AICAR stimulation on rat epitrochlearis muscle, we determined the dose and time dependency of its effects on isoform-specific AMPK activities. Pharmacological stimulation with AICAR caused a two- to threefold activation of both isoforms of AMPK (Fig. 1, *A* and *B*). The effects of AICAR stimulation on AMPK activity were not observed 10 min after the start of incubation but became prominent by 30 min. The stimulatory effect of AICAR was maximal at 40 min (Fig. 1*B*) in a dose-dependent manner (Fig. 1*A*). Therefore, we judged that stimulation with 2 mM AICAR for 40 min causes maximal AMPK activation of both α-isoforms. We also compared AICAR- and contraction-stimulated AMPK activities. Both treatments caused almost equal increases in AMPK-α_1 and -α_2 activities (Fig. 1*C*).

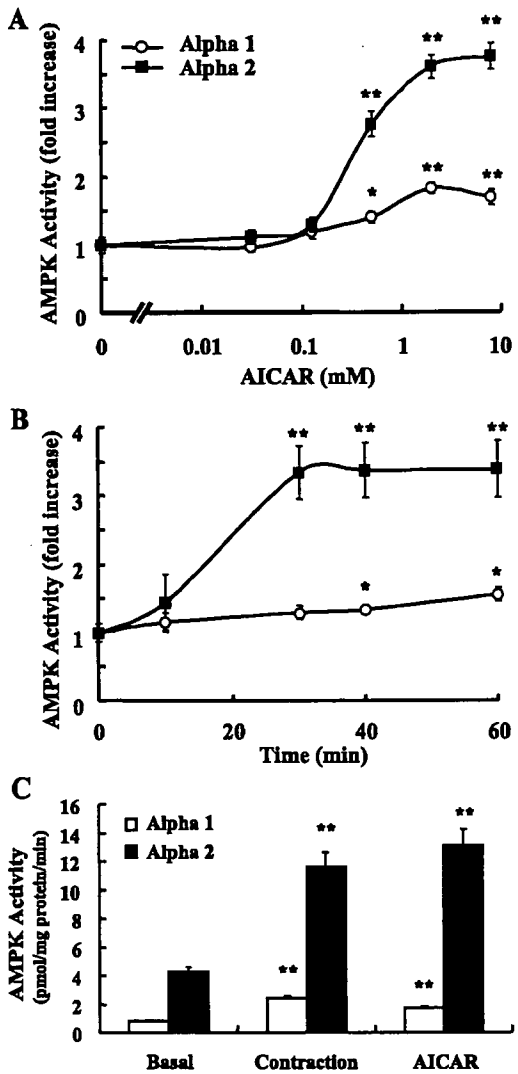

Fig. 1. Changes in α_1- and α_2-isoform-specific 5′-AMP-activated protein kinase (AMPK) activities in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by 0.03–8 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) for 40 min (*A*), 2 mM AICAR for 10–60 min (*B*), and in vitro contraction (10 min) or 2 mM AICAR for 40 min (*C*). Values are means ± SE (n = 4–6/group). *$P < 0.05$; **$P < 0.01$ vs. basal.

Contraction and AICAR activate glucose uptake to similar levels, comparable to the level achieved by a maximally effective dose of insulin. We determined whether muscle contraction and AICAR stimulation, both of which activate AMPK to similar levels (Fig. 1), increase glucose uptake. The almost identical four- to fivefold increases in 3-MG uptake stimulated by muscle contraction and AICAR (Fig. 2) are similar to that achieved by stimulation with a maximally effective dose (1 μM) of insulin.

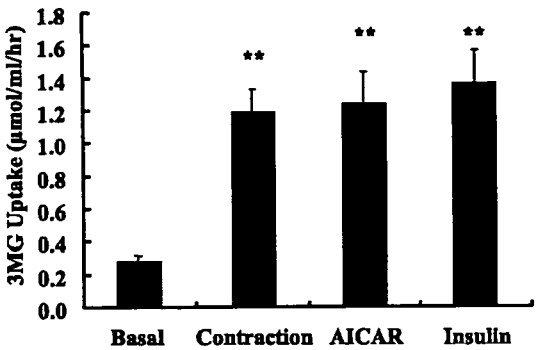

Fig. 2. 3-*O*-methylglucose (3-MG) uptake activity in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, or 1 μM insulin for 40 min. Values are means ± SE (n = 5–10/group). $^{**}P < 0.01$ vs. basal.

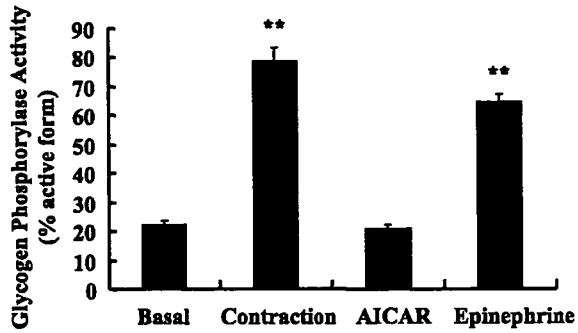

Fig. 4. Glycogen phosphorylase (GP) activity in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, or 3 μg/ml epinephrine for 15 min. Values are means ± SE (n = 6–14/group). $^{**}P < 0.01$ vs. basal.

Contraction activates, but AICAR inhibits GS activity. To determine whether the activation of AMPK affects the activity of GS, the rate-limiting enzyme of glycogen synthesis, we measured GS activity. Whereas muscle contraction caused a marked increase in GS activity, AICAR stimulation conversely decreased GS activity (Fig. 3*A*). Insulin also increased GS activity, but the effect of insulin was antagonized by the presence of AICAR [active form ratio (%) = 28.0 ± 1.0 (basal), 52.7 ± 1.5 (contraction), 24.4 ± 1.1 (AICAR), 36.1 ± 2.0 (AICAR + insulin), and 45.4 ± 1.5 (insulin); Fig. 3*A*]. AICAR-induced GS inactivation was dose dependent (Fig. 3*B*), in parallel with AMPK activity (Fig. 1*A*).

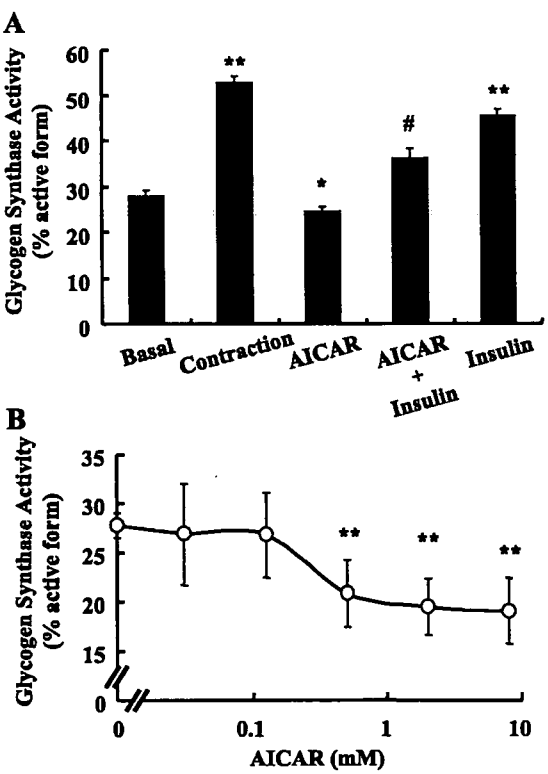

Fig. 3. Glycogen synthase (GS) activity in rat epitrochlearis muscle. *A*: isolated muscles were stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, 2 mM AICAR + 1 μM insulin for 40 min, or 1 μM insulin. Values are means ± SE (n = 12–19/group). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. basal. $^{\#}P < 0.01$ vs. insulin. *B*: muscles were incubated and stimulated by 0.03–8 mM AICAR for 40 min. Values are means ± SE [n = 4–6/group, except basal (n = 19)]. $^{**}P < 0.01$ vs. basal.

Contraction decreases, but AICAR does not alter, GP activity. To determine the effect of AMPK activation on glycogenolysis, we examined the effect of AICAR stimulation on the activity of GP, which is the rate-limiting enzyme of glycogenolysis. Whereas muscle contraction and epinephrine stimulation markedly increased GP activity, AICAR did not alter GP activity [active form ratio (%) = 22.4 ± 1.0 (basal), 78.8 ± 4.4 (contraction), 20.5 ± 1.4 (AICAR), and 64.5 ± 1.7 (epinephrine); Fig. 4].

Contraction decreases, but AICAR does not alter, muscle glycogen content. We examined the effect of AICAR-stimulated AMPK activation on the concentration of glycogen. Glycogen content was reduced in contracting and epinephrine-stimulated muscles. In contrast, glycogen was unchanged by AICAR (23.3 ± 1.3, 12.4 ± 0.9, 25.6 ± 1.3, 23.7 ± 1.2, and 13.1 ± 2.4 μmol glucosyl unit/g wet muscle wt for the basal state, contraction, AICAR, insulin, and epinephrine, respectively; Fig. 5). These results are consistent with the increase in GP activity induced by contraction or epinephrine, which was unchanged by AICAR (Fig. 4).

AICAR increases lactate release from muscle. Because AICAR stimulation resulted in the inactivation of GS, despite a marked increase in glucose uptake, we investigated the amount of lactate released into the incubation buffer to clarify

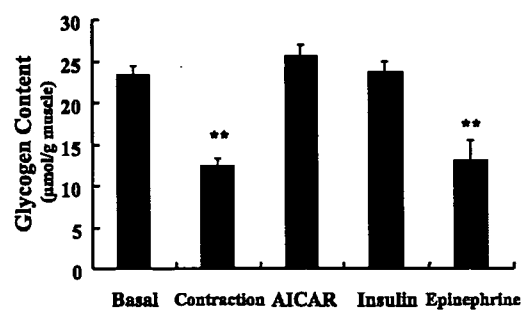

Fig. 5. Glycogen content in rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min), 2 mM AICAR for 40 min, 1 μM insulin for 40 min, or 3 μg/ml epinephrine for 15 min. Values are means ± SE [n = 16–20/group, except epinephrine (n = 4)]. $^{**}P < 0.01$ vs. basal.

whether the glucose taken up into the muscle is degraded via the glycolysis pathway. The amount of lactate was measured in buffer containing glucose, instead of pyruvate. Similar to muscle contraction, AICAR caused a significant increase in lactate release (12.7 ± 0.85, 38.6 ± 2.11, and 20.1 ± 1.14 μg lactate/mg muscle for the basal state, contraction, and AICAR, respectively; Fig. 6).

ZMP stimulates GP activity, but does not affect GS activity, in vitro. When skeletal muscle is incubated with AICAR, AICAR is taken up into the muscle. It is then phosphorylated to form the AMP-like compound ZMP (17). Consequently, it is possible that increased intracellular ZMP directly modulates GS or GP activity as an AMPK-independent effect. Therefore, we measured GS and GP activities in the presence of ZMP in vitro. GS activity was unchanged in the presence of ZMP, whereas it was markedly elevated in the presence of G-6-P, a strong allosteric activator of GS (6,669 ± 446, 36,907 ± 1,085, and 6,409 ± 267 pmol·min^{-1}·mg^{-1} without G-6-P and without ZMP, with G-6-P, and with ZMP, respectively; Fig. 7*A*). GP activity was elevated in the presence of ZMP to an extent similar to that observed with AMP, a potent allosteric activator of GP (664.2 ± 36.8, 1,335 ± 61.2, and 1,416 ± 92.4 nmol·min^{-1}·mg^{-1} without AMP and without ZMP, with AMP, and with ZMP, respectively; Fig. 7*B*).

DISCUSSION

Studies of AICAR have provided important information about the function of acute AMPK activation in muscle glucose metabolism. The specificity of AICAR as an AMPK stimulator has been established by Mu et al. (31), who blocked AMPK activity in mouse skeletal muscle with the muscle-specific expression of a dominant-negative kinase-dead form of AMPK. In that mouse, the stimulatory effects of AICAR on glucose transport (31) and GLUT4 expression (22) were abolished completely. On the basis of findings suggesting that acute AICAR stimulation in vitro activates AMPK and glucose transport in fast-twitch muscles but has no effect on the slow-twitch soleus muscle of the rat (1, 4) and that AICAR administration also has the greatest effect on the GLUT4 content of fast-twitch muscles (7, 21, 44), we analyzed the rat epitrochlearis muscle. Differential ATPase staining of rat epitrochlearis demonstrated >80% fast-twitch and only 15% slow-twitch fibers (33, 34). In the present study, AICAR

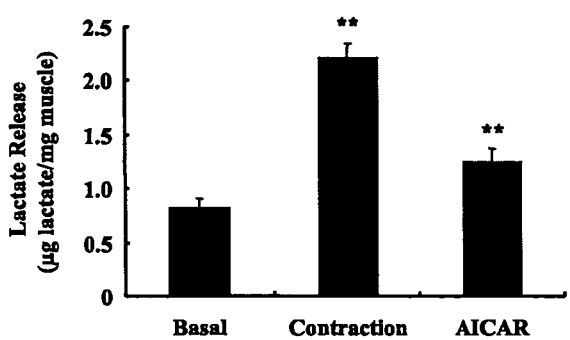

Fig. 6. Lactate release from rat epitrochlearis muscle. Isolated muscles were incubated and stimulated by in vitro contraction (10 min) or 2 mM AICAR for 40 min in glucose-containing Krebs-Ringer bicarbonate buffer, and lactate concentrations in buffer were determined. Values are means ± SE (n = 5–6/group). **P < 0.01 vs. basal.

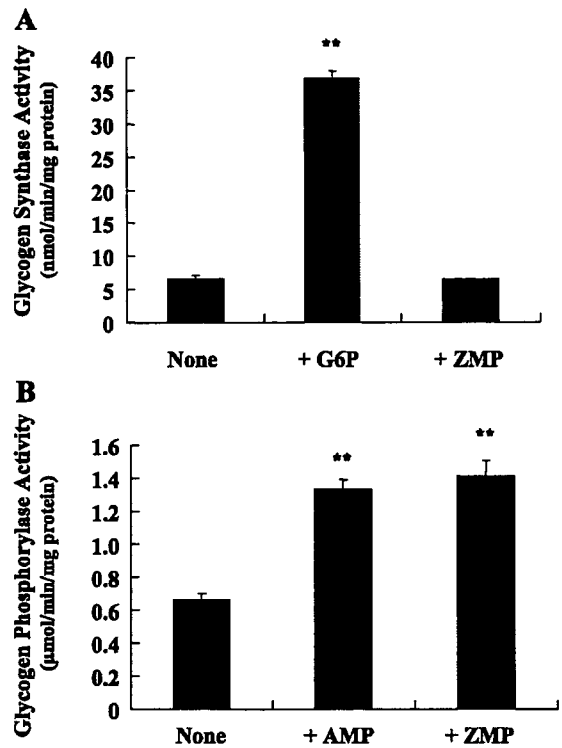

Fig. 7. Effects of ZMP on GS and GP activities in rat epitrochlearis muscle. Muscle samples in the basal state were assayed for GS activity in the presence of 10 mM glucose 6-phosphate (G-6-P) or 6 mM ZMP in vitro (*A*) and for GP activity in the presence of 6 mM AMP or 6 mM ZMP in vitro (*B*). Values are means ± SE (n = 8–9/group). **P < 0.01 vs. none.

treatment activated AMPK to the extent observed in skeletal muscle after contraction. AICAR (2 mM, 40 min) and tetanic contraction (10 s, 10 times) activated AMPK-α_1 and -α_2 (Fig. 1*C*), with a corresponding increase in the rate of 3-MG uptake to the level achieved by a maximally effective dose (1 μM) of insulin (Fig. 2).

We used isolated muscle incubated in vitro to eliminate the effects of systemic confounders, such as humoral factors and blood flow, because exercise in vivo evokes a number of dynamic changes, many of which can potentially alter fuel metabolism in contracting skeletal muscles (20). For example, exercise increases the blood concentration of epinephrine, a potent activator of glycogen breakdown (Fig. 4). Our method, using isolated muscle, made it possible to examine the direct effects of pharmacological manipulation and contraction on skeletal muscle metabolism.

In the present study, AICAR stimulation caused a decrease in GS activity, in contrast to muscle contraction (Fig. 3*A*). GS inactivation was dose dependent, in parallel with AMPK activation (Figs. 1*A* and 3*B*). Furthermore, insulin-stimulated GS activation was partially antagonized in the presence of AICAR (Fig. 3*A*). We eliminated the possibility of a direct inactivation of GS by ZMP, an intracellular metabolite of AICAR (Fig. 7*A*). Our observation is consistent with a previous report by Wojtaszewski et al. (45), who showed an inhibition of GS activity in rat hindlimb muscle after perfusion with AICAR. They also showed that the inactivation of GS was accompanied by a decrease in gel mobility and was abolished by protein

phosphatase treatment, indicating that AICAR stimulation causes GS phosphorylation (45). More recently, Jorgensen et al. (26) showed that AICAR treatment in vitro increases GS phosphorylation at site 2 (Ser^7) and decreases GS activity in mouse extensor digitorum longus muscle. Interestingly, however, Aschenbach et al. (3) showed that intraperitoneal administration of AICAR to a living rat inhibited GS activity in white gastrocnemius muscle, whereas AICAR administration also activated GS activity in red gastrocnemius muscle. They reported that in vitro incubation of the epitrochlearis and flexor digitorum brevis muscles with AICAR stimulated AMPK-α_2 but had no effect on GS activity (3). The reasons for these discrepancies in the responses of the red and white gastrocnemius muscles and between the in vivo and in vitro experiments are unclear. However, Aschenbach et al. speculate that these AICAR-stimulated changes in GS activity may be due to the secondary effects of glucose transport and glycogen accumulation, rather than the direct effects of AMPK on GS.

The idea that active AMPK prevents glycogen synthesis in skeletal muscle may appear to be inconsistent with the chronic accumulation of glycogen in rat skeletal muscle induced by repeated administrations of AICAR. Several reports have shown that once-a-day administration of AICAR for 5–28 days in vivo causes a marked increase in the glycogen concentration of rat muscles (7, 21, 44). The levels of GLUT4 and hexokinase proteins are also upregulated by AICAR (7, 21, 44). Furthermore, each AICAR treatment induces GLUT4 translocation and increases the rate of glucose transport into muscle cells (Fig. 2) (19, 27). Increased glucose transport and hexokinase expression may result in increased concentrations of G-6-P, a potent allosteric activator of GS (Fig. 7*A*). They may also override the effects of the inhibitory phosphorylation of GS by AMPK. Thus these combined effects of AICAR on protein expression and glucose transport may predominate and facilitate glycogen synthesis, despite the deactivation of GS by AICAR.

In the present study, epinephrine stimulation and contraction caused increases in GP activity (Fig. 4), with corresponding decreases in glycogen content (Fig. 5), whereas AICAR stimulation neither increased GP activity nor altered the glycogen content (Fig. 4). Therefore, acute AMPK activation is considered to have no significant effect on GP activity in skeletal muscle. Our findings are consistent with the report by Aschenbach et al. (3), who found that in vitro AICAR treatment had no effect on GP activity in isolated epitrochlearis or flexor digitorum brevis muscles, despite significant activation of AMPK-α_2. Interestingly, however, they also reported activation of GP in gastrocnemius muscle after intraperitoneal administration of AICAR in vivo, with a corresponding increase in AMPK-α_2 activity (3). The cause of this contradictory change in GP activity is unknown. However, Aschenbach et al. speculate that it may be due to secondary effects in response to in vivo AICAR treatment. Our findings also appear to be inconsistent with another report by Young et al. (47), who showed that GP is activated in rat soleus muscle incubated with AICAR in vitro. However, as mentioned above, AICAR stimulation has no effect on AMPK activity in rat soleus muscle (1, 4). Therefore, the GP activation observed in rat soleus muscle might not be related to AMPK activity. In support of this idea, AICAR did not alter the rate of glycogen synthesis in rat soleus in the basal state or a maximally stimulatory concentration of insulin (47). However, we found that AICAR decreased the basal and insulin-stimulated GS activity in rat epitrochlearis muscle (Fig. 3*A*), with a significant increase in AMPK activity (Fig. 1). Young et al. also demonstrated that the AICAR metabolite ZMP mimics the stimulatory effect of AMP, a known allosteric activator of GP, in extracts of rat soleus muscle. In the present study, we also found a marked elevation in GP activity when ZMP was added directly to muscle lysate to a level similar to that observed when AMP was added to the lysate (Fig. 7*B*). Longnus et al. (28) showed that AICAR activates GP in isolated rat myocardium in a dose-dependent manner, with no accompanying activation of AMPK. On the basis of these data, it seems reasonable that AICAR-induced GP activation is due to allosteric activation by ZMP, as in the rat myocardium.

On the basis of these observations, we propose that acute AMPK activation during muscle contraction antagonizes contraction-stimulated GS activity and that this effect consequently facilitates a glycolytic flux. Our proposal is consistent with the idea that AMPK acts as an energy sensor, switching off ATP-consuming pathways and switching on alternative pathways for ATP regeneration when cells sense low energy (17). The muscle glycogen accumulation induced by repeated AICAR stimulation may be due to the stimulatory effect of AMPK on glucose transport and on the expression of proteins such as GLUT4 and hexokinase. These effects may override the inhibitory action of AMPK on GS activity, resulting in enhanced glycogen synthesis in skeletal muscle. In conclusion, AMPK does not directly mediate contraction-stimulated GS or GP activation. However, AMPK may act as a metabolic regulator that leads to an increased glycolytic flux in contracting skeletal muscle.

ACKNOWLEDGMENTS

We thank Taro Okada (Kobe University) for technical suggestions and Yoko Koyama and Kaoru Ijiri for secretarial assistance. We also thank the Radioisotope Research Center of Kyoto University for instrumental support in radioisotope experiments.

GRANTS

This work was supported by research grants from the Japanese Ministry of Education, Science, Sports, and Culture (to T. Hayashi). T. Toyoda was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. **Ai H, Ihlemann J, Hellsten Y, Lauritzen HP, Hardie DG, Galbo H, Ploug T.** Effect of fiber type and nutritional state on AICAR- and contraction-stimulated glucose transport in rat muscle. *Am J Physiol Endocrinol Metab* 282: E1291–E1300, 2002.
2. **Arad M, Benson DW, Perez-Atayde AR, McKenna WJ, Sparks EA, Kanter RJ, McGarry K, Seidman JG, Seidman CE.** Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *J Clin Invest* 109: 357–362, 2002.
3. **Aschenbach WG, Hirshman MF, Fujii N, Sakamoto K, Howlett KF, Goodyear LJ.** Effect of AICAR treatment on glycogen metabolism in skeletal muscle. *Diabetes* 51: 567–573, 2002.
4. **Balon TW, Jasman AP.** Acute exposure to AICAR increases glucose transport in mouse EDL and soleus muscle. *Biochem Biophys Res Commun* 282: 1008–1011, 2001.
5. **Barnes BR, Marklund S, Steiler TL, Walter M, Hjalm G, Amarger V, Mahlapuu M, Leng Y, Johansson C, Galuska D, Lindgren K, Abrink M, Stapleton D, Zierath JR, Andersson L.** The 5′-AMP-activated protein kinase γ_3-isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J Biol Chem* 279: 38441–38447, 2004.
6. **Bergstrom J, Hultman E.** Muscle glycogen synthesis after exercise: an enhancing factor localized to the muscle cells in man. *Nature* 210: 309–310, 1966.

7. **Buhl ES, Jessen N, Schmitz O, Pedersen SB, Pedersen O, Holman GD, Lund S.** Chronic treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. *Diabetes* 50: 12–17, 2001.
8. **Carling D, Hardie DG.** The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. *Biochim Biophys Acta* 1012: 81–86, 1989.
9. **Chen ZP, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, Kemp BE, McConell GK.** Effect of exercise intensity on skeletal muscle AMPK signaling in humans. *Diabetes* 52: 2205–2212, 2003.
10. **Ciobanu D, Bastiaansen J, Malek M, Helm J, Woollard J, Plastow G, Rothschild M.** Evidence for new alleles in the protein kinase adenosine monophosphate-activated γ_3-subunit gene associated with low glycogen content in pig skeletal muscle and improved meat quality. *Genetics* 159: 1151–1162, 2001.
11. **Conlee RK, Hickson RC, Winder WW, Hagberg JM, Holloszy JO.** Regulation of glycogen resynthesis in muscles of rats following exercise. *Am J Physiol Regul Integr Comp Physiol* 235: R145–R150, 1978.
12. **Fiedler M, Zierath JR, Selen G, Wallberg-Henriksson H, Liang Y, Sakariassen KS.** 5-Aminoimidazole-4-carboxy-amide-1-β-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinaemia but not dyslipidaemia in KKAy-CETP mice. *Diabetologia* 44: 2180–2186, 2001.
13. **Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA, Thorell A, Goodyear LJ.** Exercise induces isoform-specific increase in 5′-AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273: 1150–1155, 2000.
14. **Garetto LP, Richter EA, Goodman MN, Ruderman NB.** Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *Am J Physiol Endocrinol Metab* 246: E471–E475, 1984.
15. **Gilboe DP, Larson KL, Nuttall FQ.** Radioactive method for the assay of glycogen phosphorylases. *Anal Biochem* 47: 20–27, 1972.
16. **Halse R, Fryer LG, McCormack JG, Carling D, Yeaman SJ.** Regulation of glycogen synthase by glucose and glycogen: a possible role for AMP-activated protein kinase. *Diabetes* 52: 9–15, 2003.
17. **Hardie DG, Carling D.** The AMP-activated protein kinase—fuel gauge of the mammalian cell? *Eur J Biochem* 246: 259–273, 1997.
18. **Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ.** Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49: 527–531, 2000.
19. **Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ.** Evidence for 5′-AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47: 1369–1373, 1998.
20. **Hayashi T, Wojtaszewski JF, Goodyear LJ.** Exercise regulation of glucose transport in skeletal muscle. *Am J Physiol Endocrinol Metab* 273: E1039–E1051, 1997.
21. **Holmes BF, Kurth-Kraczek EJ, Winder WW.** Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J Appl Physiol* 87: 1990–1995, 1999.
22. **Holmes BF, Lang DB, Birnbaum MJ, Mu J, Dohm GL.** AMP kinase is not required for the GLUT4 response to exercise and denervation in skeletal muscle. *Am J Physiol Endocrinol Metab* 287: E739–E743, 2004.
23. **Hutber CA, Hardie DG, Winder WW.** Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* 272: E262–E266, 1997.
24. **Iglesias MA, Ye JM, Frangioudakis G, Saha AK, Tomas E, Ruderman NB, Cooney GJ, Kraegen EW.** AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. *Diabetes* 51: 2886–2894, 2002.
25. **Ivy JL, Holloszy JO.** Persistent increase in glucose uptake by rat skeletal muscle following exercise. *Am J Physiol Cell Physiol* 241: C200–C203, 1981.
26. **Jorgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, Richter EA, Wojtaszewski JF.** The α_2-5′-AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* 53: 3074–3081, 2004.
27. **Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW.** 5′-AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 48: 1667–1671, 1999.
28. **Longnus SL, Wambolt RB, Parsons HL, Brownsey RW, Allard MF.** 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) stimulates myocardial glycogenolysis by allosteric mechanisms. *Am J Physiol Regul Integr Comp Physiol* 284: R936–R944, 2003.
29. **Milan D, Jeon JT, Looft C, Amarger V, Robic A, Thelander M, Rogel-Gaillard C, Paul S, Iannuccelli N, Rask L, Ronne H, Lundstrom K, Reinsch N, Gellin J, Kalm E, Roy PL, Chardon P, Andersson L.** A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. *Science* 288: 1248–1251, 2000.
30. **Mu J, Barton ER, Birnbaum MJ.** Selective suppression of AMP-activated protein kinase in skeletal muscle: update on "lazy mice." *Biochem Soc Trans* 31: 236–241, 2003.
31. **Mu J, Brozinick JT Jr, Valladares O, Bucan M, Birnbaum MJ.** A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7: 1085–1094, 2001.
32. **Nakano M, Hamada T, Hayashi T, Yonemitsu S, Miyamoto L, Toyoda T, Tanaka S, Masuzaki H, Ebihara K, Ogawa Y, Hosoda K, Inoue G, Yoshimasa Y, Otaka A, Fushiki T, Nakao K.** α_2-Isoform-specific activation of 5′-adenosine monophosphate-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside at a physiological level activates glucose transport and increases glucose transporter 4 in mouse skeletal muscle. *Metabolism* 55: 300–308, 2006.
33. **Nesher R, Karl IE, Kaiser KE, Kipnis DM.** Epitrochlearis muscle. I. Mechanical performance, energetics, and fiber composition. *Am J Physiol Endocrinol Metab* 239: E454–E460, 1980.
34. **Nesher R, Karl IE, Kipnis DM.** Epitrochlearis muscle. II. Metabolic effects of contraction and catecholamines. *Am J Physiol Endocrinol Metab* 239: E461–E467, 1980.
35. **Ojuka EO, Nolte LA, Holloszy JO.** Increased expression of GLUT-4 and hexokinase in rat epitrochlearis muscles exposed to AICAR in vitro. *J Appl Physiol* 88: 1072–1075, 2000.
36. **Price TB, Rothman DL, Taylor R, Avison MJ, Shulman GI, Shulman RG.** Human muscle glycogen resynthesis after exercise: insulin-dependent and -independent phases. *J Appl Physiol* 76: 104–111, 1994.
37. **Rasmussen BB, Winder WW.** Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. *J Appl Physiol* 83: 1104–1109, 1997.
38. **Richter EA, Garetto LP, Goodman MN, Ruderman NB.** Enhanced muscle glucose metabolism after exercise: modulation by local factors. *Am J Physiol Endocrinol Metab* 246: E476–E482, 1984.
39. **Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE.** Mammalian AMP-activated protein kinase subfamily. *J Biol Chem* 271: 611–614, 1996.
40. **Toyoda T, Hayashi T, Miyamoto L, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Inoue G, Otaka A, Sato K, Fushiki T, Nakao K.** Possible involvement of the α_1-isoform of 5′-AMP-activated protein kinase in oxidative stress-stimulated glucose transport in skeletal muscle. *Am J Physiol Endocrinol Metab* 287: E166–E173, 2004.
41. **Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, Ruderman NB.** Contraction-induced changes in acetyl-CoA carboxylase and 5′-AMP-activated kinase in skeletal muscle. *J Biol Chem* 272: 13255–13261, 1997.
42. **Winder WW.** Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *J Appl Physiol* 91: 1017–1028, 2001.
43. **Winder WW, Hardie DG.** Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am J Physiol Endocrinol Metab* 270: E299–E304, 1996.
44. **Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M, Holloszy JO.** Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol* 88: 2219–2226, 2000.
45. **Wojtaszewski JF, Jorgensen SB, Hellsten Y, Hardie DG, Richter EA.** Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. *Diabetes* 51: 284–292, 2002.
46. **Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D.** LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13: 2004–2008, 2003.
47. **Young ME, Radda GK, Leighton B.** Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR—an activator of AMP-activated protein kinase. *FEBS Lett* 382: 43–47, 1996.
48. **Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL.** Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. *J Appl Physiol* 91: 1073–1083, 2001.

0013-7227/08/$15.00/0
Printed in U.S.A.

Endocrinology 149(2):483–491

doi: 10.1210/en.2007-1094

Therapeutic Potential of Atrial Natriuretic Peptide Administration on Peripheral Arterial Diseases

Kwijun Park, Hiroshi Itoh, Kenichi Yamahara, Masakatsu Sone, Kazutoshi Miyashita, Naofumi Oyamada, Naoya Sawada, Daisuke Taura, Megumi Inuzuka, Takuhiro Sonoyama, Hirokazu Tsujimoto, Yasutomo Fukunaga, Naohisa Tamura, and Kazuwa Nakao

Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

Peripheral arterial diseases are caused by arterial sclerosis and impaired collateral vessel formation, which are exacerbated by diabetes, often leading to leg amputation. We have reported that an activation of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway accelerated vascular regeneration and blood flow recovery in murine legs, for which ischemia had been induced by a femoral arterial ligation as a model for peripheral arterial diseases. In this study, ip injection of carperitide, a human recombinant atrial natriuretic peptide, accelerated blood flow recovery with increasing capillary density in ischemic legs not only in nondiabetic mice but also in mice kept upon streptozotocin-induced hyperglycemia for 16 wk, which significantly impaired the blood flow recovery compared with nondiabetic mice. Based on these findings, we tried to apply the administration of carperitide to the treatment of peripheral arterial diseases. The study group comprised a continuous series of 13 patients with peripheral arterial diseases (Fontaine's classification I, one; II, five; III, two; and IV, five), for whom conventional therapies had not accomplished appreciable results. Carperitide was administrated continuously and intravenously for 2 wk to Fontaine's class I–III patients and for 4 weeks to class IV patients. The dose was gradually increased to the maximum, with the patient's systolic blood pressure being kept above 100 mm Hg. Carperitide administration improved the ankle-brachial pressure index, intermittent claudication, rest pain, and ulcers. In conclusion, this study showed a therapeutic potential of carperitide to treat peripheral arterial diseases refractory to conventional therapies. (*Endocrinology* 149: 483–491, 2008)

LOWER EXTREMITY PERIPHERAL artery disease (PAD), which consists of arteriosclerosis thrombosis and thromboangitis obliterans, is caused by the altered structure and function of the arteries that supply the lower limbs. Numerous pathophysiological processes can contribute to the creation of stenoses or aneurysms of peripheral artery circulation. Among them, diabetes mellitus is one of the most important causes of PAD. According to the Centers for Disease Control and Prevention's National Center for Chronic Disease Prevention and Health Promotion, 82,000 people have diabetes-related leg, foot, or toe amputations each year in the United States. World Diabetes Day announced that up to 70% of leg amputation cases are patients with diabetes. In PAD patients with diabetes, collateral vessel formation is impaired (1), and intricately modified angiogenesis contributes to a large variety of complications including diabetic gangrene (2). Mechanisms that alter angiogenesis in diabetes are largely unknown. It is reported, however, that either inappropriate production or action of nitric oxide (NO) may play important roles in vascular insufficiencies with diabetes (3). NO activates soluble guanylyl cyclase (GC) followed by the cGMP signal transduction cascade (4). Significant reverse correlation between the urinary cGMP excretion rate and the disease grade according to Fontaine's classification observed in PAD patients seems to imply the impact of diminished cGMP production in PAD (5).

Natriuretic peptides (NPs) consist of atrial NP (ANP), brain NP (BNP), and C-type NP (CNP) and elicit various biological effects by activating particulate GCs: GC-A is a receptor selective for ANP and BNP, and GC-B is a receptor selective for CNP (4, 6–8). One of the major mediators of cGMP signaling is cGMP-dependent protein kinase (cGK) (4). ANP and BNP are secreted mainly from the atrium and ventricle of the heart, respectively, and act as cardiac hormones (4, 6, 7). The clinical significance of NPs is already recognized in the diagnosis and treatment of congestive heart failure (CHF). Recombinant human ANP and BNP are used for treating CHF, with the main expectation of diuretic and natriuretic effects (9, 10).

Recently, NPs have been revealed to have various effects on cell survival, proliferation, and differentiation. We reported that ANP at a physiological concentration induces endothelial regeneration in the human coronary artery and umbilical vein through the activation of ERK and phosphatidylinositol 3-kinase/Akt pathways (11). We used genetically engineered mice that overexpress BNP and type I cGK (cGKI), or otherwise lack cGKI, and demonstrated that BNP can promote vascular regeneration and accelerate the restoration of blood flow after the removal of a hind-limb artery in mice through the activation of the GC-A/cGMP/cGKI

First Published Online November 8, 2007

Abbreviations: ABI, Ankle-brachial pressure index; ADMA, asymmetric dimethylarginine; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; cGK, cGMP-dependent protein kinase; cGKI, type I cGMP-dependent protein kinase; CHF, congestive heart failure; CNP, C-type natriuretic peptide; EC, endothelial cell; ESRD, end-stage renal disease; GC, guanylyl cyclase; NP, natriuretic peptide; PECAM, platelet endothelial cell adhesion molecule-1; SMC, smooth muscle cell; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

pathway (12–14). Meanwhile, CNP, which is secreted from endothelial cells (ECs) and acts as an endothelium-derived relaxing peptide (15), also induces redifferentiation of vascular smooth muscle cells (SMCs) while accelerating reendothelialization and suppressing neointimal hyperplasia in vein grafting or balloon injuries in rabbits, which simulate atherosclerotic lesions in humans (16, 17). These observations indicate that GC-A/cGMP and GC-B/cGMP signaling cascades have potential to promote vascular regeneration in PAD and to inhibit the progression of atherosclerotic lesions. On the other hand, we have reported previously that endothelial CNP expression is progressively reduced in accordance with the severity of human coronary atherosclerosis (18), which indicates that not only NO/soluble GC/cGMP signaling but also CNP/GC-B/cGMP signaling might be impaired in PAD. Therefore, the restoration of intracellular cGMP levels by the activation of GC-A, the third signaling pathway using cGMP as the second messenger in vascular SMCs and ECs, could improve PAD.

In this context, we hypothesized that an administration of ANP or BNP could, at least partly, compensate for impaired angiogenesis due to diminished intracellular cGMP levels in PAD patients by an activation of GC-A. In Japan, carperitide, a recombinant human ANP, is already approved and widely used for the treatment of CHF. By contrast, nesiritide, a recombinant human BNP, has not been approved in Japan, and it cannot be applied to rodent models because amino acid sequences and molecular forms of BNP are quite different between humans and rodents. In the present study, we therefore examined the effect of carperitide on vascular regeneration in animal models with diabetes, and we further tried to determine safety and to investigate any possible therapeutic effects of carperitide in PAD patients.

Materials and Methods

Animals

C57BL/6 male mice (CLEA Japan, Inc., Tokyo, Japan) were used for experiments. Diabetes was induced in the mice by repetitive (once a day for 4–6 consecutive days) ip injections of streptozotocin (STZ) (Nacalai Tesque Inc, Kyoto, Japan; 65–100 mg/kg body weight in 200 μl of 10 mM sodium citrate buffer, pH 4.0) at 8 wk of age. Blood glucose concentrations were monitored weekly after STZ treatment with Dexter-ZII (Bayer Medical Ltd., Tokyo, Japan). Animals with blood glucose levels above 220 mg/dl at 2 wk after the first STZ injection were used as STZ-diabetic mice. Control mice received an equal volume of citrate buffer. Mice were used for experiments of limb ischemia at 4, 16, and 26 wk after the first injection of STZ or vehicle.

An animal model of limb ischemia was made by a ligation of one femoral artery. The blood flow in both legs was assessed with a laser Doppler perfusion image analyzer (Moor Instruments, Devon, UK), and the blood flow recovery was assessed by the ischemic limb to normal limb ratio of blood flow, as we described previously (14).

To assess the effect of carperitide, a recombinant human ANP (Daiichi Asubio Pharma Co., Ltd., Tokyo, Japan), on angiogenesis in ischemic limbs, the femoral artery ligation was carried out at 16 wk after the first injection of STZ or vehicle, and carperitide at a dose of 2.2 μg/kg·min or equal volume of water (vehicle) was administered continuously and ip via a microosmotic pump (Alzet model 1002D; Alzet Pharmaceuticals, Palo Alto, CA), which was implanted ip at 3 d after the femoral artery ligation. Pumps were renewed at d 14 after primary implantation. At 28 d from the femoral artery ligation, mice were euthanized by an overdose of pentobarbital injection, and the ischemic hind limb was isolated for the histological analysis.

All experimental procedures were performed according to Kyoto University standards for animal care.

Histological analysis

After fixation with 4% paraformaldehyde, ischemic lower legs were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at −80 C. Cryostat sections (4–8 μm thick) of the tissue were stained with a rat antimouse platelet EC adhesion molecule-1 (PECAM-1) antibody (item 553370; PharMingen, San Diego, CA). Four random fields on two different sections (3 mm apart) from each mouse were photographed with a digital camera (Olympus, Tokyo, Japan). By computer-assisted analysis using NIH IMAGE, capillary density was calculated as the mean number of capillaries stained with PECAM-1, as we described previously (14).

Patients

Participants were a series of 13 Japanese patients including 11 males and two females, aged 38–92 yr, who had already been diagnosed with PAD and hospitalized in our department from June 2003 to August 2005 (Table 1). Patients classified as Fontaine's classes II–IV or with characteristic symptoms of PAD were included. Diseases accompanying PAD were defined as follows: type 2 diabetes mellitus, following the diagnostic criteria of Japan Diabetes Society; hypertension, blood pressure is equal to or greater than 140/90 mm Hg; end-stage renal disease, chronic renal failure on indispensable renal replacement therapy; ischemic heart disease, history of angina pectoris or myocardial ischemia with or without present medication; CHF, past diagnosis of CHF with or without present medication; hyperlipidemia, low-density lipoprotein-cholesterol is equal to or greater than 140 mg/dl, or triglyceride is equal to or greater than 150 mg/dl; obesity, body mass index is greater than 25 kg/m^2. Exclusion criteria were contraindications for carperitide: possibility of immediate surgery, suffering from malignancy, febrility, an inability to declare subjective symptoms, pregnancy, or other unfavorable statuses. The study was conducted in accordance with the guidelines in the Declaration of Helsinki. The study protocol was approved by the Ethics Committee Graduate School and Faculty of Medicine, Kyoto University. Patients were fully informed of the aim of the study, and their written informed consent was obtained.

Procedure of carperitide administration to patients

Carperitide was administered continuously and iv for 2 wk for Fontaine I–III patients and for 4 wk for Fontaine IV patients in principle. The starting dose of 0.006 μg/kg·min was gradually increased as long as the systolic blood pressure remained above 100 mm Hg. The range of final

TABLE 1. Patients' characteristics

Characteristic	n
Sex	
Male	11
Female	2
Diagnosis	
Arteriosclerosis obliterans	12
Thromboangitis obliterans	1
Gangrene or ulcer(s)	4
Fontaine's classification	
I	1
II	5
III	2
IV	5
Other disorders	
Hypertension	12
Type 2 diabetes	11
ESRD	7
CHF	5
Ischemic heart disease	4
Hyperlipidemia	4
Obesity (BMI > 25)	3

Patients' mean ± SD age was 72 ± 15 yr. BMI, Body mass index.

doses of carperitide used in this study was 0.003–0.1 μg/kg·min. Drugs for injection such as prostaglandins were avoided during the carperitide administration. The administration was stopped and standard remedy performed if any unfavorable symptoms appeared.

Pain was assessed when present with a numerical rating scale from 0–10; grade 0 indicated no pain and grade 10 the strongest pain the patient could imagine. The ankle-brachial pressure index (ABI) was assessed by an automated measurement device (BP-203RPEII; Colin Medical Technology Corp., Aichi, Japan). An exercise tolerance test was carried out weekly for patients with intermittent claudication. Pain-free walking distance on a flat ground was assessed. A stair-climb test was performed when walking on flat ground did not induce claudication. The test assessed how many floors a patient could climb without pain on the stair of our internal medicine ward building. Blood sampling was performed immediately before the beginning of carperitide administration and weekly during the administration for routine blood examination. It was also performed to determine the plasma levels of ANP, cGMP, and vascular endothelial growth factor (VEGF).

Analysis of blood samples

The blood samples from mice were withdrawn in an ice-cold tube containing 0.5 M Na_2EDTA final concentration and mixed well. Aprotinin was added at 500 U/ml when a sample was used for human ANP measurement. The plasma was immediately isolated by a centrifugation and stored at −20 C until further processing. Plasma concentrations of cGMP, VEGF, and human ANP were analyzed by SRL, Inc. (Tokyo, Japan).

Statistical analysis

Results are presented as mean ± SEM unless otherwise indicated. The statistical significance of differences in means was evaluated by ANOVA supplemented with Fisher's least-significant difference in comparisons among three or more groups in animal experiments and by paired *t* tests between before and after the carperitide administration in the human study. A P value < 0.05 was considered significant.

Results

Animal experiments

Angiogenesis was impaired in diabetic mice

Blood glucose levels in STZ-diabetic mice, on which the hind-limb ischemia was induced at 4, 16, and 26 wk after STZ injections, were 354 ± 151 mg/dl (n = 9), 354 ± 38 mg/dl (n = 9), and 308 ± 23 mg/dl (n = 9), respectively, on the day of surgery. In control nondiabetic mice, blood glucose levels at 4 wk after the injection of vehicle were 139 ± 4 mg/dl (n = 6), 132 ± 2 mg/dl (n = 9), and 131 ± 4 mg/dl (n = 9) for mice operated at 4, 16, and 26 wk, respectively, after the vehicle injection.

At 4 wk after the induction of diabetes, blood flow recovery of the STZ-diabetic group was similar to that of nondiabetic controls (Fig. 1A). But after a long-term hyperglycemic state of 16 or 26 wk, recovery was suppressed in the STZ-diabetic group by 26 or 32%, respectively, when compared with the control mice (Fig. 1, B and C).

ANP administration restored angiogenesis in diabetic mice

To investigate whether ANP can improve the impairment of blood flow recovery, carperitide was administered to C57BL/6 mice in which femoral artery ligation was made after a 16-wk exposure to hyperglycemia.

Blood glucose levels at femoral artery ligation were 116 ± 4 mg/dl in the vehicle-treated nondiabetic group, 122 ± 3 mg/dl in the carperitide-treated nondiabetic group, 343 ± 42 mg/dl in the vehicle-treated STZ-diabetic group, and 366 ± 42 mg/dl in the carperitide-treated STZ-diabetic group. In nondiabetic mice, the carperitide administration significantly accelerated blood flow recovery compared with the vehicle-treated group. The ischemic/normal limb blood flow ratio measured at 21 d after the surgery was 0.58 ± 0.03 in the vehicle-treated nondiabetic group (n = 13) and was significantly augmented in the carperitide-treated nondiabetic group (0.74 ± 0.06, n = 7; P < 0.05). The accelerating effect of carperitide on blood flow recovery was also seen in STZ-diabetic mice. The ischemic/normal limb blood flow ratio at

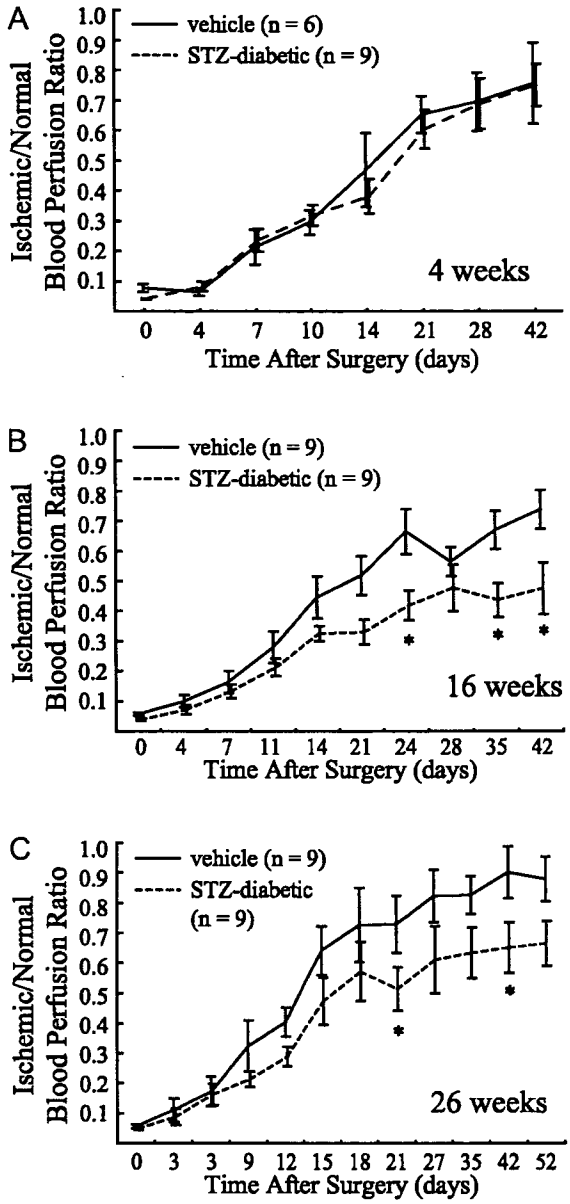

FIG. 1. Impairment of ischemia-induced blood flow recovery in mice with diabetes. Blood flow recovery after femoral artery ligation assessed by an ischemic/normal blood perfusion ratio was not altered 4 wk after STZ administration (A) but was significantly delayed 16 wk (B) and 26 wk (C) after induction of diabetes compared with vehicle-treated nondiabetic controls.*, P < 0.05 *vs.* vehicle-treated mice at each time point by ANOVA.

21 days after surgery was 0.52 ± 0.05 in the carperitide-treated STZ-diabetic group (n = 8) and significantly higher than that in the vehicle-treated STZ-diabetic group (0.37 ± 0.06, n = 7; $P < 0.05$) (Fig. 2B). The time course of blood flow recovery in each group was shown in Fig. 2A.

In the vehicle-treated STZ-diabetic group, the capillary density was 907 ± 69 counts/mm^2 (n = 6) and was more significantly reduced than in the vehicle-treated nondiabetic group (1406 ± 98 counts/mm^2, n = 6; $P < 0.05$) (Fig. 2, C and D). The capillary density tended to be higher in the carperitide-treated nondiabetic group (1604 ± 108 counts/mm^2, n = 6) than in the vehicle-treated nondiabetic group. Among STZ-diabetic mice, the carperitide administration significantly increased the capillary density to 1180 ± 95 counts/mm^2 (n = 6; $P < 0.05$).

In this study, 4-wk administration of carperitide to mice increased plasma human ANP levels from under the detection limit (10 pg/ml) to 156 ± 79 pg/ml (n = 5 each) and plasma cGMP levels from 8.9 ± 1.1 nM (n = 7) to 20.0 ± 2.9 nM (n = 6, $P < 0.05$). The carperitide administration altered blood pressure from 106 ± 3/73 ± 3 mm Hg to 94 ± 4/62 ± 4 mm Hg (n = 4 each; $P < 0.05$).

Human study

All patients had characteristic symptoms of PAD (Fontaine's class: I, one; II, five; III, two; and IV, five) (Table 2). A patient who was Fontaine's class I had a cold sensation in the lower extremities. The diagnosis was confirmed by ABI measurement, ultrasound velocity spectroscopy, or magnetic resonance angiography.

Hypertension and diabetes were the two most frequent underlying diseases among participants (Table 1). Among diabetic subjects, HbA1c levels were 7.7 ± 0.5%, and disease duration was 16.5 ± 2.1 yr. Seven patients suffered from end-stage renal diseases and were on hemodialysis. Eight patients had a past history of an ischemic heart disease, CHF, or both, and all of them were in stable condition with or without medication Plasma ANP levels were 315 ± 130 pg/ml, and ejection fractions measured by ultrasonic echocardiography were 49.9 ± 6.2%.

Plasma ANP levels were elevated from 224 ± 93 pg/ml at baseline to 400 ± 125 pg/ml during the administration (n = 12; $P < 0.05$; data were lacking in patient 5). Plasma cGMP levels were elevated from 14.4 ± 3.5 to 24.0 ± 4.5 nM (n =

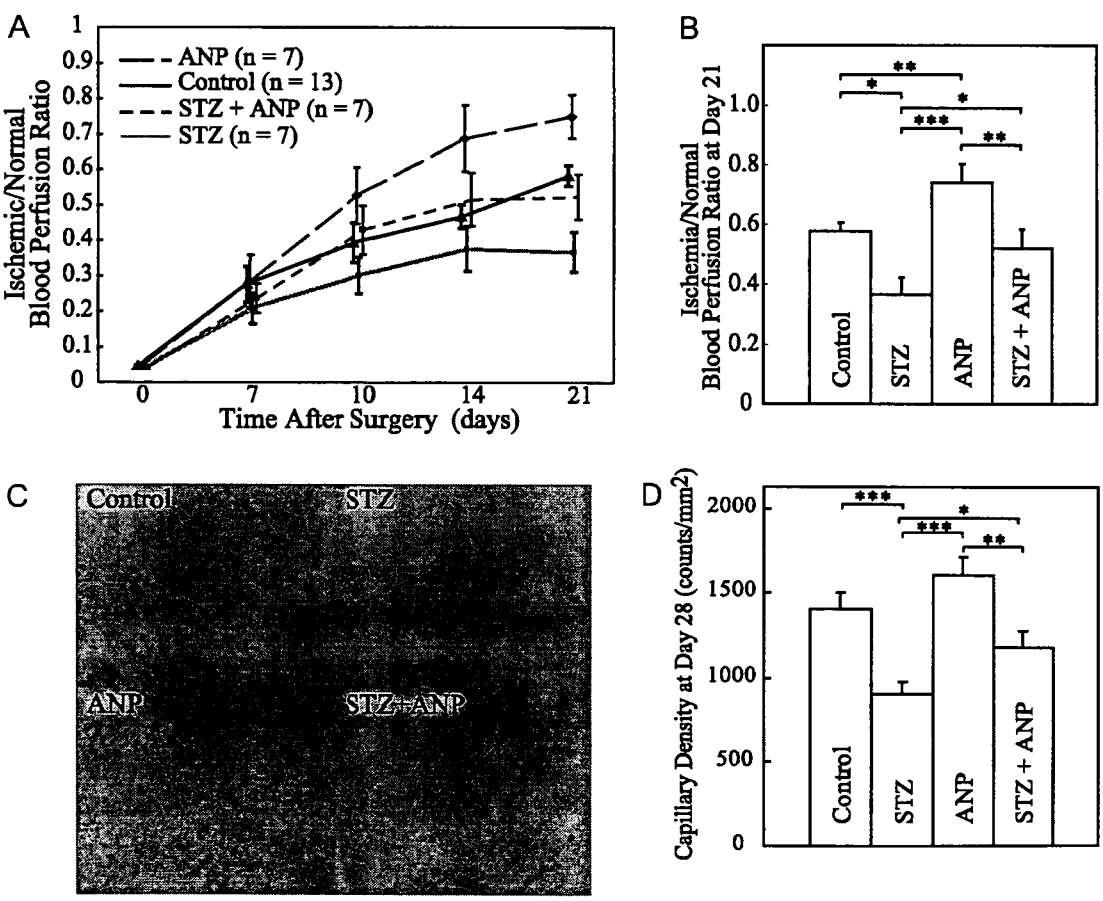

FIG. 2. Acceleration of ischemia-induced vascular regeneration by continuous ip administration of carperitide in nondiabetic and diabetic mice. A, Time course of ischemic/normal blood perfusion ratios measured by laser Doppler imaging; B, Calculated ischemic/normal blood perfusion ratios on d 21; C, immunostaining of the ischemic hind-limb tissue with anti-PECAM-1 antibody (*bright red*) at 28 d after the induction of ischemia; D. quantitative analysis of capillary density assessed by the immunostaining of PECAM-1. Control, Vehicle-treated nondiabetic; STZ, vehicle-treated STZ-diabetic; ANP, carperitide-treated nondiabetic; STZ + ANP, carperitide-treated STZ-diabetic. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2. Detailed patients' characteristics

Patient no.	Diagnosis	Age (yr)/sex	Fontaine's class	Accompanying disease	Symptoms	RP rating	Exercise tolerance	Plasma ANP levels (pg/ml)	Medication
1	ASO	69/M	III	ESRD, DM, HT, IHD	RP	3	NA	79	Ap, P, C
2	TAO	38/F	II	DM, Ob	IC	NA	290	<5	Ap, P, V
3	ASO	82/F	I	DM, HT, Ob, HL, CHF	CS	NA	NA	16	An, Ap, P, V
4	ASO	77/M	IV	ESRD, HT, CHF	UI/RP	5	NA	668	An, Ap, P
5	ASO	90/M	IV	DM, HT	UI/RP	4	NA	152	P, V
6	ASO	85/M	IV	ESRD, DM, HT	UI/RP	NA	NA	51	An, C, N, V
7	ASO	76/M	II	DM, HT, Ob, HL	IC	NA	240	22	An, Ap, V
8	ASO	75/M	II	DM, HT, HL, IHD	IC	4	200	36	An, Ap, P, V
9	ASO	63/M	III	ESRD, HT	RP	NA	NA	97	An, Ap, P, V
10	ASO	92/M	II	ESRD, DM, HT, CHF	IC	NA	100	922	Ap, N, P
11	ASO	71/M	IV	ESRD, DM, HT, CHF	UI	NA	NA	645	Ap
12	ASO	57/M	II	DM, HT, HL, IHD	IC	3	5F	14	Ap, C, V
13	ASO	55/M	IV	ESRD, DM, HT, CHF, IHD	UI/RP		NA	137	An, Ap, P, V

For patient 12, exercise tolerance was assessed by a stair-climb test, the floor number of stair-climbing without pain was 5. Medications were continued during carperitide injection without a change. An, Angiotensin-converting enzyme inhibitor or angiotensin receptor blocker; Ap, antiplatelet; ASO, arteriosclerosis obliterans; C, cilostazaol; CS, cold sensation of the peripheral; DM, type 2 diabetes mellitus; F, female; 5F, five floors; HL, hyperlipidemia; HT, hypertension; IC, intermittent claudication; IHD, ischemic heart disease; M, male; N, nitrate; NA, not applicable; Ob, obesity; P, prostanoid; RP, rest pain; TAO, thromboangitis obliterans; UI, gangrane or non-healing ulcer(s); V, vasodilator.

9; $P < 0.01$). No significant differences were seen in plasma VEGF levels: 92.2 ± 25.4 pg/ml at the baseline and 65.2 ± 11.1 pg/ml in the course of administration (n = 8). The blood pressure of patients (excepting those on hemodialysis) fell from 143 ± 8/74 ± 2 mm Hg to 123 ± 7/69 ± 3 mm Hg (n = 5; $P < 0.05$). An excessive decrease in systolic blood pressure to less than 90 mm Hg was observed in a few patients on hemodialysis and could be quickly reversed by reducing the carperitide infusion rate. Medications except for injections were continued during carperitide injection without any changes. Details of medications especially for PAD are shown in Table 2. Alprostadil (prostaglandin E) had been iv administrated daily for a week to patients 2 and 3, and for a month to patients 6 and 11, and was stopped at least 3 d before the beginning of carperitide administration. Phosphodiesterase inhibitors other than cilostazaol were not used in patients enrolled in this study. Smoking status was not changed in five never-smokers (patients 1, 3, 5, 12, and 13) and seven former smokers (patients 2, 4, 7, 8, 9, 10, and 11) during this study. One patient (no. 6) was a current smoker (20 cigarettes/d) at the enrollment and stopped smoking 7 d before the administration.

The ABI of the affected limb (or worse side when both limbs affected) was significantly elevated from 0.61 ± 0.08 at the baseline to 0.72 ± 0.09 on the 14th day of administration (n = 12; $P < 0.05$) except for patient 5, for whom the administration was stopped within a week (Table 3 and Fig. 3b). Brachial systolic blood pressure values for ABI calculations before and on the 14th day of administration were 140 ± 10 and 132 ± 8 mm Hg, respectively (n = 12; $P = 0.5$). Ankle systolic blood pressure values at affected limb were 84 ± 13 mm Hg before administration and were increased to 94 ± 11 mm Hg on the 14th day of administration (n = 12; $P = 0.4$).

Pain was assessed with a numerical rating scale in six patients who complained of rest pain (Table 2). Rest pain disappeared in three of the six patients (patient 6, 4/0; patient 9, 4/0; and patient 13, 3/0, as before/after the administration of carperitide) and was reduced in another patient (no. 1, 3/1). In patient 4, although the pain once worsened in the early phase of administration (from 4 to 6), the injections were continued, and the pain was reduced to level 1 within a week. In another patient (no. 5), the carperitide infusion was stopped at d 7 because rest pain had worsened (4 to 6) (Fig. 3A). All patients who felt the rating score of rest pain reduced could stop to use pain relievers or hypnotics.

Exercise performance was carried out on all patients with intermittent claudication except for those who could not walk as a result of rest pain or weakness (patients 2, 7, 8, 10, and 12) (Fig. 3C). The pain-free walking distance was assessed in four patients and prolonged in all of them after the carperitide administration (patient 2, 290 to 380 m; patient 7, 240 to 560 m; patient 8, 200 to 800 m; patient 10, 100 to 200 m). In another patient with a stair-climb test, the floor number of pain-free stair climbing was increased from five to seven.

Five patients had multiple foot ulcers, and dermatologists in our hospital had recommended foot amputation. Al-

TABLE 3. Changes in ABI by 14 d administration of carperitide

Patient no.	Systolic BP (mm Hg)				ABI	
	Brachial		Ankle			
	Before	2 wk	Before	2 wk	Before	2 wk
1	96	182	35	106	0.36	0.58
2	141	101	115	89	0.82	0.88
3	159	140	69	81	0.43	0.58
4	88	115	83	140	0.94	1.22
5	138	NA	86	NA	0.62	NA
6	176	151	188	154	1.07	1.02
7	150	123	112	108	0.75	0.88
8	113	117	97	78	0.86	0.67
9	162	100	0	0	0	0
10	101	99	60	90	0.59	0.91
11	143	161	66	111	0.46	0.69
12	153	132	80	83	0.52	0.63
13	201	164	107	91	0.53	0.55
Mean	140	132	84	94	0.61	0.72
(SEM)	10	8	13	11	0.08	0.09

Values of brachial and ankle brachial pressure and ABI in each patient before and on the d 14 of administration. The administration was interrupted on the d 7 in patient 5. Data of patient 5 are excluded for the calculation of mean and SEM. NA, Not assessed.

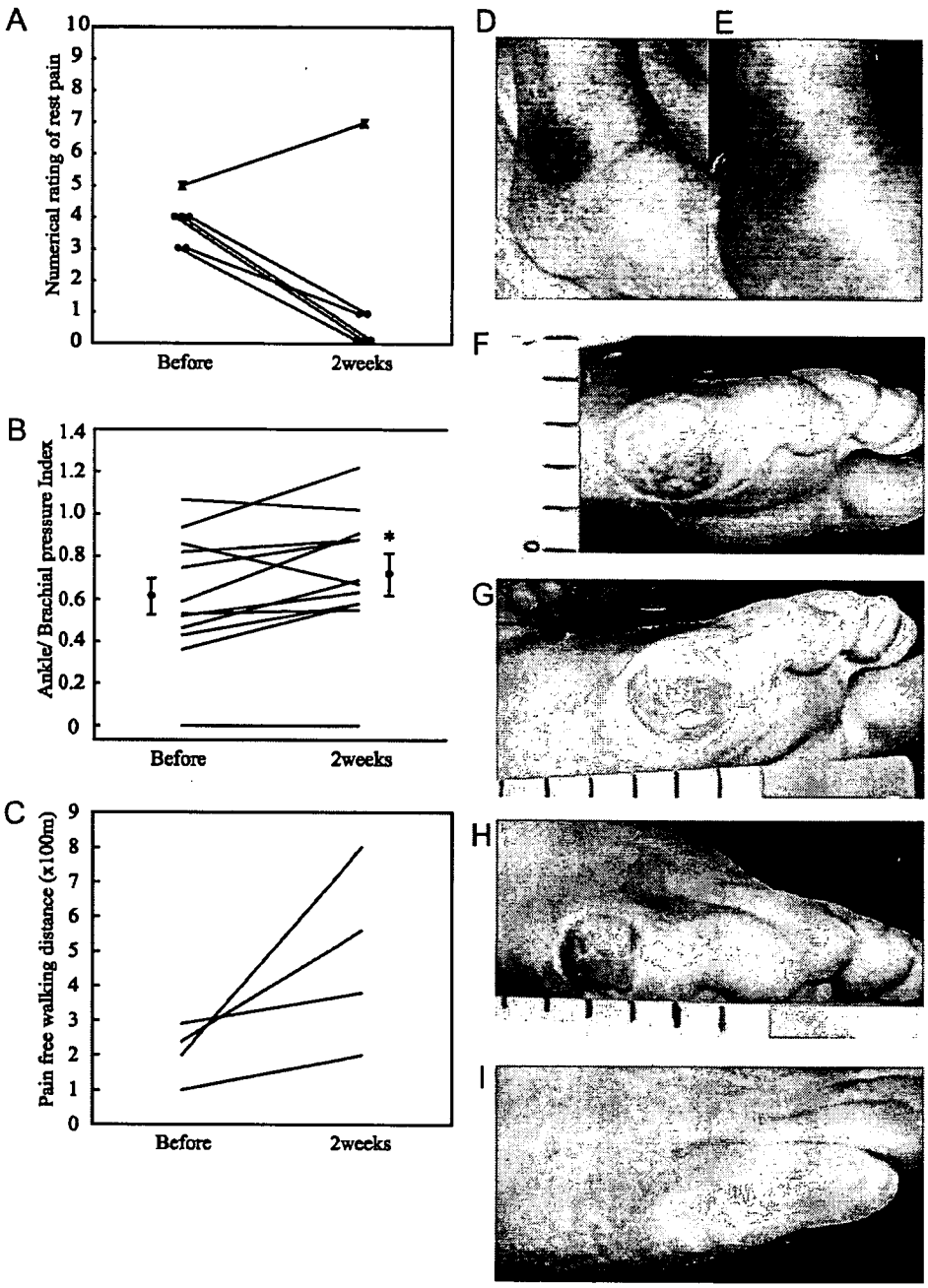

FIG. 3. Changes in symptoms resulting from carperitide infusion. A, Changes of 11-grade numerical rating of rest pain; B, changes in ABI of affected or worse side limb in each patient. Mean values are shown together with *error bars* (SEM) before and 2 wk after the carperitide administration. n = 12; *, $P < 0.05$. The administration was interrupted on the seventh day in patient 5, and ABI was undetectable in the affected limb of patient 9. C, Change in exercise tolerance assessed by pain-free walking distance; D–I, improvement of foot ulcer in patients 4 and 13. Pictures are before (D) and after 8-wk administration of carperitide (E) in patient 4 and before (F) and after 3 (G) and 6 (H) wk administration of carperitide and 4 months after leaving hospital (I) in patient 13. Pitting foot edema was observed in patient 4 (E).

though the ulcers did not change in severity in two cases (patients 5 and 6), they improved in another three cases (patients 4, 11, and 13) for whom foot amputations could be avoided. A representative case is shown in Fig. 3, D–I.

Other changes observed during administration were as follows: hot sensation in lower extremities in eight patients (nos. 1, 2, 4, 5, 6, 7, 8, and 13), transient flush and slight nausea in one patient (no. 2), pitting edema in both feet in five patients on hemodialysis (nos. 1, 4, 6, 11, and 13), and an increase in menstrual bleeding in a patient (no. 2).

Discussion

Diabetic foot is one of the most severe complications of diabetes mellitus and often results in leg amputation. Because it has been shown that an impairment of angiogenesis in patients with diabetes mellitus is a major cause of diabetic gangrene, we tried to generate a mouse model to investigate the mechanism of the impaired angiogenesis in diabetes. We induced diabetes in mice with STZ injections, and the mice were subjected to a femoral artery ligation after exposure to diabetic conditions (a blood glucose level higher than 220 mg/dl) for 4–26 wk. Although a 4-wk exposure to the diabetic condition did not affect blood flow recovery after the femoral artery ligation, exposure to high blood glucose for longer periods (16 or 26 wk) significantly impaired the blood flow recovery. This observation suggests that a quite long period of high blood glucose level is required to impair ischemia-induced collateral vessel formation. We therefore

selected 16 wk after the STZ induction of diabetes as the time point when the femoral artery ligation was performed on mice.

We showed here that carperitide, a recombinant human ANP, significantly accelerated blood flow recovery in a mouse model of ischemia-induced angiogenesis in both nondiabetic and diabetic conditions. The blood flow recovery in carperitide-treated diabetic mice was improved to a level similar to that in vehicle-treated nondiabetic mice. A histological analysis revealed that capillary density in the muscle of the ischemic limb was reduced in diabetic mice. The carperitide infusion significantly recovered capillary density in diabetic mice to the level in vehicle-treated nondiabetic mice. These observations indicate that carperitide can improve ischemia-induced angiogenesis, which accelerates blood flow recovery in diabetic conditions. We have shown that an increase of circulating BNP levels by targeted overexpression of the murine BNP gene in the liver or an overexpression of cGK throughout the body by the transgenic technology can accelerate the restoration of blood flow in limb ischemia experimentally generated by a femoral artery ligation, which results from the promotion of ischemia-induced angiogenesis through the activation of the ERK cascade (14). We have also shown that ANP at a physiological concentration induces proliferation and migration of ECs and enhances endothelial regeneration via activating ERK1/2 and phosphatidylinositol 3-kinase/Akt pathways in an *in vitro* wound healing assay using the cells from either coronary arteries or umbilical veins of humans (11). CNP, another member of the NP family, was shown to enhance migration of ECs and to accelerate reendothelialization in vein grafts after an arterial bypass surgery, although CNP inhibits proliferation and migration of vascular SMCs (16, 17). NPs use particulate GCs as their signaling receptors and share cGMP signaling pathways, especially signaling through cGKI, with NO, which activates soluble GC to generate cGMP (4). It is known that NO is a mediator of VEGF, which is a potent mitogen for vascular ECs and induces angiogenesis (19). A significant portion of VEGF-induced human EC proliferation is reportedly mediated by cGKI (20). In diabetes, hyperglycemia induces formation of reactive oxygen species, which decrease the bioavailability of NO (21). Taken together, deterioration of cGMP signaling appears to be a key process leading to the impaired angiogenesis and PAD in diabetes. In this study, the administration of carperitide could overcome the impairment of cGMP signaling in diabetic conditions, and it would be a new, therapeutic approach to PAD with diabetes. Because the urinary cGMP excretion rate is inversely correlated with the grade of Fontaine's classification in PAD patients (5), an impairment of cGMP signaling appears to be a common feature of PAD. We therefore investigated the therapeutic potential of carperitide administration in PAD patients.

We did not assign participants to a vehicle-treated group for an ethical reason; most cases of participants had been treated with conventional therapies, which had not accomplished appreciable effects. The carperitide administration significantly increased ABI, effectively relieved symptoms including intermittent claudication and rest pain, and promoted the healing of foot ulcers in PAD patients. The dosage of carperitide we used in the human study was optimized for each patient according to the maximum permissible dosage, which is the highest dose possible without causing an excessive fall in systolic blood pressure, because sensitivity to exogenously administered ANP differs among patients depending, presumably, upon basal plasma ANP levels. Although doses of carperitide administration were lower than those usually given in the treatment of CHF, plasma cGMP levels were increased twice as much as basal levels, and relief from the characteristic signs and symptoms of PAD became possible. This observation suggests that a blood pressure fall would not limit the therapeutic use of carperitide for PAD patients.

It is reported that asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial NO synthase, is accumulated in patients with end-stage renal disease (ESRD) and a high plasma ADMA level is a strong indicator of risks for all-cause mortality and cardiovascular events (22). It might be speculated that responses to the carperitide administration are better in ESRD patients than in non-ESRD patients, because carperitide is supposed to restore cGMP signaling, which is impaired by ADMA, via an activation of GC-A. Considering heterogeneity of patients' clinical characteristics, a larger number of participants will be needed to address this issue.

All patients, for whom exercise tolerance was evaluated, had been treated with conventional therapies using per os and per cutaneous medications under hospitalization and been encouraged to walk for at least 3 wk without any increases of pain-free walking distances. A 2-wk carperitide administration was then added to the conventional therapies and resulted in significantly improved exercise tolerance. The improvement, therefore, cannot be explained by a training effect only.

NPs have various biological effects on vascular functions other than the promotion of angiogenesis, and some of them appear favorable to treating PAD. NPs regulate vascular tone, and CNP, especially, is a candidate for endothelial-derived hyperpolarizing factor, which plays a fundamental role in the regulation of local blood flow and systemic blood pressure (23). In the clinical investigation of this paper, changes in symptoms and ABI appeared within a few days or a week of the administration. The effect of carperitide on symptoms in the early phase might be due to a vasodilatory action of ANP to some extent, because the changes appeared too early to be regarded as effects of vascular regeneration. On the other hand, the elongation of pain-free walking distance persisted after the cessation of the administration was ceased, and ABI remained elevated for several months after the end of administration. If the vasodilatory action of ANP is the only mechanism of the improvement, the effects of carperitide should disappear promptly at the cessation of the infusion, because the half life of ANP in circulation is a couple of minutes (24).

In patients with advanced arteriosclerosis, severe calcification of arterial walls in lower extremities can cause an overestimation of ankle blood pressure. Where vasodilators such as carperitide were used in such patients, ABI might be increased solely due to a decrease in brachial blood pressure. In this study, we observed slight decreases in brachial blood

pressure, but we could observe increases in ankle blood pressure although the changes were not statistically significant. Increases in ABI, therefore, should not be false and should be, at least in part, the result of blood flow recovery.

The improvement in exercise tolerance and ABI might, therefore, be achieved by modifying vascular endothelial structure or promoting vascular regeneration. Plasma VEGF levels were not significantly elevated by the carperitide infusion in this study, indicating that VEGF is not an essential mediator of carperitide's effects on PAD symptoms. It is reported that NPs elicit antiinflammatory and antithrombotic effects in animals (17, 25, 26), and further investigation will be needed to see whether such effects of NPs are clinically significant.

Carperitide is often used to treat CHF patients in Japan, and its safety is clinically proven. No critical side effects were observed in this study. An increase in menstrual bleeding observed in a participant could be accidental or a result of ANP's vasodilatory action, because the symptom faded soon after the cessation of the infusion. There are, however, several reports indicating the physiological significance of CNP/GC-B signaling in the control of ovarian cycling (27, 28). A close observation would be needed where carperitide infusion would be applied to women of reproductive age for a long duration (more than 2 wk). Leg edema appeared in three patients, who were in relatively serious states of the foot disease. Many PAD patients develop postoperative edema after surgeries of revascularization (29), which indicates that they have circulatory inadequacy for autoregulating blood hydrostatic pressure. Because ANP reportedly plays an essential role in maintaining vascular permeability via GC-A on vascular ECs (30), edema might result from this direct action on vascular endothelium.

Conclusion

This study revealed that a long-duration diabetic condition impaired ischemia-induced angiogenesis and blood flow recovery in a mouse model of hind-limb ischemia and that ANP as a therapeutic agent for CHF can restore the ischemia-induced angiogenesis in diabetic mice. Based on this observation, we applied carperitide administration to 13 PAD patients and found that carperitide infusion at doses lower than those for CHF could safely improve signs and symptoms. Carperitide administration, therefore, can be a new therapeutic strategy for PAD, and it appears effective in patients for whom conventional therapies do not work well.

Acknowledgments

We thank our colleagues in Kyoto University Hospital for referring patients and assistance; the staff of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University for animal care; Ms. Akane Nonoguchi for technical assistance; and Ms. Ayumi Ishida and Ms. Shiho Takada for secretarial assistance.

Received August 7, 2007. Accepted November 1, 2007.

Address all correspondence and requests for reprints to: Hiroshi Itoh, M.D., Ph.D., Department of Internal Medicine, Keio University School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: hiito@kuhp.kyoto-u.ac.jp.

This work was supported by the Kyoto University 21st Century Centers of Excellence Program "Integration of Transplantation Therapy and Regenerative Medicine"; Grant-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology and the Japan Smoking Foundation; Grant of Regenerative Medicine Realization Project of the Ministry of Education, Culture, Sports, Science, and Technology; Fifth Grant for Clinical Vascular Function from the Kimura Memorial Heart Foundation; Grant from Takeda Medical Foundation; Grant from Japan Foundation of Applied Enzymology; and the Research Grant for Cardiovascular Disease 19C-7 from the Ministry of Health, Labor, and Welfare.

Disclosure Statement: The authors of this manuscript have nothing to declare.

References

1. Rivard A, Silver M, Chen D, Kearney M, Magner M, Annex B, Peters K, Isner JM 1999 Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. Am J Pathol 154:355–363
2. Martin A, Komada MR, Sane DC 2003 Abnormal angiogenesis in diabetes mellitus. Med Res Rev 23:117–145
3. Tesfamariam B, Cohen RA 1992 Free radicals mediate endothelial cell dysfunction caused by elevated glucose. Am J Physiol 263:H321–H326
4. Tamura N, Chrisman TD, Garbers DL 2001 The regulation and physiological roles of the guanylyl cyclase receptors. Endocr J 48:611–634
5. Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC 1997 Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation 95:2068–2074
6. Sugawara A, Nakao K, Morii N, Yamada T, Itoh H, Shiono S, Saito Y, Mukoyama M, Arai H, Nishimura K, Obata K, Yasue H, Ban T, Imura H 1988 Synthesis of atrial natriuretic polypeptide in human failing hearts. Evidence for altered processing of atrial natriuretic polypeptide precursor and augmented synthesis of β-human ANP. J Clin Invest 81:1962–1970
7. Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, Shirakami G, Jougasaki M, Obata K, Yasue H, Kambayashi Y, Inoue K, Imura H 1991 Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. J Clin Invest 87:1402–1412
8. Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K 1992 Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130:229–239
9. Colucci WS, Elkayam U, Horton DP, Abraham WT, Bourge RC, Johnson AD, Wagoner LE, Givertz MM, Liang CS, Neibaur M, Haught WH, LeJemtel TH 2000 Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group. N Engl J Med 343:246–253
10. Suwa M, Seino Y, Nomachi Y, Matsuki S, Funahashi K 2005 Multicenter prospective investigation on efficacy and safety of carperitide for acute heart failure in the 'real world' of therapy. Circ J 69:283–290
11. Kook H, Itoh H, Choi BS, Sawada N, Doi K, Hwang TJ, Kim KK, Arai H, Baik YH, Nakao K 2003 Physiological concentration of atrial natriuretic peptide induces endothelial regeneration in vitro. Am J Physiol Heart Circ Physiol 284:H1388–H1397
12. Ogawa Y, Itoh H, Tamura N, Suga S, Yoshimasa T, Uehira M, Matsuda S, Shiono S, Nishimoto H, Nakao K 1994 Molecular cloning of the complementary DNA and gene that encode mouse brain natriuretic peptide and generation of transgenic mice that overexpress the brain natriuretic peptide gene. J Clin Invest 93:1911–1921
13. Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M, Hirneiss C, Wang GX, Korth M, Aszodi A, Andersson KE, Krombach F, Mayerhofer A, Ruth P, Fassler R, Hofmann F 1998 Defective smooth muscle regulation in cGMP kinase I-deficient mice. EMBO J 17:3045–3051
14. Yamahara K, Itoh H, Chun TH, Ogawa Y, Yamashita J, Sawada N, Fukunaga Y, Sone M, Yurugi-Kobayashi T, Miyashita K, Tsujimoto H, Kook H, Feil R, Garbers DL, Hofmann F, Nakao K 2003 Significance and therapeutic potential of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway in vascular regeneration. Proc Natl Acad Sci USA 100:3404–3409
15. Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H 1992 Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. Possible existence of "vascular natriuretic peptide system". J Clin Invest 90:1145–1149
16. Doi K, Ikeda T, Itoh H, Ueyama K, Hosoda K, Ogawa Y, Yamashita J, Chun TH, Inoue, M, Masatsugu K, Sawada N, Fukunaga Y, Saito T, Sone M, Yamahara K, Kook H, Komeda M, Ueda M, Nakao K 2001 C-type natriuretic peptide induces redifferentiation of vascular smooth muscle cells with accelerated reendothelialization. Arterioscler Thromb Vasc Biol 21:930–936
17. Ohno N, Itoh H, Ikeda T, Ueyama K, Yamahara K, Doi K, Yamashita J, Inoue M, Masatsugu K, Sawada N, Fukunaga Y, Sakaguchi S, Sone M, Yurugi T, Kook H, Komeda M, Nakao K 2002 Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. Circulation 105:1623–1626

18. Naruko T, Ueda M, van der Wal AC, van der Loos CM, Itoh H, Nakao K, Becker AE 1996 C-type natriuretic peptide in human coronary atherosclerotic lesions. Circulation 94:3103–3108
19. Morbidelli L, Chang CH, Douglas JG, Granger HJ, Ledda F, Ziche M 1996 Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. Am J Physiol 270:H411–H415
20. Hood J, Granger HJ 1998 Protein kinase G mediates vascular endothelial growth factor-induced Raf-1 activation and proliferation in human endothelial cells. J Biol Chem 273:23504–23508
21. Ceriello A, Motz E 2004 Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arterioscler Thromb Vasc Biol 24:816–823
22. Kielstein JT, Zoccali C 2005 Asymmetric dimethylarginine: a cardiovascular risk factor and a uremic toxin coming of age? Am J Kidney Dis 46:186–202
23. Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ 2003 Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. Proc Natl Acad Sci USA 100:1426–1431
24. Ingwersen SH, Jorgensen PN, Eiskjaer H, Johansen NL, Madsen K, Faarup P 1992 Superiority of sandwich ELISA over competitive RIA for the estimation of ANP-270, an analogue of human atrial natriuretic factor. J Immunol Methods 149:237–246
25. Kiemer AK, Vollmar AM 2001 The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. Ann Rheum Dis 60(Suppl 3):68–70
26. Scotland RS, Cohen M, Foster P, Lovell M, Mathur A, Ahluwalia A, Hobbs AJ 2005 C-type natriuretic peptide inhibits leukocyte recruitment and platelet-leukocyte interactions via suppression of P-selectin expression. Proc Natl Acad Sci USA 102:14452–14457
27. Tamura N, Doolittle LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL 2004 Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci USA 101:17300–17305
28. Acuff CG, Huang H, Steinhelper ME 1997 Estradiol induces C-type natriuretic peptide gene expression in mouse uterus. Am J Physiol 273:H2672–2677
29. Coats P, Wadsworth R 2005 Marriage of resistance and conduit arteries breeds critical limb ischemia. Am J Physiol Heart Circ Physiol 288:H1044–H1050
30. Sabrane K, Kruse MN, Fabritz L, Zetsche B, Mitko D, Skryabin BV, Zwiener M, Baba HA, Yanagisawa M, Kuhn M 2005 Vascular endothelium is critically involved in the hypotensive and hypovolemic actions of atrial natriuretic peptide. J Clin Invest 115:1666–1674

Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Effects of Candesartan Compared With Amlodipine in Hypertensive Patients With High Cardiovascular Risks

Candesartan Antihypertensive Survival Evaluation in Japan Trial

Toshio Ogihara, Kazuwa Nakao, Tsuguya Fukui, Kohshiro Fukiyama, Kenji Ueshima, Koji Oba, Tosiya Sato, Takao Saruta; for the Candesartan Antihypertensive Survival Evaluation in Japan Trial Group

Abstract—The Candesartan Antihypertensive Survival Evaluation in Japan Trial was designed to compare the long-term effects of the angiotensin II receptor blocker candesartan and the calcium channel blocker amlodipine on the incidence of cardiovascular events, represented as a composite of sudden death and cerebrovascular, cardiac, renal, and vascular events in high-risk Japanese hypertensive patients. We conducted a prospective, randomized, open-label study with blinded assessment of the end point in 4728 Japanese hypertensive patients (mean age: 63.8 years; mean body mass index: 24.6 kg/m^2). Patients were followed for an average of 3.2 years. Blood pressure was well controlled with both treatment-based regimens (systolic blood pressure/diastolic blood pressure: 136.1/77.3 mm Hg for candesartan-based regimens and 134.4/76.7 mm Hg for amlodipine-based regimens after 3 years). Primary cardiovascular events occurred in 134 patients with both the candesartan- and amlodipine-based regimens. The 2 treatment-based regimens produced no significant differences in cardiovascular morbidity or mortality in the high-risk Japanese hypertensive patients (hazard ratio: 1.01; 95% CI: 0.79 to 1.28; P=0.969). In each primary end point category, there was no significant difference between the 2 treatment-based regimens. New-onset diabetes occurred in fewer patients taking candesartan (8.7/1000 person-years) than in those taking amlodipine (13.6/1000 person-years), which resulted in a 36% relative risk reduction (hazard ratio: 0.64; 95% CI: 0.43 to 0.97; P=0.033). We disclosed that candesartan-based and amlodipine-based regimens produced no statistical differences in terms of the primary cardiovascular end point, whereas candesartan prevented new-onset diabetes more effectively than amlodipine. (***Hypertension.* 2008;51:393-398.**)

Key Words: antihypertensive therapy ■ hypertension ■ cardiovascular diseases ■ angiotensin II ■ calcium channel blockers ■ clinical trials

Angiotensin II receptor blockers (ARBs) and calcium channel blockers (CCBs) have proven to be important advances for the treatment of hypertension.[1,2] These agents have been shown to be as effective or sometimes better than other antihypertensive drugs in terms of cardiovascular morbidity and mortality and associated adverse events.[3–5] Clinical trials have shown significant effects from treatment with CCBs or angiotensin-converting enzyme inhibitors for preventing cardiovascular morbidity and mortality in high-risk populations.[2,6,7] In the Valsartan Antihypertensive Long-term Use Evaluation (VALUE) Trial, the ARB valsartan was compared with the CCB amlodipine in Europe and the United States.[8] The VALUE Trial concluded that the main outcome (cardiac disease) did not differ between the groups, whereas unequal reductions in blood pressure may have accounted for the observed differences between the groups in the cause-specific outcomes. Thus, it is still unclear whether there are differences in the efficacies of ARBs and CCBs.

The event rates of cardiovascular disease in Japan differ from those in Europe and the United States. Mortality from ischemic heart disease in Japan is one third of that in the United States, and mortality from cerebrovascular disease in Japan is ≈1.5 times higher than that in the United States.[9] These differences may be partly explained by differences in the lifestyles of Japanese and Western populations, which are reflected in body mass index (BMI) (mean BMI: 23 to 25 kg/m^2 and 28 to 30 kg/m^2, respectively).[10,11] In this context, the Candesartan Antihypertensive Survival Evaluation in Japan (CASE-J) Trial was designed to evaluate the efficacies of the ARB candesartan cilexetil and the CCB amlodipine besylate for reducing the incidences of cardiovascular morbidity and mortality (primary and secondary end points), as

Received July 23, 2007; first decision August 7, 2007; revision accepted November 29, 2007.

From the Osaka University Graduate School of Medicine (T.O.), Osaka, Japan; EBM Research Center (K.N., K.U., K.O.) and Department of Medicine and Clinical Science (K.N.), Kyoto University Graduate School of Medicine, Kyoto, Japan; St Luke's International Hospital (T.F.), Tokyo, Japan; Japan Seaman's Relief Association Moji Hospital (K.F.), Fukuoka, Japan; Department of Biostatistics (T.Sato), Kyoto University School of Public Health, Graduate School of Medicine, Kyoto, Japan; and Keio University Graduate School of Medicine (T.Saruta), Tokyo, Japan.

This trial has been registered at www.clinicaltrials.gov (identifier NCT00125463).

Correspondence to Kazuwa Nakao, Department of Medicine and Clinical Science and EBM Research Center, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawaracho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail nakao@kuhp.kyoto-u.ac.jp

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.107.098475

well as new-onset diabetes (prespecified end point) in high-risk Japanese hypertensive patients.

Methods

Study Design

The CASE-J Trial was a prospective, multicenter, randomized, open-label, active-controlled, 2-arm parallel-group comparison in Japan with a response-dependent dose titration and blinded assessment of the end points in high-risk hypertensive patients. The random assignment, data collection, and analyses were performed by the EBM Research Center of Kyoto University. The rationale and complete design of CASE-J Trial have been published elsewhere.[12] In addition, the end point of new-onset diabetes was prespecified at the 28th Annual Meeting of the Japanese Society of Hypertension on September 17, 2005.

The Ethics Committee at the Kyoto University Graduate School of Medicine approved the CASE-J Trial protocol according to the principles of the Helsinki Declaration. After obtaining informed consent, the patients were randomly assigned to the treatment groups. Enrolled patients were given 1 of 2 medications: candesartan cilexetil or amlodipine besylate. The former was administered orally at a dose of 4 to 8 mg/d. When the patient's blood pressure (BP) did not reach the targets for controlled BP, the dose was increased to 12 mg/d. The latter was administered orally at a dose of 2.5 to 5.0 mg/d and was increased to 10.0 mg/d when necessary. Once a patient was given the assigned medication, the use of other ARBs, CCBs, and all of the angiotensin-converting enzyme inhibitors was prohibited. Patients already being treated with diuretics, α-blockers, β-blockers, or α- and β-blockers before enrollment were allowed to continue taking these medications. According to the guideline proposed by Japanese Society of Hypertension, ≥2 consecutive BP measurements were taken from each patient in a sitting position at a clinic.[13] The targets for the control of BP were as follows: <60 years old, systolic BP (SBP)/diastolic BP (DBP) <130/85 mm Hg; 60 to 69 years old, SBP/DBP <140/90 mm Hg; 70 to 79 years old, SBP/DBP <150/90 mm Hg; and ≥80 years old, SBP/DBP <160/90 mm Hg.[13]

Population and Treatment

Patients with high-risk hypertension (SBP ≥140 mm Hg or DBP ≥90 mm Hg in patients <70 years old or SBP ≥160 mm Hg or DBP ≥90 mm Hg in patients ≥70 years old) were enrolled in the study. As reported previously,[12] high-risk patients were defined by the presence of any of the following factors: (1) severe hypertension (SBP ≥180 mm Hg or DBP ≥110 mm Hg); (2) type 2 diabetes mellitus; (3) a history of stroke or transient ischemic attack >6 months before the screening; (4) left ventricular hypertrophy, which was defined by the thickness of the left ventricular posterior wall or the interventricular septum wall ≥12 mm on echocardiography or Sv1+Rv5 ≥35 mm on electrocardiography, angina pectoris, or a history of myocardial infarction >6 months before the screening; (5) proteinuria or a serum creatinine concentration ≥1.3 mg/dL; or (6) arteriosclerotic peripheral artery obstruction. The exclusion criteria have also been reported elsewhere.[12] The event evaluation was performed independently by the event evaluation committee, which was blinded to the assigned treatment groups and adjudicated according to the protocol criteria. Adverse events and prespecified safety parameters were monitored by the data and safety monitoring board. The CASE-J Trial was closed on January 1, 2006.

Outcome Measures

The primary end point, which was the first fatal/nonfatal cardiovascular event, the secondary end points, and the prespecified end point are listed in Table 1. For the analysis of new-onset diabetes, we excluded all of the patients with type 2 diabetes mellitus at baseline from the analysis. Individual case report forms and adverse-event databases were monitored for any information reporting that the patients began to use antidiabetic drugs and/or for newly apparent cases of type 2 diabetes.

Table 1. Outcome Measures

Primary end points (composite of the following events)
Sudden death: unexpected death that happened within 24 hours without external causes
Cerebrovascular events: stroke or transient ischemic attack
Cardiac events: heart failure, angina pectoris, or acute myocardial infarction
Renal events: serum creatinine concentration ≥4.0 mg/dL, doubling of the serum creatinine concentration (however, creatinine ≤2.0 mg/dL is not regarded as an event), or end-stage renal disease
Vascular events: dissecting aortic aneurysm or arteriosclerotic occlusion of a peripheral artery
Secondary and prespecified end points
All-cause deaths
New-onset diabetes
Discontinuance of treatment because of adverse events

Statistical Methods

Based on previous results from studies of CCBs,[2,14,15] the CASE-J Trial was designed to detect a 40% relative risk reduction in the cardiovascular incidence rate in patients taking candesartan-based regimens with a 2-sided α level of 0.05 and 90% power.[16] Assuming a 20% loss to follow-up, we required a minimum of 3200 patients in total, and each patient was enrolled during a 1.5-year period and was followed for ≥3 consecutive years. An interim analysis was conducted 1 year after the completion of enrollment (December 2003). An O'Brien-Fleming spending function was used to adjust the α level.[17]

The incidence proportions were calculated using the Kaplan–Meier method and were compared with a log-rank test stratified by diabetic status at baseline. The hazard ratio (HR) and 95% CI were also estimated using Cox regression analysis. The P value and CI were adjusted for sequential testing of the results of the primary end point. These analyses were performed based on the intention-to-treat principle. If there were inequalities in BP levels during the follow-up, the imbalance in the BPs was adjusted using Cox regression analysis with SBP or DBP as the time-dependent covariate.

Exploratory subgroup analyses were prespecified to assess the primary, secondary, and prespecified results corrected for the baseline characteristics (diabetes; sex; age; SBP and DBP; systolic hypertension; BMI; CCB, angiotensin-converting enzyme inhibitor or ARB use before starting the CASE-J Trial; creatinine clearance; and history of cerebrovascular events, cardiac events, or renal events). Cox regression analysis was used to identify the treatment effect in these subgroups. Cox regression analysis was also used to identify the clinically relevant interactions between the treatment and these subgroups.

The safety population was grouped according to the treatment actually received. Differences in the frequency of adverse events were analyzed with the χ^2 test. All of the statistical tests were 2-sided with an α level of 0.05 and were performed using SAS version 9.1 (SAS Institute) and East 4.1 (Cytel).

Results

Study Profile and Baseline Characteristics

Between September 2001 and December 2002, 4728 patients with a mean age of 63.8 years and a mean BMI of 24.6 kg/m^2 were assigned to the 2 treatment-based regimens. As shown in Figure 1, 4703 randomly assigned patients were included in the analysis, and 136 patients (2.9%) were lost to follow-up. Table 2 summarizes the characteristics of the patients at baseline. There was a statistical difference between the sex ratios for the 2 treatment-based regimens (46.4% and 43.2% of the subjects were female for the candesartan-based regimens and

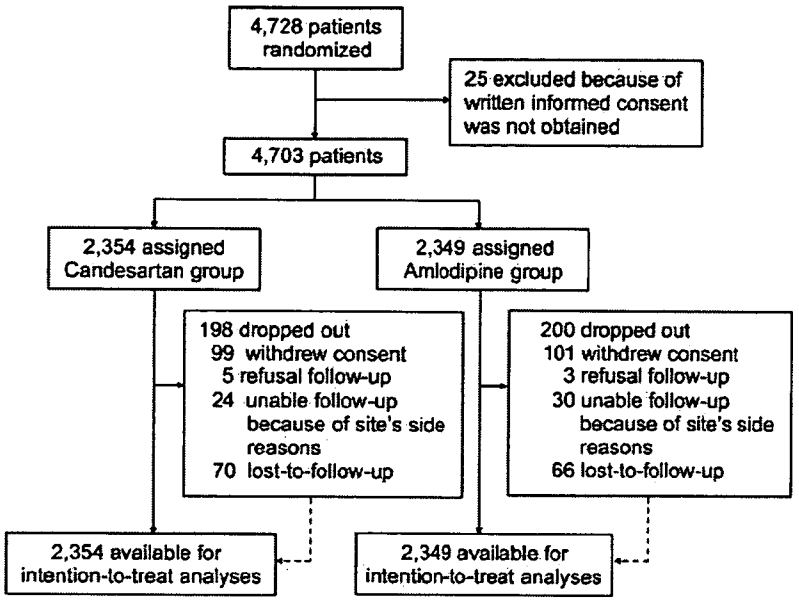

Figure 1. Trial profile of the CASE-J Trial.

amlodipine-based regimens, respectively), whereas there were no differences in terms of the other clinical parameters.

Duration of Follow-Up and Adherence to the Treatment

For both treatment-based regimens, the mean follow-up periods were 3.2 years, and the median values were 3.4 years.

Table 2. Baseline Characteristics of Trial Participants

Baseline Characteristics	Candesartan (n=2354)	Amlodipine (n=2349)
Women	1092 (46.4)	1014 (43.2)
Age	63.8±10.5	63.9±10.6
BMI, kg/m^2	24.6±3.7	24.5±3.6
SBP, mm Hg	162.5±14.2	163.2±14.2
DBP, mm Hg	91.6±11.0	91.8±11.4
Current smokers	489 (20.8)	536 (22.8)
Severe hypertension: SBP ≥180 mm Hg or DBP ≥110 mm Hg	454 (19.3)	493 (21.0)
Type 2 diabetes mellitus*	1011 (42.9)	1007 (42.9)
History of cerebrovascular events†	248 (10.5)	225 (9.6)
History of cardiac events‡	1007 (42.8)	1023 (43.6)
History of renal events§	572 (24.3)	543 (23.1)
Arteriosclerotic peripheral arterial obstruction	29 (1.2)	24 (1.0)

Data are shown as the No. of patients (%) or the mean±SD.

*Type 2 diabetes mellitus was defined by fasting blood glucose levels ≥126 mg/dL, casual blood glucose levels ≥200 mg/dL, HbA1c ≥6.5%, 2-hour blood glucose levels in the 75-g oral glucose tolerance test ≥200 mg/dL, or current treatment with a hypoglycemic agent at baseline.

†History of cerebrovascular events includes cerebral hemorrhage, cerebral infarction, and transient ischemic attack.

‡History of cardiac events includes left ventricular hypertrophy, angina pectoris, and myocardial infarction.

§History of renal events includes proteinuria and serum creatinine levels ≥1.3 mg/dL.

The fifth to 95th percentile interval of the follow-up periods was 0.9 to 4.1 years for the candesartan-based regimens and 1.0 to 4.2 years for the amlodipine-based regimens. The study accumulated 15 175 person-years of follow-up (7563 person-years and 7612 person-years for the candesartan- and amlodipine-based regimens, respectively). The percentages of patients who received >80% of the allocated drugs during the follow-up were 96.5% and 96.0% in the candesartan- and amlodipine-based regimens, respectively. The percentage of the candesartan-treated patients who received other antihypertensive drugs was larger than that of the amlodipine-treated patients (54.5% and 42.7%, respectively; $P<0.001$; Table 3). After 3 years, the mean number of antihypertensive drugs used, including the allocated drugs, was 1.54 for patients treated with candesartan-based regimens and 1.37 for those treated with amlodipine-based regimens.

Table 3. No. of Patients Using Additional Drugs Throughout the Follow-Up Period

Additional Drugs	Candesartan (n=2354), n (%)	Amlodipine (n=2349), n (%)	P*
Antihypertensive drugs	1282 (54.5)	1003 (42.7)	<0.001
Diuretics	580 (24.6)	323 (13.8)	<0.001
α-Blockers	610 (25.9)	391 (16.6)	<0.001
β-Blockers	524 (22.3)	397 (16.9)	<0.001
α- and β-Blockers	193 (8.2)	146 (6.2)	0.009
Others	100 (4.2)	47 (2.0)	<0.001
Antihyperlipidemics	1050 (44.6)	1032 (43.9)	0.644
Antidiabetics (including insulin)	874 (37.1)	900 (38.3)	0.402
Antithrombotics	652 (27.7)	620 (26.4)	0.314
Antianginal	264 (11.2)	280 (11.9)	0.450
Antiarrhythmic	113 (4.8)	122 (5.2)	0.536

*P values were obtained using χ^2 tests.

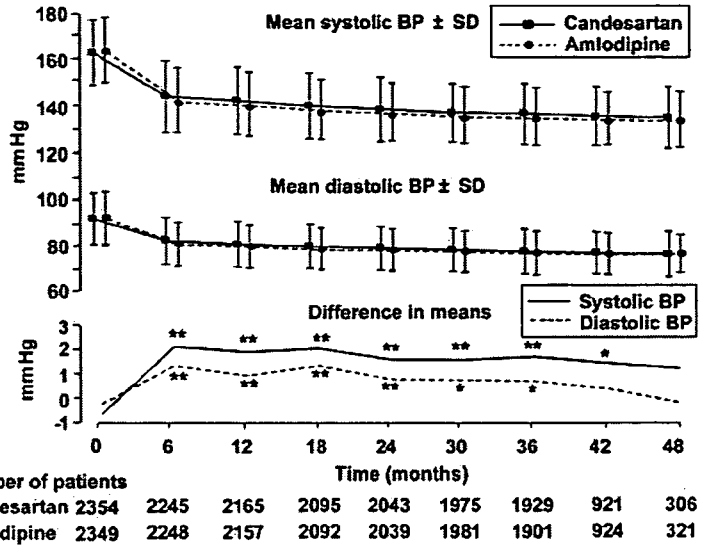

Figure 2. Changes in the SBP and DBP, as well as differences (candesartan−amlodipine) during the follow-up period. Mean SBP and mean DBP measured in the treatment groups and differences between the means. $^{**}P<0.01$; $^{*}P<0.05$.

Effects on BP

The SBP and DBP were well controlled in the CASE-J Trial. SBP/DBP was 162.5/91.6 mm Hg (SD: 14.2/11.0) at baseline and 136.1/77.3 mm Hg (SD: 12.9/9.6) after 3 years for candesartan-based regimens. SBP/DBP was 163.2/91.8 mm Hg (SD: 14.2/11.4) at baseline and 134.4/76.7 mm Hg (SD: 12.1/9.3) after 3 years for amlodipine-based regimens (Figure 2). Both the SBP and DBP were significantly lower in amlodipine-treated patients compared with candesartan-treated patients; after 3 years, the SBP and DBP were 1.7 mm Hg ($P<0.001$) and 0.6 mm Hg ($P=0.028$) lower in the amlodipine-treated patients, respectively.

Primary Outcome

Primary cardiovascular events occurred in 134 patients with both the candesartan- and amlodipine-based regimens. The 2 treatment-based regimens produced no significant difference in cardiovascular morbidity or mortality in the high-risk hypertensive patients (HR: 1.01; 95% CI: 0.79 to 1.28; $P=0.969$; Figure 3). In each primary end point category, there was no significant difference between the 2 treatment-based regimens (Figure 4). The HR for primary composite end point after an adjustment for the baseline characteristics (sex, age, CCB use, angiotensin-converting enzyme inhibitor or ARB use, creatinine clearance rate, and history of cerebrovascular, cardiac, and renal events) was 1.00 (95% CI: 0.78 to 1.27), and HRs after an adjustment using Cox regression analysis with SBP and DBP as the time-dependent covariates were 0.98 and 1.02 (95% CI: 0.77 to 1.25 and 0.80 to 1.30), respectively. The primary result did not change after these adjustments. In addition, we also evaluated the time-specific interval risk ratios of cardiovascular events every 6 months. There were no statistically significant time-specific interval risk ratios between the 2 treatment-based regimens.

Secondary and Prespecified Outcomes

For the secondary end points, 73 candesartan-treated patients (9.4/1000 person-years) and 86 amlodipine-treated patients (11.1/1000 person-years) died during the follow-up period. Neither the all-cause death rates nor the death rates because of cardiovascular events differed significantly between the 2 regimens. At baseline, 1343 candesartan-treated patients (mean age:

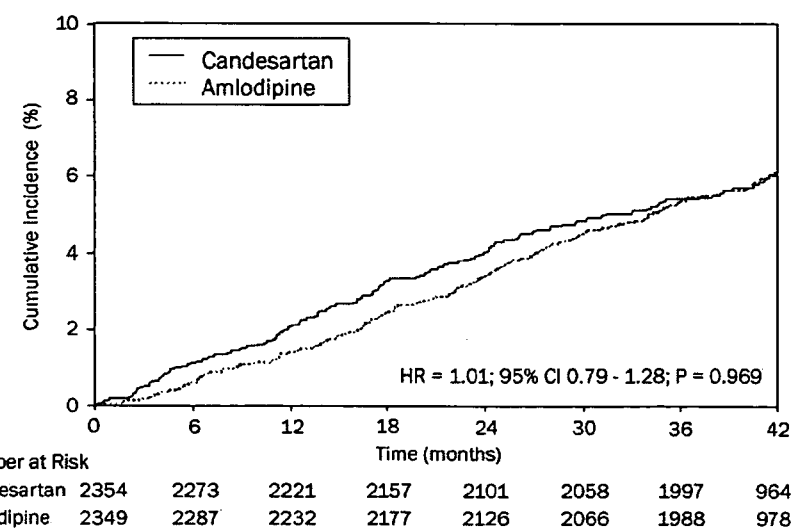

Figure 3. Kaplan–Meier curves for the primary composite end point. The primary end point was the time to the first cardiovascular event.

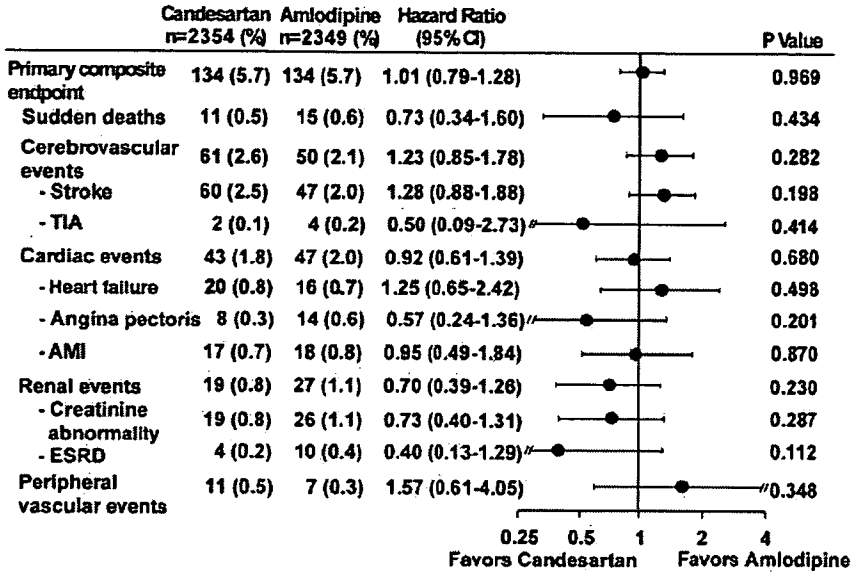

Figure 4. Comparisons of the primary composite end point and each cardiovascular event. The first event for each category was counted, including the number of each event, HRs and the corresponding 95% CIs, and *P* values. TIA indicates transient ischemic attack; AMI, acute myocardial infarction; ESRD, end-stage renal disease. Creatinine abnormality was defined as a serum creatinine concentration ≥4.0 mg/dL or doubling of the serum creatinine concentration. Any creatinine concentration ≤2.0 mg/dL, however, was not regarded as an event.

63.6; mean BMI: 24.2 kg/m^2) and 1342 amlodipine-treated patients (mean age: 63.8; mean BMI: 24.0 kg/m^2) did not have diabetes. With the exception of a small imbalance in the sex ratios, there were no significant differences between the 2 treatment groups for the other clinical parameters. New-onset diabetes occurred in significantly fewer patients treated with candesartan-based regimens (8.7/1000 person-years) than in those treated with amlodipine-based regimens (13.6/1000 person-years). A 36% relative risk reduction was observed for the incidence of new-onset diabetes with the candesartan-based regimens (HR: 0.64; 95% CI: 0.43 to 0.97; P=0.033). The number needed to treat to prevent an additional new-onset diabetic patient with the candesartan-based regimens compared with the amlodipine-based regimens was 63 patients during the average 3.2-year follow-up.

Exploratory Subgroup Analyses

We assessed the prespecified subgroup analysis of the primary end point. There were no significant interactions between the 2 treatment-based regimens for each of the baseline characteristics. In the prespecified subgroup analyses of all-cause death, candesartan-based regimens were favorable for the subgroup with BMI ≥27.5 kg/m^2 (HR: 0.33; 95% CI: 0.14 to 0.79). In addition, a 48% (HR: 0.52; 95% CI: 0.29 to 0.95) relative risk reduction of new-onset diabetes was observed in candesartan-based regimens compared with amlodipine-based regimens in the subgroup with BMI ≥25.0 kg/m^2 (mean BMI: 27.7 kg/m^2).

Safety Parameters

A total of 125 patients treated with candesartan-based regimens (5.4%) and 134 patients treated with amlodipine-based regimens (5.8%) discontinued treatment because of adverse events. Hyperkalemia was noted more often in candesartan-treated patients (1.0% for candesartan-based regimens versus 0.3% for amlodipine-based regimens), and flushing was observed more frequently in amlodipine-treated patients (0.0% versus 0.2%). In addition, pneumonia was more frequently reported with amlodipine-based regimens (0.1% versus 0.5%) (Table S1, available at http://hyper.ahajournals.org).

Discussion

The CASE-J Trial demonstrates no statistically significant difference between candesartan-based and amlodipine-based regimens in terms of the primary composite end point in high-risk Japanese hypertensive patients, although the BP level achieved with candesartan treatment was not as low as that achieved with amlodipine; the differences in SBP and DBP were 1.7 and 0.6 mm Hg after 3 years, respectively. Because BP is a crucial prognostic factor for cardiovascular events, the influence of BP differences on the primary composite end point is not negligible. When we adjusted for the imbalance in SBP or DBP levels using Cox regression analysis, we obtained similar results. Accordingly, it is likely that the failure to achieve similar levels of BP control did not influence the outcomes in the CASE-J Trial. Furthermore, it is notable that the BP levels achieved in the CASE-J Trial (<140/80 mm Hg) were lower than those reported in previous antihypertensive trials.[3,8,18] These findings suggest that strict BP control is important for the treatment of high-risk hypertensive patients.

The CASE-J Trial also shows that the incidence of new-onset diabetes was significantly lower in patients treated with candesartan-based regimens compared with patients treated with amlodipine-based regimens. The relative risk reduction for new-onset diabetes was 36% in the CASE-J Trial, although the incidence of new-onset diabetes in the amlodipine-treated patients in the CASE-J Trial (13.6/1000 person-years) was approximately one third of that in VALUE Trial (41.1/1000 person-years).[8] The mean BMI for patients without diabetes in the CASE-J Trial was 24.1 kg/m^2, whereas that in VALUE Trial was 28.0 kg/m^2.[19] In addition, the relative risk reduction of new-onset diabetes in the CASE-J Trial was 48% in the subgroup with BMI ≥25 kg/m^2, in which the mean BMI (27.7 kg/m^2) was similar to that in the VALUE Trial. The more favorable effect profile of candesartan in the CASE-J Trial compared with that of valsartan in the VALUE Trial may be explained by the smaller patient population taking additional diuretics in the CASE-J Trial, as well as the potentially beneficial effects of candesartan. Because the number of patients with diabetes and metabolic syndrome is increasing in Eastern coun-