may influence glycemic control (18). Furthermore, AngII is known to affect other hormones that are related to the regulation of blood pressure, for example, by promoting the release of aldosterone and vasopressin. It has been suggested that changes in catecholamine production by genetic disruption of AT1R in mice may affect glycemic control (19). Diminished tissue perfusion and lowered blood potassium concentration caused by AngII may also be involved in the development of insulin resistance. These findings indicate that various mechanisms are likely to be involved in AngII-induced insulin resistance. However, the effect of AngII on muscle mitochondrial content and subsequent influence on glycemic control has not yet been elucidated.

In a previous study, we showed that cGMP was involved in the regulation of mitochondrial content and function of cultured C2C12 myotubular cells by altering the expressions of the genes related to mitochondrial biogenesis and the antioxidant system (20). Interestingly, cGMP is an intracellular second messenger of the vasodilating substances, natriuretic peptides and nitric oxide, both of which exert antagonistic effects to AngII actions. In the current study, we therefore focused on the effects of AngII on muscle mitochondria, especially on the regulation of their biogenesis, and their relationship with the pathogenesis of glucose intolerance.

RESEARCH DESIGN AND METHODS

Cell culture. C2C12 cells (RIKEN BioResource Center, Tsukuba, Japan) were grown in a low-glucose (100 mg/dl) medium, as described previously (20). Cells were fully differentiated, grown to overconfluence, and treated in a 24-well dish with or without 10^{-6}–10^{-8} mol/l AngII (Sigma, St. Louis, MO); an AT1R blocker, RNH6270 (Olmesartan, 10^{-5} mol/l, a gift from Daiichi-Sankyo, Tokyo, Japan); or an AT2R blocker, PD123319 (10^{-5} mol/l; Sigma). Unless indicated otherwise, total DNA, RNA, and proteins were extracted from the cells after 48 h of treatment.

Transient overexpression of angiotensin receptors in vitro and RNA interference. We constructed angiotensin receptor overexpressing vectors by fusing the chicken β-actin promoter-driven vector, pCAGGS (21) (a gift from Dr. J. Miyazaki, Osaka University, Japan) with cDNA of mouse AT1R (GenBank accession no. NM_177322) or AT2R (NM_007429). C2C12 cells were transiently transfected with these vectors (0.1 μg/well) using the Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We then generated small interfering RNAs (siRNAs) for genetic blockade of AT1R and AT2R. C2C12 cells were transfected with these siRNAs, or scrambled RNA as negative control (Stealth RNAi Negative Control, Invitrogen), by means of a Lipofectamine RNAi Max Reagent (Invitrogen) according to the manufacturer's instructions.

Quantification of mitochondrial DNA copy number and gene expressions by real-time PCR. Quantitative PCR analysis was performed by standard methods. Details of the methods used are provided in the supplemental materials (available in an online-only appendix at (http://diabetes.diabetesjournals.org/cgi/content/full/db08-0949.

Quantification of mitochondrial mass, ROS production, and membrane potential and ATP content. Mitochondrial mass, mitochondrial ROS production, and membrane potential of the C2C12 cells were determined, respectively, with the aid of the fluorescent dyes: MitoTracker Green FM, MitoSOX Red, and JC-1 (Molecular Probes, Eugene, OR), following the same procedures as described previously (20). The fluorescent intensity was estimated by a multi-plate reader (Wallac ARVO SX; Perkin Elmer, Wellesley, MA). ATP content of the cells was determined with the chemiluminescence method (ATP bioluminescence Assay Kit HS II; Roche Diagnostics, Basel, Switzerland). For microscopic analysis, C2C12 cells were stained with the fluorescent probes and observed with a confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

Quantification of protein levels by Western blotting. Western blotting was performed by standard methods. Details of the methods used are provided in the supplemental materials.

Animal experiments. All animal experiments were performed in accordance with the animal care and use guidelines of Keio University. Male C57bl/6 mice (Nihon Clea, Tokyo, Japan) were maintained under specific pathogen-free conditions, and exogenous AngII was infused at a dose of 0.1 μg/g body wt/day, which is recognized as a subpressor dose that does not influence blood pressure (22). The infusion was carried out via an osmotic mini-pump (Alzet model 1002; Durect, Cupertino, CA) subcutaneously implanted when the mice were 8 weeks old. The AngII-infused mice were treated with or without the AT1R blocker CS-866 (Olmesartan medoxomil, a precursor of olmesartan with a longer effect in vivo than olmesartan, and a gift from Daiichi-Sanko) or the AT2R blocker PD-123319, four times per week by oral administration (10 mg/kg body wt) with a stomach sonde. On day 10, the mice were killed for tissue harvest.

Glucose tolerance test, indirect calorimetry, and quantification of serum insulin concentration, muscle triglyceride, and enzyme activity of mitochondria. For the glucose tolerance test, the mice were fasted for 8 h and intraperitoneally injected with glucose at 2.0 g/kg body weight. Blood samples were collected from the tail vein, and the blood glucose level was promptly determined with the glucose dehydrogenase method (ACCU-CHEK Aviva, Roche Diagnostics). Oxygen consumption and fat oxidation in mice were determined by means of indirect calorimetry, which was performed for 24 h from 2000 until the next day (ARCO-2000; Arco Systems, Kashiwa, Japan). Serum insulin concentration was determined with an enzyme-linked immunosorbent assay kit (Ultra-Sensitive Mouse Insulin ELISA kit, 200716; Morinaga, Yokohama, Japan). Lipids in quadriceps were extracted with the Folch method (23), and the triglyceride level was determined with a commercially available kit (Triglyceride E-test; Wako, Osaka, Japan). We performed Oil-red-O staining of the muscle, and the method is provided in the supplemental materials. Enzyme activity of the mitochondrial proteins, cytochrome C oxidase (COX) and β-hydroxyacyl-CoA dehydrogenase (β-HAD), was determined in the skeletal muscle by a commercially available assay kit (Mitochondria Activity Assay Kit, Bio Chain, Hayward, CA) and a standard method (24), respectively.

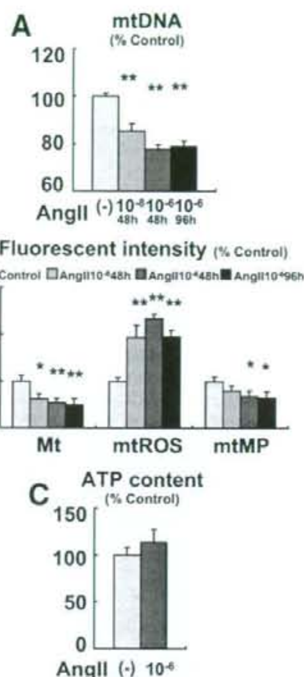

FIG. 1. AngII reduces mitochondrial content in association with an increase in mitochondrial ROS production in C2C12 cells. C2C12 cells were incubated with or without AngII (at the concentrations indicated: 10^{-8} or 10^{-6} mol/l) for 48 or 96 h. *A*: Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis (n = 12 per group). *B*: Mitochondrial density (Mt), mitochondrial ROS production (mtROS), and mitochondrial membrane potential (mtMP) estimated with the aid of fluorescent probes (n = 12). *C*: ATP content determined with the chemiluminescence method (n = 12). The values were standardized to those for the control (no AngII treatment). *$P < 0.05$, **$P < 0.01$ vs. control.

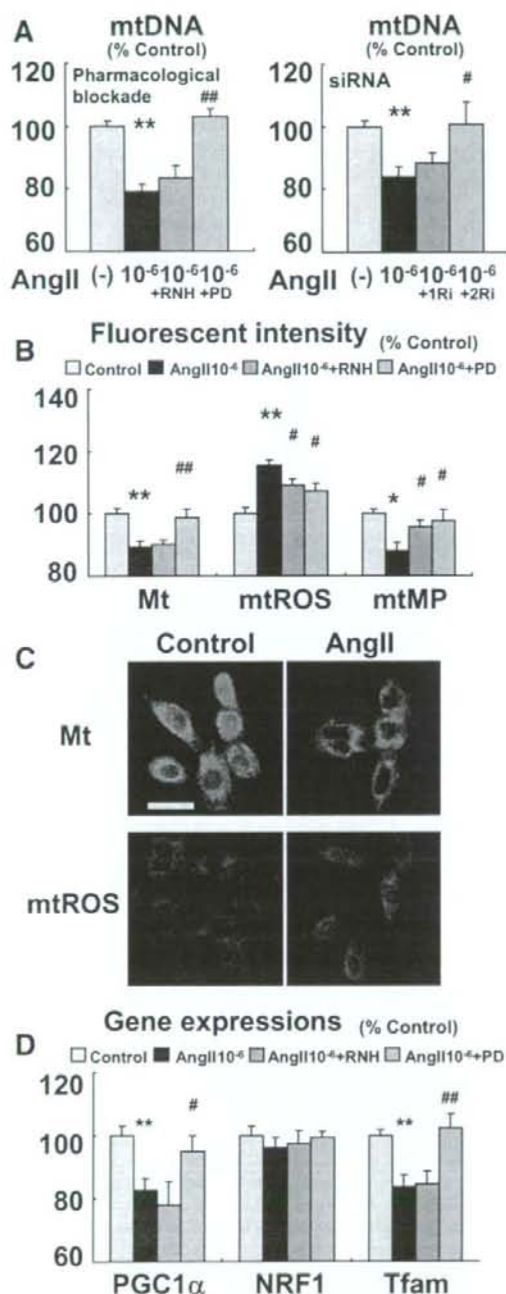

FIG. 2. Pharmacological blockade or targeted silencing of AT2R but not AT1R in C2C12 cells recovers mitochondrial biogenesis reduced by AngII. The AT1R blocker, RNH-6270 (RNH, 10^{-5} mol/l), or the AT2R blocker, PD-123319 (PD, 10^{-5} mol/l), was added to C2C12 cells previous to the treatment with AngII (10^{-6} mol/l) for 48 h (*A–D*). Targeted disruption of AT1R (1Ri) or AT2R (2Ri) by small interfering RNA (siRNA) was also performed (*A*). *A*: Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis (n = 12 per group). *B*: Mitochondrial density (Mt), mitochondrial ROS production (mtROS), and mitochondrial membrane potential (mtMP) estimated with the aid of fluorescent probes (n = 12). *C*: Microscopic analysis of the cells. C2C12 cells were stained with MitoTracker Green (green, a probe for mitochondria) or MitoSOX Red (red, a probe for mitochondria-derived ROS) and observed with a confocal microscope. Scale bar: 100 μm. *D*: Expression of genes involved in mitochondrial biogenesis (n = 12). The values were standardized to those for the control. *P < 0.05, **P < 0.01 vs. control. #P < 0.05, ##P < 0.01 vs. the AngII-treated group. (Please see http://diabetes.diabetesjournals.org/cgi/content/full/db08-0949 for a high-quality digital representation of this figure.)

Microarray analysis. Microarray analysis of the skeletal muscle of AngII-infused mice was performed. Details of the methods used are provided in the supplemental materials.

Statistical analysis. Statistical analysis was performed by standard methods. Details of the methods used are provided in the supplemental materials.

RESULTS

AngII reduces mitochondrial content in association with increased mitochondrial ROS production and lowers mitochondrial membrane potential in C2C12 cells. To investigate the effect of AngII on mitochondrial content, ROS production, membrane potential, and ATP production, we treated C2C12 myotubular cells with or without 10^{-8}–10^{-6} mol/l AngII for 48–96 h. Mitochondrial DNA copy number, which is considered to be a surrogate marker of mitochondrial content, showed a dose-dependent reduction (22% reduction at 10^{-6} mol/l, n = 12, P < 0.01, Fig. 1*A*) in the AngII-treated groups. The magnitude of reduction was similar for 48 and 96 h of incubation with AngII. Mitochondrial mass, estimated by means of fluorescent staining, also decreased as a result of AngII treatment (9% reduction at 10^{-6} mol/l, Fig. 1*B*), in association with a significant increase in mitochondrial ROS (mtROS) (27% increase, Fig. 1*B*). Mitochondrial membrane potential (mtMP) was lowered (6% decrease, Fig. 1*B*) by AngII, whereas cellular ATP content was not significantly changed (Fig. 1*C*).

Blockade of AT2R but not AT1R reverses mitochondrial reduction in C2C12 cells caused by AngII, whereas blockade of either AT1R or AT2R suppresses AngII-induced changes in mtROS and mtMP. To determine the receptor responsible for the effects of AngII on mitochondria, C2C12 cells were subjected to pharmacological and genetic blockade of their receptors. Pharmacological blockade of AngII by the AT1R blocker RNH-6270 (10^{-5} mol/l) or AT2R blocker PD-123319 (10^{-5} mol/l) revealed that the decline in mitochondrial content was completely reversed by blockade of AT2R (Fig. 2*A* and *B*). Consistent results were obtained by genetic blockade of these receptors by siRNA in that the silencing of AT2R completely reversed the decrease in mitochondrial content, whereas that of AT1R did not affect it (Fig. 2*A*). We confirmed that the siRNAs achieved >80% reduction in the expression and protein levels of the receptors by using quantitative PCR and Western blotting (data not shown). On the other hand, the increase in mtROS and the decrease in mtMP induced by AngII were partially, but significantly, suppressed by blockade of either AT1R or AT2R (Fig. 2*B*). Using a confocal microscope, we were able to confirm that the fluorescent probes were distributed specifically in mitochondria (Fig. 2*C*).

We also examined the expressions of genes involved in mitochondrial biogenesis (PGC1α, NRF1, and mitochondrial transcription factor A [Tfam]) and found that both PGC1α and Tfam were decreased in the AngII-treated group (18% and 16% decrease, respectively, n = 12, P < 0.01, Fig. 2*D*); however, expression of NRF1 was not affected by AngII. Consistent with its effects on mitochon-

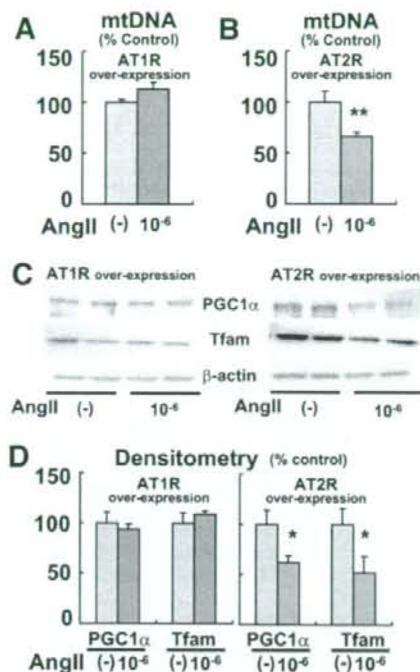

FIG. 3. Overexpression of AT2R in C2C12 cells augments AngII-induced reduction in mitochondrial biogenesis. Angiotensin II receptors (AT1R or AT2R) were overexpressed in C2C12 cells by CAG promoter–driven expression vectors previous to the treatment with AngII (10^{-6} mol/l) for 48 h (*A–D*). Mitochondrial DNA copy number (mtDNA) estimated by quantitative PCR analysis in C2C12 cells overexpressing AT1R (*A*) or AT2R (*B*) (n = 12 per group). *C*: Western blots of proteins that are related to mitochondrial biogenesis (PGC1α and Tfam). *D*: Densitometry of the Western blots. The density of the blots for PGC-1α and Tfam was normalized by that for the internal control (β-actin) (n = 8). The values were standardized to those for the control. *$P < 0.05$, **$P < 0.01$ vs. control.

drial content (Fig. 2*A* and *B*), AT2R blockade by PD-123319 completely reversed the AII-induced decrease in PGC1α and Tfam, but AT1R blockade by RNH-6270 did not affect their expressions (Fig. 2*D*).

These results indicate that, under the present experimental condition using C2C12 cells, AT1R-dependent signal pathways have effects on mtROS and mtMP without any change in mitochondrial content, whereas AT2R-dependent pathways influence mitochondrial biogenesis, mtROS, and mtMP. The expression levels of the receptors can thus be expected to determine the effect of AngII on mitochondria.

Overexpression of AT2R in C2C12 cells augments AngII-induced reduction in mitochondrial biogenesis. Next, we performed overexpression of the receptors by using the CAG promoter–driven expression vectors in C2C12 cells (Fig. 3*A–D*). In the AT1R overexpression group, mtDNA was not significantly changed as a result of treatment with AngII (Fig. 3*A*); however, in the AT2R overexpression group, mtDNA showed a major decrease as the result of treatment with AngII (44% decrease, n = 12, $P < 0.01$, Fig. 3*B*). Western blot analysis confirmed that PGC1α and Tfam protein levels in the AT2R overexpression group were significantly diminished by AngII (Fig. 3*C* and *D*). These results were also compatible with those for

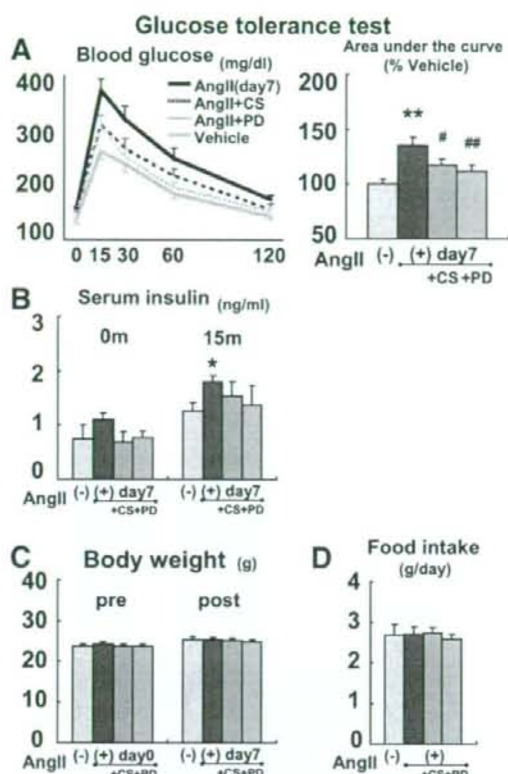

FIG. 4. Exogenous administration of AngII in mice for 1 week provokes glucose intolerance without changes in body weight or food intake. The 8-week-old male C57bl/6 mice were treated with a subpressor dose (0.1 μg/g body wt/day) of AngII or PBS (vehicle) by means of a subcutaneously implanted osmotic pump. The AT1R blocker, CS-866 (CS), or the AT2R blocker, PD-123319 (PD), was orally administered (*A–D*). *A*: Blood glucose levels (mg/dl) at 0, 15, 30, 60, and 120 min after intraperitoneal injection of 2.0 g/kg body wt glucose and area under the curve of the glucose level (% vehicle) (n = 18 per group). *B*: Serum insulin levels during glucose tolerance test at 0 and 15 min (0m, 15m). *C*: Body weight of mice before and 1 week after the implantation of osmotic pumps (pre, post). *D*: Food intake of mice during the experiment. *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. the AngII-treated group.

their gene expressions under pharmacological blockades (Fig. 2*D*).

Exogenous administration of AngII in mice for 1 week provokes glucose intolerance without changes in body weight or food intake. To explore the effects of AngII on muscle mitochondria and glycemic control in vivo, C57bl/6 mice were subjected to chronic infusion of AngII by means of an osmotic pump, combined with the pharmacological blockade of AT1R by CS-866 or of AT2R by PD-123319. We compared the effect on the four groups: control (vehicle implanted), AngII infusion, AngII infusion with AT1R blockade, and AngII infusion with AT2R blockade. In the AngII-treated groups, glucose levels after the glucose challenge were significantly higher than in the control (33% elevation in area under the curve of the glucose level, n = 18, $P < 0.01$; Fig. 4*A*), and the AngII-induced change in glycemic control was significantly suppressed by blockade of either AT1R or AT2R (13% or 17% suppression, n = 18, $P < 0.05$ and 0.01, respectively; Fig.

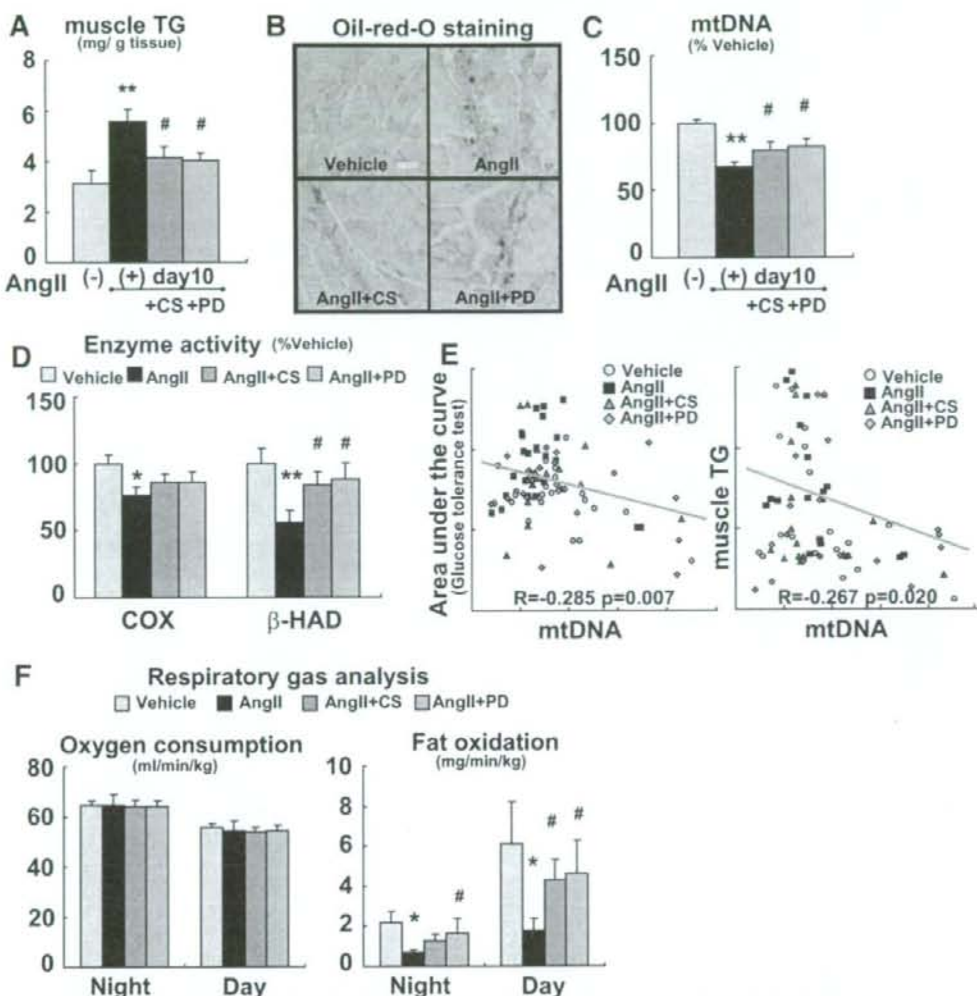

FIG. 5. AngII reduces mitochondrial content and increases triglyceride (TG) content in the skeletal muscle in both AT1R- and AT2R-dependent manners. *A*: Muscle triglyceride content (muscle triglyceride) measured in the quadriceps ($n = 8$ per group). *B*: Histological analysis of AngII-induced lipid accumulation in muscle by Oil-red-O staining. Neutral lipids were stained in red. Scale bar: 100 μm. *C*: mtDNA of the quadriceps estimated by quantitative PCR analysis ($n = 12$). *D*: Enzyme activity in the muscle of the mitochondrial proteins, cytochrome C oxidase (COX), and β-hydroxyacyl-CoA dehydrogenase (β-HAD) ($n = 12$). *E*: The relationship between area under the curve of the glucose levels in the glucose tolerance test and mtDNA, or between muscle triglyceride and mtDNA. Symbols were distinguished according to the treatment. *F*: Oxygen consumption and fat oxidation analyzed by indirect calorimetry. $*P < 0.05$, $**P < 0.01$ vs. control; $\#P < 0.05$ vs. the AngII-treated group. (Please see http://diabetes.diabetesjournals.org/cgi/content/full/db08-0949 for a high-quality digital representation of this figure.)

4*A*). In the AngII-infused groups, the serum insulin concentrations at 0 and 15 m of the glucose challenge showed a parallel increase with glucose levels (Fig. 4*B*). There were no significant changes in body weight or food intake in any of the four groups (Fig. 4*C* and *D*).

AngII reduces mitochondrial content and increases triglycerides in the skeletal muscle in both AT1R- and AT2R-dependent manners. We then examined the muscle triglyceride level in the quadriceps of the mice and found that they were higher in the AngII group (78% increase, $n = 8$, $P < 0.01$, Fig. 5*A*) than in the control group. Pharmacological blockade of either AT1R or AT2R caused a significant reduction in the muscle triglyceride level compared with that in the AngII group (26% or 28% reduction, respectively, $n = 8$, $P < 0.05$, Fig. 5*A*). To confirm that lipids were accumulated in the intramyocellular region, we performed Oil-red-O staining and found that AngII infusion increased intramyocellular lipids, and the increase was suppressed by blockade of either AT1R or AT2R (Fig. 5*B*). We also found that the ceramide content in the muscle was parallel to the triglyceride level (data not shown). The copy number of muscle mitochondrial DNA was reduced in the AngII group, and this change was also significantly diminished by blockade of either AT1R or AT2R (Fig. 5*C*). Furthermore, COX and β-HAD activities in the muscle, which represent electron transport and β-oxidative function of mitochondria, respectively, were significantly reduced in the AngII group, and

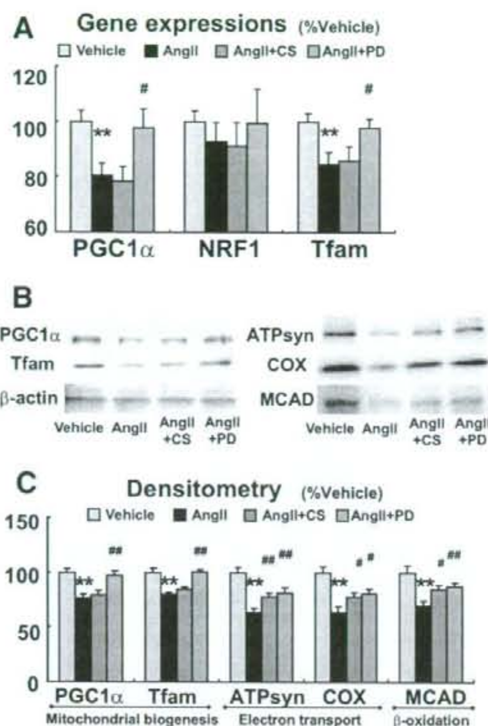

FIG. 6. Blockade of AT2R but not AT1R in AngII-infused mice reverses reduction in expression and protein levels of molecules involved in mitochondrial biogenesis. *A*: Expression of genes involved in mitochondrial biogenesis in the skeletal muscle of the mice ($n = 12$ per group). *B*: Western blots of proteins that are related to mitochondrial biogenesis (PGC1α and Tfam), electron transport (ATPsyn and COX), and β-oxidation (MCAD). *C*: Densitometry of the Western blots. The density of the blot was normalized by that for the internal control (β-actin) ($n = 8$). The values were standardized to those for the control. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the AngII-treated group.

this change was diminished by blockade of either AT1R or AT2R, in a manner that was parallel to the mitochondrial content (Fig. 5*D*). These experiments also showed that mtDNA correlated significantly with the area under the curve of the glucose tolerance test ($R = -0.285$, $n = 72$, $P < 0.01$) and muscle triglyceride ($R = -0.267$, $n = 72$, $P < 0.05$) in the quadriceps (Fig. 5*E*). Respiratory gas analysis was used to estimate oxygen consumption and fat oxidation. Although oxygen consumption measured over 24 h showed no significant changes in the four groups (Fig. 5*F*), a dramatic decrease in fat oxidation was observed in the AngII-infused group (69% at night and 70% during the day, $n = 8$, $P < 0.05$, Fig. 5*F*), and this change was abrogated by blockade of either AT1R or AT2R and to a greater extent by the AT2R blockade (41% or 46% recovery at night, respectively, $n = 8$, $P < 0.05$, Fig. 5*F*).

Blockade of AT2R but not AT1R in AngII-infused mice reverses reduction in expression and protein levels of molecules involved in mitochondrial biogenesis. Consistent with the result for C2C12 cells (Fig. 2*D*), AT2R blockade by PD-123319 in AngII-infused mice reversed the reduction in the expression in PGC1α and Tfam, but blockade of AT1R by CS-866 did not affect their expressions (Fig. 6*A*). Western blot analysis and densitometry of the blots confirmed this result (Fig. 6*B* and *C*). On the other hand, the AngII-induced reduction in mitochondrial proteins involved in electron transport or fatty acid oxidation (ATP synthase [ATPsyn], COX, and medium-chain fatty acyl-CoA dehydrogenase [MCAD]) were all suppressed by blockade of either AT1R or AT2R (Fig. 6*B* and *C*), and the manner of changes in the protein levels was parallel to that seen in mtDNA (Fig. 5*B*).

These results indicate that the AngII-induced reduction in muscle mitochondrial content in mice is caused by AT2R-dependent suppression of mitochondrial biogenesis and also by AT1R-dependent mechanisms that are not directly related to their biogenesis.

Microarray analysis of the skeletal muscle of AngII-infused mice. Detailed results of microarray analysis are presented in the supplemental materials.

DISCUSSION

In the study reported here, we found that AngII reduced mitochondrial content in cultured myotubular cells and skeletal muscle of mice. In addition, the AngII-infused mice showed a decrease in fat oxidation that was associated with an increase in intramuscular triglyceride content and impaired glucose tolerance. These findings imply that the cardiovascular hormone AngII, which has been thought previously to act mainly on the cardiovascular system, may have novel roles in the regulation of mitochondria and lipid metabolism in the skeletal muscle.

To determine the receptor responsible for the effects of AngII on mitochondrial content, we used pharmacological blockade and RNA interference of the receptors in C2C12 myotubes, which exhibited substantial expressions of both AT1R and AT2R. We found that the decrease in mitochondrial content induced by treatment with AngII for 48 h could be reversed by the pharmacological and genetic blockades of AT2R but not by those of AT1R. Consistent with this finding, AngII decreased the expression levels of PGC1α and Tfam, which positively regulate mitochondrial biogenesis, in an AT2R-dependent manner. We further confirmed that this reduction was attributable to AT2R but not to AT1R, by means of overexpression of the receptors in C2C12 cells. In the skeletal muscle of AngII-infused mice, on the other hand, the pharmacological blockade of either AT1R or AT2R partially but significantly reversed the AngII-induced reduction in mitochondrial content. The change in muscle mitochondrial content was parallel to the mitochondrial protein levels of ATPsyn, COX, and MCAD and the enzyme activity of COX and β-HAD. While still in this in vivo situation, the AngII-induced decrease in expression and protein levels of PGC1α and Tfam was prevented by the pharmacological blockade of AT2R but not by that of AT1R. Therefore, the difference in the protein levels under the AT1R blockade was observed between PGC1α/Tfam and ATPsyn/COX/MCAD.

These results imply that the in vitro and in vivo regulation of mitochondria by AngII was somewhat different under the present experimental conditions. AngII reduced mitochondrial content predominantly via an AT2R-dependent direct suppression of mitochondrial biogenesis in the cultured myocytes. On the other hand, mitochondrial content in the skeletal muscle in mice appeared to be determined by a complex combination of factors. Cytokines and hormones released from other tissues, ROS production, and nutritional availability are all known to influence mitochondrial DNA copy number in vivo (25).

For example, the plasma level of the insulin-sensitizing hormone adiponectin from adipose tissue has been reported to be decreased by AngII infusion via AT1R (26). Because adiponectin has been shown to increase mitochondrial number (27), decreased adiponectin levels via AT1R might affect mitochondria in the in vivo condition. These kinds of mechanisms can explain the difference, which we found in the present study, between the results for AT1R blockade under the in vitro and in vivo experimental conditions.

The finding that the expression and protein levels of molecules involved in mitochondrial biogenesis in skeletal muscle of AngII-infused mice were not modulated by AT1R suggests that AT1R-dependent pathways reduce mitochondrial content by a way other than via the reduction of mitochondrial biogenesis. Therefore, we propose that mitochondrial degradation was involved in the regulation of mitochondrial content in the present study. Mitochondria are degraded in lysosomes by a process known as "mitophagy" (28). Previous studies have shown that AT1R-dependent pathways augment ROS production, which is known to promote mitophagy and reduce mitochondrial content in rat kidney (29,30). Moreover, in our experiments, an AT1R-dependent increase in lysosomes has been observed in AngII-treated C2C12 cells (data not shown). These findings together suggest that the AngII-induced ROS production via AT1R would augment muscle mitophagy and that an AT1R blockade can be expected to protect muscle mitochondria from various insults that lead to mitophagy.

Chronic AngII infusion in rodents has been shown to reduce glucose uptake in muscle and provoke insulin resistance (31). Consistent with this finding, our study demonstrated that 1-week exogenous administration of a subpressor dose of AngII with a subcutaneously implanted osmotic pump exacerbated glycemic control in C57bl/6 mice without causing changes in food intake or suppression in insulin secretion. The AngII-induced glucose intolerance in mice was accompanied by a reduction in mitochondrial content and an increase in triglyceride levels in the skeletal muscle. Previous studies have shown a strong relationship between accumulation of intramuscular triglycerides and insulin resistance (32,33). Although triglycerides themselves are thought to be biologically inactive, accumulating muscle triglyceride levels lead to an increase in intramuscular fatty acids, which has been shown to inhibit insulin signaling via phosphorylation of serine residues in insulin receptor substrate 1 (34). Other lipid metabolites, such as long-chain fatty acyl coenzyme A, diacylglycerols, and ceramides, have also been shown to impair muscle insulin signal directly (35).

Muscle triglycerides are believed to increase in association with a reduction in fat oxidation; indeed, a significant relationship between reduced fat oxidation and increased triglyceride levels has been demonstrated by means of percutaneous biopsy of the vastus lateralis muscle in insulin-resistant subjects (36). Moreover, reduced muscle mitochondrial content has been shown to correlate with decreased fat oxidation and insulin resistance in nondiabetic subjects with a family history of type 2 diabetes (37). The fact that PGC1α-dependent pathways shift fuel substrates for oxidation from carbohydrates to lipids, in addition to promoting mitochondrial biogenesis, can explain the relationship between mitochondrial content and fat oxidation in muscle (38). These studies point to the importance of mitochondrial content in skeletal muscle as an upstream element in the pathogenesis of intramuscular lipids and insulin resistance.

The findings in the present study lead us to hypothesize that administration of AngII in mice causes glucose intolerance at least partly by reducing the mitochondrial content in skeletal muscle, which results in reduced fat oxidation and subsequent accumulation of intramuscular lipids. In support of this notion, we identified significant relationships among mitochondrial content in quadriceps on the one hand, and intramuscular triglyceride and the index of glucose intolerance, expressed as area under the curve of glucose levels after glucose challenge, on the other. The results of the in vivo energy expenditure, which showed that the AngII infusion in mice did not change the oxygen consumption, indicate that the reduction in muscle mitochondrial content by AngII was not mediated through changes in chronic physical activity. However, it is possible that AngII first impairs glycemic control and subsequently reduces mitochondrial content, because hyperglycemia itself has been known to decrease mitochondrial content (39,40). Future studies should specifically focus on the time course of the AngII-induced decrease in mitochondrial content and deterioration of glycemic control, as well as their causal relationship.

To summarize, we have demonstrated that AngII causes a reduction in mitochondrial content in cultured myotubular cells and the skeletal muscle in mice. Exogenous administration of AngII with an osmotic pump in mice provoked glucose intolerance, which is associated with reduced mitochondrial content, decreased fat oxidation, and increased intramuscular triglyceride levels. Putting these findings together suggests that the cardiovascular hormone AngII, which has been thought to act mainly on the cardiovascular system, can also regulate mitochondrial content and lipid metabolism in the skeletal muscle, and thus affect glycemic control. AngII-infused mice are likely to reduce muscle mitochondrial content through both AT1R and AT2R by different mechanisms: through AT1R-dependent augmentation of mitochondrial degradation and AT2R-dependent direct suppression of their biogenesis.

ACKNOWLEDGMENTS

No potential conflicts of interest relevant to this article were reported.

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Hypertension

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Kamon Iigaya, Hiroo Kumagai, Toru Nabika, Yuji Harada, Hiroshi Onimaru, Naoki Oshima, Chie Takimoto, Tadashi Kamayachi, Takao Saruta and Hiroshi Itoh

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Relation of Blood Pressure Quantitative Trait Locus on Rat Chromosome 1 to Hyperactivity of Rostralventrolateral Medulla

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Abstract—Genetic factors that induce essential hypertension have been examined using genome-wide linkage analyses. A quantitative trait locus (QTL) region that is closely linked to hypertension has been found on chromosome 1 in stroke-prone spontaneously hypertensive rats (SHRSPs). We used 2 congenic rats in which the blood pressure QTL on rat chromosome 1 was introgressed from SHRSP/Izm to Wistar-Kyoto (WKY)/Izm (WKYpch1.0) and from WKY/Izm to SHRSP/Izm (SHRSPwch1.0) rats by repeated backcrossing. Previous studies reported that the intermediate phenotype of this QTL for hypertension is characterized by the hyperactivity of the sympathetic nervous system in response to physiological and psychological stress. We performed intracellular patch-clamp recordings of rostral ventrolateral medulla (RVLM) neurons from WKY, WKYpch1.0, SHRSPwch1.0, and SHRSPs and compared the basal electrophysiological activities of RVLM neurons and the responses of these neurons to angiotensin II. The basal membrane potential of RVLM neurons from WKYpch1.0 was significantly "shallower" than that of the neurons from WKY. The depolarization of RVLM neurons from WKYpch1.0 in response to angiotensin II was significantly larger than that in neurons from WKY rats, whereas the depolarization of RVLM neurons from SHRSPwch1.0 was significantly smaller than that in neurons from SHRSPs. The response to angiotensin II of RVLM neurons from WKYpch1.0 and SHRSPs was sustained even after the blockade of all of the synaptic transmissions using tetrodotoxin. The QTL on rat chromosome 1 was primarily related to the postsynaptic response of RVLM bulbospinal neurons to brain angiotensin II, whereas both the QTL and other genomic regions influenced the basal activity of RVLM neurons. **(*Hypertension.* 2009;53:42-48.)**

Key Words: sympathetic nervous system ■ congenic rat ■ angiotensin II ■ stress ■ RVLM neurons

The stroke-prone spontaneously hypertensive rat (SHRSP) is a useful model for the study of human essential hypertension.[1] Previous genome-wide analyses identified a potent quantitative trait locus (QTL) on rat chromosome 1 (Chr-1) that is responsible for hypertension in SHRSPs; this trait was confirmed in congenic strains for the QTLs.[2–5] Further analyses of the congenic strains suggested that this QTL harbored a gene (or genes) that regulated sympathetic responses to various stresses, such as restraint, cold, and air-jet stress.[5–7] Because the stressors used were either physical or emotional in nature, we hypothesized that a common pathway regulating sympathetic responses to stress might be responsible for this phenomenon. In this regard, the genetic effects of the Chr-1 QTL on the neuronal activity of the rostral ventrolateral medulla (RVLM), which is thought to determine the basal sympathetic nervous tone in response to various inputs from higher brain centers,[8,9] were explored in this study. In addition, among various modulators of RVLM activity, we particularly focused on the role of angiotensin II (Ang II) based on the following observations: physiological studies on rabbits showed that cardiovascular responses to air-jet stress were attenuated by the infusion of Ang II receptor blockers into the RVLM, implying a pivotal role of angiotensinergic neurons in the RVLM on the responsiveness to emotional stress.[10] Thus, we studied the stimulatory effect of Ang II on RVLM neurons in a previous study using brain stem-spinal cord preparations from neonatal rats.[11]

In the present study, we used reciprocal congenic strains constructed to evaluate the effect of the blood pressure QTL on Chr-1 on the electrophysiological activities of RVLM neurons in the absence of any influence from blood pressure and the higher brain center in the hypothalamus. The basal activity and response to Ang II superfusion were recorded in a single neuron using the whole-cell patch-clamp technique.

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From the Division of Endocrinology, Metabolism, and Nephrology (K.I., N.O., C.T., T.K., T.S., H.I.), Department of Internal Medicine, Keio University School of Medicine, Tokyo; Department of Nephrology (H.K.), National Defense Medical College, Tokorozawa, Saitama; Department of Pathology (T.N., Y.H.), Shimane University School of Medicine, Izumo; and Department of Physiology (H.O.), Showa University School of Medicine, Tokyo, Japan.

Correspondence to Kamon Iigaya, Discipline of Physiology, F13, University of Sydney, New South Wales 2006, Australia. E-mail kamon@physiol.usyd.edu.au

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Materials and Methods

Animals

Two congenic rat strains, WKY.SHRSP-(D1Wox29-D1Arb21)/Izm (abbreviated as WKYpch1.0) and SHRSP.WKY-(D1Wox29-D1Arb21)/Izm (abbreviated as SHRSPwch1.0) were provided through the National BioResource Project for the Rat.[12] These strains were established by introgressing the chromosomal segment from SHRSP/Izm into Wistar-Kyoto (WKY)/Izm rats and vice versa.[5,13] The congenic region between D1Wox29 and D1Arb21 covered a 100:1 CI for the blood pressure QTL. The genotyping of >150 simple sequence-length markers confirmed that the background genome was homozygous for the original WKY and SHRSPs in the WKYpch1.0 and SHRSPwch1.0 strains, respectively.[3,5] Information on the congenic strains is available at the Web site of the National BioResource Project for the Rat (http://www.anim.med.kyoto-u.ac.jp/nbr/home.htm).

WKY/Izm and SHRSP/Izm rats were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). The genomic constructs of the 4 strains used in this study are shown in References 4 and 5. The congenic rats were maintained in the Keio University School of Medicine Animal Laboratory Center. All of the rats were fed a standard laboratory chow and tap water ad libitum and kept in a room maintained at a constant temperature of 25°C. The experimental protocols were approved by the Keio University School of Medicine Animal Research Committee, in compliance with Japanese Law (No. 105).

Recording of Electrophysiological Activities

Experiments were performed on brain stem-spinal cord preparations obtained from 0- to 4-day-old rats. Under deep ether anesthesia, the brain stem and spinal cord were isolated and sectioned at the second thoracic nerve root (Th_2) level, as described previously.[11,14,15] The preparation was continuously superfused at 2 to 3 mL/min with a standard solution consisting of (in mmol/L) 124.0 NaCl, 5.0 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26.0 $NaHCO_3$, 1.2 KH_2PO_4, and 30.0 D-glucose and equilibrated with 95% O_2 and 5% CO_2 (pH7.4), at 26°C to 27°C.

Intracellular recordings using the whole-cell patch-clamp technique were performed as follows: a patch electrode was filled with the following pipette solution (mmol/L): 130 K-gluconate, 10 EGTA, 10 HEPES, 2 Na_2-ATP, 1 $CaCl_2$, 1 $MgCl_2$, and 0.5% lucifer-yellow (Aldrich Chemical [pH 7.2 to 7.3], adjusted with KOH). A patch-clamp amplifier (Axopatch 1D, Axon Instruments) was used to record the membrane potential. Before starting the intracellular recordings, the firing patterns were checked using extracellular recordings. RVLM neurons exhibiting discharges that were synchronized with the simultaneously recorded phrenic nerve activity were assumed to be respiratory neurons and were excluded from the study. The membrane potential was recorded using the current-clamp technique (20-pA increments from −100 to 20 pA, 500-ms duration) and was corrected for the junctional potential at the tip of the pipettes (−-11 mV). The basal membrane potential and the firing rate were recorded over 10 minutes. The input resistance was calculated from the current-voltage curve. The membrane potential was shifted to −50 mV because of the negative current. One RVLM neuron per preparation was used in the experiment.

To evaluate the responses to Ang II, we superfused the brain stem-spinal cord preparation for 20 minutes with Ang II (6 μmol/L, Sigma-Aldrich) dissolved in a standard solution and recorded the changes in the membrane potential and in the input resistance of the RVLM neurons. Li and Guyenet[16] used a bath application of 0.3 to 1.0 μmol/L of Ang II in their slice preparations. In an earlier study from our laboratory, we used 1, 3, and 12 μmol/L of Ang II to examine the dose responsiveness of RVLM neurons in a brain stem-spinal cord preparation, in which the target neurons were located 100 μm from the surface of the preparation.[11] Based on these results, we selected the dosage of 6 μmol/L of Ang II, because the distance from the surface of the preparation to the RVLM neurons makes it difficult for Ang II to reach the target neurons.

We then performed superfusion with a mixture of Ang II (6 μmol/L) and tetrodotoxin (50 μmol/L; Wako Pure Chemical Industries). Tetrodotoxin was used to block every synaptic input to the RVLM neurons. The intermediolateral cell column neurons were stimulated with a stainless-steel electrode (5 to 15 V, 100 ms, single pulse) to identify the RVLM bulbospinal neurons. RVLM bulbospinal neurons showing antidromic action potentials after intermediolateral cell column stimulation were used in the experiments.[17] Lucifer yellow was allowed to diffuse spontaneously or by iontophoresis into the neurons during the intracellular recordings to verify the location of the neurons examined.[18] All of the data were recorded and analyzed using PowerLab (AD Instruments). After the addition of tetrodotoxin, we first confirmed the disappearance of phrenic nerve activity (≈15 to 20 minutes after the addition of tetrodotoxin) and then added Ang II to the superfusate. Superfusion with Ang II induced depolarization in the bulbospinal neurons of the RVLM after a 1- or 2-minute latency period.

Statistics

All of the data were represented as the means±SDs. Differences between the WKY and WKYpch1.0 rats or between the SHRSPwch1.0 and SHRSP rats were examined using independent *t* tests. A value of $P<0.05$ was considered statistically significant.

Results

Basal Electrophysiological Activities of the RVLM Neurons

The RVLM neurons are classified into 3 types: regularly firing neurons, irregularly firing neurons, and silent-type neurons; however, the physiological roles of these neurons have not yet been fully elucidated.[14] The irregularly firing neurons exhibited many excitatory postsynaptic potentials, whereas the regularly firing neurons rarely showed such behavior (Figures 1 and 2).

In the regularly firing neurons, the basal membrane potential was significantly less negative in the WKYpch1.0 rats (−46.4±2.3 mV) than in the WKY rats (−55.4±5.6 mV). The basal membrane potential of the SHRSPwch1.0 rats (−47.8±2.6 mV) was similar to that of the SHRSP rats (−45.4±3.6 mV). The firing rate did not differ between the WKYpch1.0 and WKY rats or between the SHRSPwch1.0 and SHRSP rats.

As shown in Figure 2, the same trend was observed in the irregularly firing neurons of the RVLM; the basal membrane potential was significantly less negative in the WKYpch1.0 rats (−49.3±3.0 mV) than in the WKY rats (−57.0±1.0 mV; $P<0.01$). The basal membrane potential of the SHRSPwch1.0 rats (−51.3±3.2 mV) was similar to that of the SHRSP rats (−50.0±0.0 mV). The firing rate did not differ between the WKYpch1.0 and WKY rats or between the SHRSPwch1.0 and SHRSP rats.

The input resistance of the regularly and irregularly firing neurons was also not significantly different among the 4 strains (data not shown).

Response to Ang II

Superfusion with Ang II (6 μmol/L) induced depolarization in the bulbospinal neurons of the RVLM after a 1- or 2-minute latency period. Repolarization toward the basal level was then observed over the next 2 or 3 minutes; this repolarization was considered to represent the desensitization of the neurons. We, thus, quantified the magnitude of the maximal depolarization.

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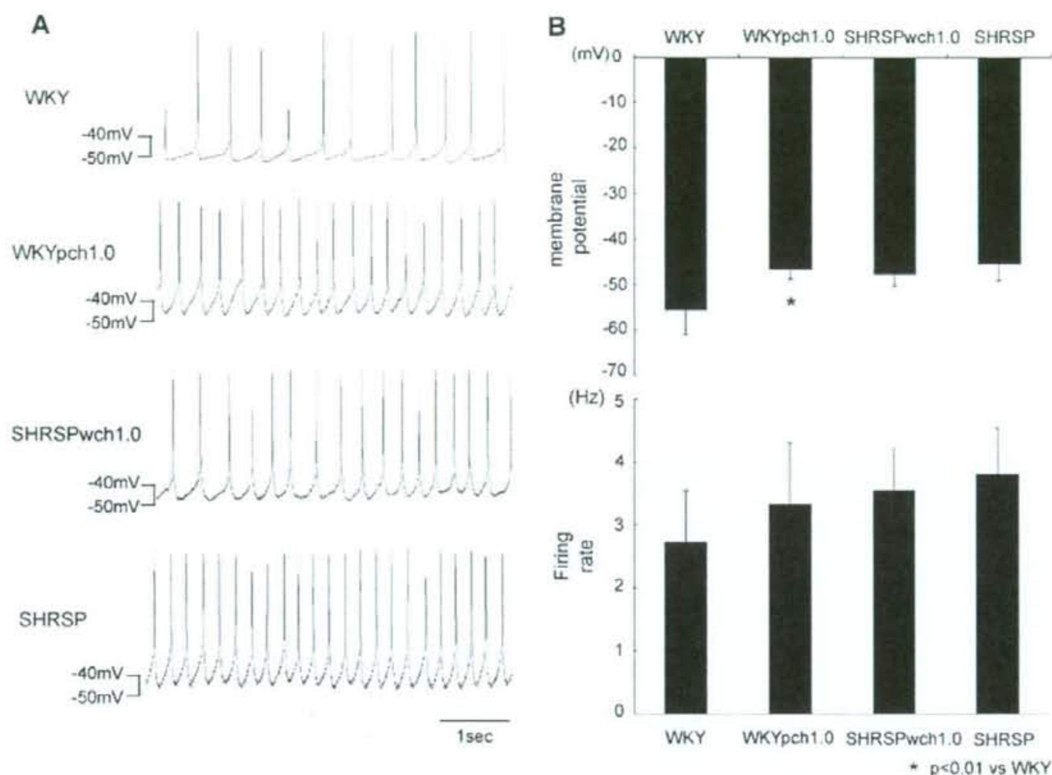

Figure 1. Basal membrane potential and firing rate of regularly firing neurons in the RVLM of 4 rat strains. A, Representative traces of neuronal activity in RVLM from the 4 strains, recorded using the intracellular whole-cell patch-clamp technique. B, Top and bottom panels show the basal membrane potential and the firing rate (mean±SD of 7 neurons from different rats of each strain).

The magnitude of the depolarization of the RVLM neurons from WKYpch1.0 rats during Ang II superfusion was +4.1±2.3 mV, which was significantly larger than that of the neurons from WKY rats (Figure 3). The depolarization of the RVLM neurons from SHRSPs was significantly larger than that of the neurons from SHRSPwch1.0 rats. Of note, the depolarization was statistically significant in the 2 strains (WKYpch1.0 and SHRSP) that possess the SHRSP-derived fragment of the Chr-1 QTL.

In the next experiment, which used a potent inhibitor of synaptic transmission, tetrodotoxin, we examined whether the Ang II effect was mediated through presynaptic or postsynaptic pathways. After the administration of tetrodotoxin, we first confirmed the disappearance of phrenic nerve activity and then added Ang II to the superfusate. The average magnitude of the depolarization of the neurons from WKYpch1.0 rats during superfusion with tetrodotoxin and Ang II was significantly larger than that of the neurons from WKY rats (Figure 4). Meanwhile, the average magnitude of the depolarization of the neurons from SHRSPs was also significantly larger than that of the neurons from SHRSP-wch1.0 rats. These differences in depolarization were basically the same as those obtained in the experiments without tetrodotoxin, and the neurons from SHRSPs and WKYpch1.0 rats showed a significantly greater depolarization than those from the respective counterpart rats.

Discussion

The major finding of the present study was that the QTL on rat Chr-1 affected the electrophysiological activity of RVLM neurons, particularly their responsiveness to Ang II. Because isolated brain stem-spinal cord preparations from neonatal rats were used in this study, we would like to emphasize that the results were not caused by the secondary effects of hypertension or by the influence of the higher brain centers, such as the hypothalamus. Therefore, this observation strongly suggests that a gene (or genes) responsible for the difference in the activity of the RVLM neurons is (are) located in this genomic region.

In regularly firing neurons, the basal membrane potential differed significantly between neurons from WKY and WKYpch1.0 rats (Figure 1). Considering the genomic composition of the congenic strain (WKYpch1.0), this observation suggests that genes responsible for this interstrain difference are located both inside and outside of the QTL. Furthermore, the lack of a difference between neurons from SHRSPs and SHRSPwch1.0 rats implies that the effects of these genes are not additive, suggesting that they may be

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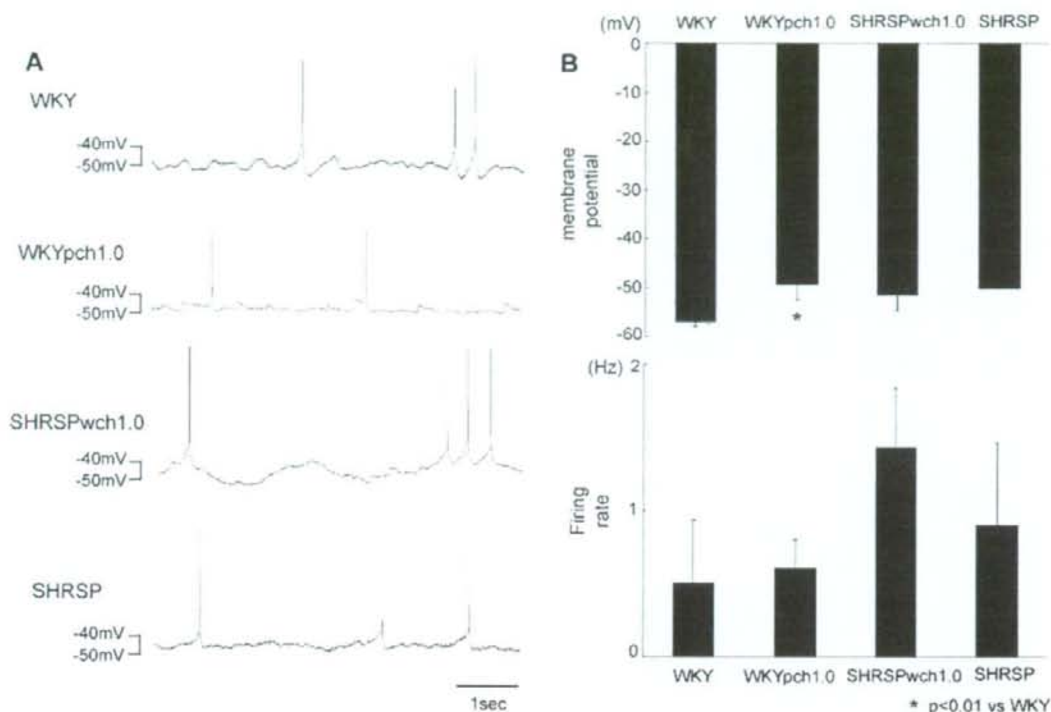

Figure 2. Basal membrane potential and firing rate of irregularly firing neurons in the RVLM. A, Representative traces from the 4 strains. B, Top and bottom panels show the basal membrane potential and the firing rate (mean±SD of 8 neurons from different rats).

involved in the same biological process, just as in the case of the blood pressure QTLs in Dahl salt-sensitive rats.[19]

In the irregularly firing neurons, we obtained a similar result regarding the difference in the basal membrane potential and no differences in the firing rate (Figure 2). These results can probably be explained by the fact that a larger number of synaptic inputs modulates the firing rate in irregularly firing neurons.[14] Nevertheless, a similar interstrain difference in the membrane potential was observed in neurons with different properties, suggesting that this genetic effect is ubiquitous in RVLM neurons.

The Ang II–induced depolarization and increase in the firing rate are shown in Figure 3. Of particular importance, these responses depended largely on the genotype of the congenic fragment, irrespective of the background genome; the 2 strains with the congenic fragment originating from SHRSPs (WKYpch1.0 and SHRSP) showed a significantly greater depolarization than the rats containing the congenic fragment originating from WKY rats (WKY and SHRSPwch1.0). This observation suggests that the responsiveness of the RVLM neurons to Ang II was largely determined by the gene(s) located in the QTL.

WKYpch1.0 rats have been shown repeatedly to have exaggerated sympathetic responses to different types of stress.[5–7] Considering a recent observation that the Ang II type 1 receptor in the RVLM mediates activating the sympathetic nervous system by emotional stress, resulting in an increase in blood pressure in conscious rabbits,[10] it is quite attractive to hypothesize that a gene (or genes) in the Chr-1 QTL affects (affect) the responsiveness of the sympathetic nervous system to stress through an Ang-II–mediated system in the RVLM. Supporting this hypothesis, Yamazato et al[7] reported that the hyperresponsiveness of blood pressure and renal sympathetic nerve activity to air-jet stress in WKYpch1.0 rats was inhibited by the intracerebroventricular injection of an Ang II receptor blocker.

Based on the results of the present study, we suspect that the basal membrane potential in RVLM neurons is affected primarily by age and background genome, whereas the response to Ang II in RVLM neurons depends on the blood pressure QTL of Chr-1. The depolarization of the RVLM neurons by Ang II superfusion was statistically significant in the neonatal WKYpch1.0 and SHRSPs but not in the neonatal WKY and SHRSPwch1.0 rats. Our data are supported by the results reported by Yamazato et al,[7] who showed that increases in renal sympathetic nerve activity in response to air-jet stress were significantly larger in 4-week–old WKYpch1.0 and SHRSPs than in 4-week–old WKY rats and that the sympathoexcitation of WKYpch1.0 rats was similar to that of SHRSPs. The depolarization of RVLM neurons by Ang II shown in our study may account for the increases in sympathetic nerve activity and blood pressure in response to air-jet stress, because the intracerebroventricular injection of Ang II receptor blocker candesartan reduced the sympatho-

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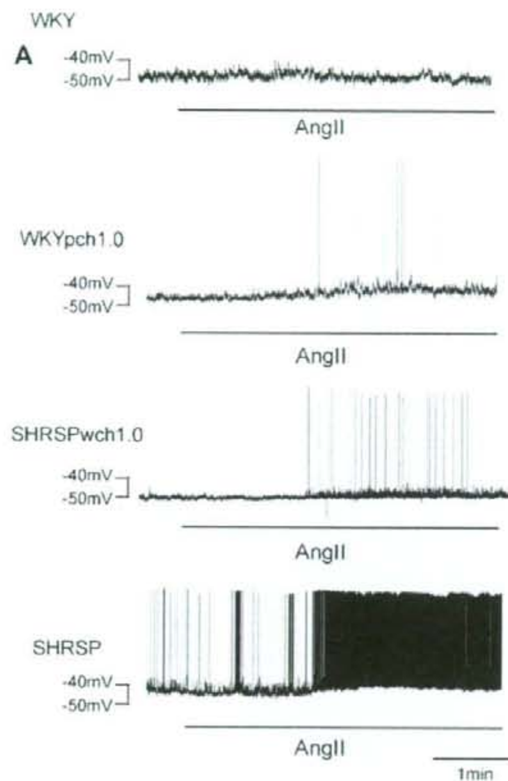

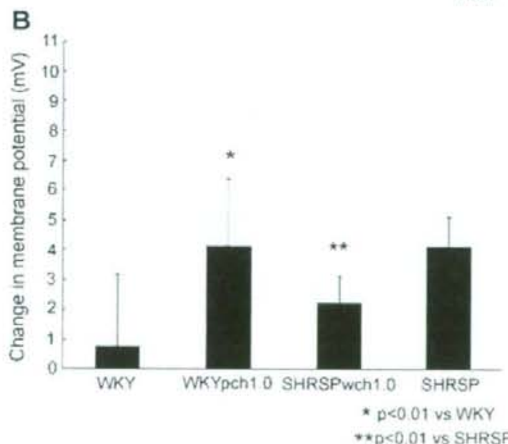

Figure 3. Effect of Ang II (6 μmol/L) superfusion on membrane potential of RVLM neurons. A, Representative traces from each strain. B, Changes in membrane potential (depolarization) in response to Ang II superfusion (mean±SD of 8 neurons from different rats of each strain).

excitatory and pressor responses.[7] Also, a report from Cui et al[5] demonstrated that the increase in systolic blood pressure in response to restraint stress in 16- to 20-week-old WKYpch1.0 rats was larger than that of WKY rats. These compatible data for different rat ages and from 3 different laboratories strongly suggest that the genes in the Chr-1 QTL of SHRSPs are responsible for the depolarization response of RVLM neurons to Ang II in neonatal WKYpch1.0 and SHRSPs, the increase in peripheral sympathetic activity in response to air-jet stress in 4-week-old WKYpch1.0 and SHRSPs, and the pressor response to restraint stress in 16- to 20-week-old WKYpch1.0 rats. However, these data do not suggest that age is responsible for the stress responses. In contrast, the basal blood pressures of WKY and WKYpch1.0 rats seem to be determined largely by age and also by genes both inside and outside the QTL.

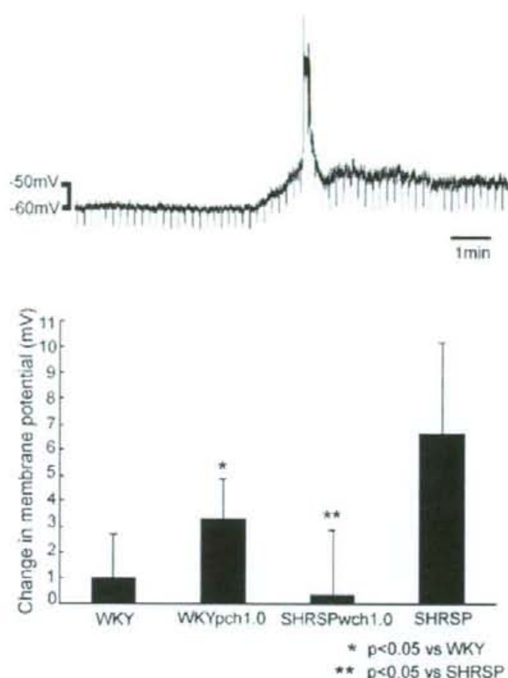

Figure 4. Top, Representative tracing of membrane potential in response to Ang II superfusion in the presence of tetrodotoxin during the application of a single current pulse (500 ms) for constructing a current-voltage (iv) curve every 10 seconds. A significant depolarization was detected as the result of a calcium influx through voltage-dependent calcium channels. Bottom, Changes in membrane potential in response to Ang II (6 μmol/L) superfusion in the presence of tetrodotoxin (50 μmol/L; mean±SD of 5 neurons from different rats).

In some cases, the effects of the QTL region on blood pressure were asymmetrical in reciprocal congenic strains for hypertensive QTLs. As Rapp[20] pointed out previously, strains with congenic fragments from the Dahl salt-sensitive rat on a background of normotensive strains did not show a significant increase in blood pressure, whereas congenic rats with the reversed genotype showed an obvious reduction. Consistent with this finding, WKYpch1.0 rats showed little, if any, increase in blood pressure, whereas SHRSPwch1.0 rats showed a large decrease.[3,13] This asymmetrical nature of the effects of QTLs on blood pressure was probably caused by gene-gene interactions, which convoluted the analyses. In

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contrast, reciprocal genetic effects like the one observed in the present study suggest a mendelian control of phenotypes: the genotype (or the haplotype) of a single gene (or a cluster of genes) had a major effect on the phenotype, independent of the genetic background.[21,22] Such a phenotype may be regulated by the gene(s) in a more direct manner and may be useful for speculating the functions of the gene(s).

Because tetrodotoxin blocks all synaptic transmissions, the observation that the addition of tetrodotoxin did not change the responsiveness to Ang II further implied that the interstrain difference in the responsiveness was intrinsic (or postsynaptic) in the RVLM neurons. A previous study of Sumners et al[23] reported that the exaggerated reactivity of RVLM neurons to Ang II in spontaneously hypertensive rats was because of an increase in the number of Ang II receptors on the RVLM neurons, which then induced oxidative stress and activated signal transduction via G proteins. It would be interesting to investigate the underlying intracellular mechanisms of the Ang II–dependent activation of RVLM neurons using congenic strains.

The blood pressure QTL on rat Chr-1 is quite large and contains hundreds of genes and expressed sequence tags.[5,13] Among them, however, several interesting candidates were found when the putative roles of the genes in the sympathetic nervous system were considered. These genes include Arix, a transcription factor regulating the development of the sympathetic nervous system, as well as the expression of dopamine β-hydroxylase[24–26]; Ntrk3, a receptor for neurotropin 3[27]; Arrb1, a cofactor regulating the internalization of the β-adrenergic receptors and angiotensin receptors[28]; Nox4, a subunit of NADPH oxidase[29]; and Homer 2, a regulator of the metabolic glutamate receptors.[30,31] In future studies, it will be necessary to reduce the number of candidate genes using both their positional and functional information to identify the responsible gene(s) and to confirm the roles of increased RVLM activity in the pathogenesis of hypertension.

The main limitation of this study is the lack of data regarding how RVLM neuron activity may (or may not) differ between strains in older animals and how this possible difference may be correlated with blood pressure. We hope to measure the RVLM neuron activity of both young and old rats in the future. To demonstrate that these early differences in RVLM neuron activity are causally related to stress-related differences in blood pressure, we should have compared the RVLM activity of older congenic and parental rats. Ideally, the electrophysiological properties of RVLM and the blood pressure, heart rate, and renal sympathetic nerve activity should be simultaneously examined in neonatal and older rats in vivo, and the changes in these parameters in response to various stressors should be determined. However, because the contact between the targeted neurons and the patch pipette is impaired by the increase in glial cells and astrocytes that occurs in rats older than 2 weeks of age, intracellular patch-clamp recordings of RVLM neurons are technically impossible to perform in older rats.

Perspectives

Several lines of evidence have indicated that sympathetic nerve activity differs significantly between spontaneously hypertensive rats/SHRSPs and WKY rats, and this difference is one of the putative causes of hypertension in spontaneously hypertensive rats/SHRSPs. Because membrane potential of RVLM neurons determines the sympathetic tone, the present observation suggests that a gene (or genes) in the Chr-1 QTL is (are) responsible for the greater sympathetic tone observed in SHRSPs. Identification of the responsible gene(s) will improve our understanding of the role of the sympathetic nervous system in the pathogenesis of hypertension and promote the development of new therapeutic and preventive strategies for essential hypertension.

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Disclosures

None.

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"Pulse" Treatment With High-Dose Angiotensin Blocker Reverses Renal Arteriolar Hypertrophy and Regresses Hypertension

Kimiko Ishiguro, Kaori Hayashi, Hiroyuki Sasamura, Yusuke Sakamaki and Hiroshi Itoh

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"Pulse" Treatment With High-Dose Angiotensin Blocker Reverses Renal Arteriolar Hypertrophy and Regresses Hypertension

Kimiko Ishiguro, Kaori Hayashi, Hiroyuki Sasamura, Yusuke Sakamaki, Hiroshi Itoh

Abstract—One ultimate goal of hypertension therapy is to cause permanent reversal ("regression") of already established hypertension. Our aim was to examine whether high-dose "pulse" treatment with a renin-angiotensin system inhibitor could cause regression of established hypertension and to link this action to reversal of arteriolar hypertrophy and changes in vascular matrix metalloproteinase activities. First, 16-week-old male spontaneously hypertensive rats (n=60) were pulse treated for 2 weeks with high-dose angiotensin-converting enzyme inhibitor (enalapril), angiotensin receptor blocker (candesartan), calcium channel blocker (nifedipine), or vasodilator (hydralazine) with or without salt restriction, and the long-term effects on blood pressure were examined. Second, spontaneously hypertensive rats were treated with angiotensin receptor blocker or calcium channel blocker, and the effects on renal gene expressions, arteriolar structure, and vascular matrix metalloproteinase were compared. Treatment of spontaneously hypertensive rats with different antihypertensive agents caused apparently similar reductions in blood pressure during the course of the pulse treatment, within the limitations of the tail-cuff method. After cessation of medications, blood pressure in the rats treated with renin-angiotensin system inhibitor remained reduced by >30 to 40 mm Hg for 4 months. No such effect was seen with calcium channel blocker or vasodilator. The 2-week angiotensin receptor blocker treatment induced a marked reversal of the arteriolar hypertrophy specifically in the small (30 to 100 μm) renal arterioles, together with increased expression and activity of matrix metalloproteinase-13. In conclusion, transient high-dose pulse treatment with angiotensin receptor blocker caused changes in vascular matrix metalloproteinase activity, specific reversal of renal arteriolar hypertrophy, and regression of hypertension in spontaneously hypertensive rats. **(*Hypertension*. 2009;53:83-89.)**

Key Words: angiotensin receptor blocker ■ calcium channel blocker ■ regression ■ spontaneously hypertensive rat ■ MMP ■ renal arteriolar hypertrophy

It has been estimated that ≈26.4% of the adult world population in the year 2000 had hypertension, and the number was projected to increase to 29.2% by the year 2025.[1] Because hypertension is a major risk factor for diseases such as stroke, coronary artery disease, heart failure, kidney disease, and vascular disease, the medical, economic, and social consequences of the current epidemic of hypertension are considerable.[2]

One strategy for managing this disease is "prevention" of the development of hypertension. Previous studies by Harrap et al,[3] Richer et al,[4] and other groups, including our own[5–7], have shown that treatment of young (4- to 6-week-old) prehypertensive spontaneously hypertensive rats (SHRs) with a renin-angiotensin system (RAS) inhibitor is effective in permanently attenuating the later development of hypertension. In other words, transient administration of a RAS inhibitor, if given before hypertension was fully established, was found to be effective for hypertension prevention in SHR. The feasibility of using transient RAS inhibition to prevent the development of hypertension in human patients has been confirmed recently by Julius et al[8] in the landmark Trial of Preventing Hypertension.

A different strategy would be to aim for "regression" of already established hypertension. Importantly, Smallegange et al[9] reported that transient treatment of adult SHRs with a high-dose angiotensin-converting enzyme inhibitor (ACEI), together with a low-salt diet, was effective in causing a sustained reduction of blood pressure even if administration of the drug was started at 16 weeks, well after hypertension was established in the SHR model. These results suggested that high-dose RAS inhibition could indeed be effective in the reversal or regression of already established hypertension. Potentially, this could have a great clinical benefit, because it could mean that patients with established hypertension could well be "cured" by appropriate transient therapy.

At present, it is unclear whether regression of hypertension is an effect that is specific to RAS inhibitors or is generally

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From the Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan.
The first 2 authors contributed equally to this study.
Correspondence to Hiroyuki Sasamura, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail sasamura@sc.itc.keio.ac.jp
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seen with high doses of other antihypertensive agents, such as calcium channel blockers (CCBs) or vasodilators. The molecular mechanism is also undefined, in particular, the relationship with renal arteriolar hypertrophy, which is thought to play an important role in the maintenance of hypertension in SHRs.[10,11]

The objective of this study was, therefore, to test the hypothesis that high-dose pulse treatment with a RAS inhibitor but not a CCB would cause regression of established hypertension and to link this action to reversal of arteriolar hypertrophy and changes in vascular matrix metalloproteinase (MMP) activities. The specific aim of the first experiment was to compare the efficacy of a total of 8 antihypertensive pulse regimens (with and without a low-salt diet) in inducing regression. In the second experiment, we examined whether high-dose pulse treatment could affect the renal arteriolar hypertrophy found in SHRs, as well as cause changes in activities of the vascular matrix metalloproteinases (MMPs). Our results suggest that high-dose pulse treatment with a RAS inhibitor causes changes in arteriolar MMP activity, leading to reversal of arteriolar hypertrophy and, ultimately, to regression of hypertension in the SHR model.

Methods

Animal Treatment Protocols

The studies were conducted using 16-week-old male Wistar-Kyoto (WKY) rats (WKY/Izm) and SHRs (SHR/Izm) obtained from Sankyo Laboratory Services (Tokyo, Japan). All of the experiments were approved by the institutional review committee and performed in accordance with the Keio University School of Medicine Animal Experimentation Guidelines.

Experiment 1

SHRs were randomly divided into 10 groups as follows (n=6 per group). Rats in group 1 were control SHRs. Rats in groups 2 to 5 were treated from 16 to 18 weeks with the ACEI enalapril maleate in drinking water (20 mg/kg per day), the ARB candesartan cilexetil dissolved in the drinking water[7] (50 mg/kg per day), the vasodilator hydralazine (25 mg/kg per day), or the CCB nifedipine in chow (50 mg/kg per day). Rats in groups 6 to 10 were treated identically to groups 1 to 5 but were also treated from 16 to 18 weeks with a low-salt diet (0.05% Na). All of the interventions were discontinued at age 18 weeks, and the rats were observed without any medication for a further 18 weeks, then euthanized at age 36 weeks.

Experiment 2

WKY rats and SHRs were randomly divided into 4 groups as follows (n=6 per group). Rats in group 1 were control WKY rats. Rats in group 2 were control SHRs. Rats in groups 3 and 4 (ARB and CCB groups) were treated with either the ARB candesartan (50 mg/kg per day) or the CCB nifedipine (50 mg/kg per day), as described above, then euthanized at the end of the 2-week pulse treatment at age 18 weeks.

Assays

The systolic blood pressure and heart rate of awake animals were measured by tail-cuff plethysmography using a Natsume KN-210 manometer (Natsume, Inc). Twenty-four-hour urine collection was performed in metabolic cages, and urine albumin excretion was determined by a direct competitive ELISA (Nephrat). Other biochemical assays are described in the online data supplement (available at http://hyper.ahajournals.org).

Histological Studies

The kidneys and thoracic aortas were removed and fixed in 4% paraformaldehyde, then embedded in paraffin blocks. In experiment 2, tissue samples were also obtained from the mesentery, heart, and brain, for the examination of mesenteric, cardiac, and cerebral arterioles. Details of the histological assessment are described in the online data supplement.

Preparation of RNA and Real-Time RT-PCR and Microarray Analysis

Kidney RNA was prepared for real-time RT-PCR and microarray analysis, as described in detail in the online data supplement.

In Situ Zymography and Immunofluorescence Staining

High-resolution, high-sensitive zymography was performed using the protocol of Ahmed et al,[12] with minor modifications. Immunofluorescence staining of vascular MMP expression was performed using standard protocols (for details, see the online data supplement).

Statistics

Results were expressed as the means±SEMs. Statistical comparisons were made by ANOVA, followed by Scheffe's posthoc test. *P* values <0.05 were considered statistically significant.

Results

Experiment 1

Effects of Pulse Treatment With Antihypertensive Agents on Systolic Blood Pressure in SHRs

The changes in systolic blood pressure in the different groups are shown in Figure 1. At age 16 weeks, before the initiation of the pulse treatment, hypertension had been fully established, ie, the blood pressure in the different groups had reached the plateau value of ≈220 mm Hg. Treatment with the different antihypertensive agents caused a decrease in blood pressure to ≈150 mm Hg during the duration of the 2-week pulse therapy. After discontinuation of the antihypertensive medication, the blood pressure rapidly reverted to control values in the CCB- and vasodilator-treated groups. In clear contrast, the blood pressure in the ACEI- and ARB-treated groups were maintained at values of ≤180 mm Hg (a difference of >30 to 40 mm Hg). Essentially similar results were found in groups 6 to 10, which had been exposed to a low-salt diet from age 16 to 18 weeks (Figure 1B).

Effects of Pulse Treatment With Antihypertensive Agents on Cardiovascular Hypertrophy, Parameters of Renal Function, and Plasma Renin Activity/Plasma Aldosterone Concentration at Age 36 Weeks in SHRs

As expected from the sustained decrease in blood pressure, the heart weight:body weight ratios, aortic media:lumen ratios, and renal arteriolar media:lumen ratios were decreased at the end of the study (age 36 weeks) in the rats previously treated with pulse ACEI or ARB (data not shown). No significant differences in blood urea nitrogen, plasma creatinine, plasma renin activity, plasma aldosterone concentration, or the oxidative marker plasma lipid peroxides were found in the different groups at age 36 weeks.

Experiment 2

Short-Term Effects of Pulse Treatment With ARB or CCB on Cardiovascular Hypertrophy and Parameters of Renal Function at Age 18 Weeks in WKY Rats and SHRs

In experiment 2, we examined the short-term effects of the 2-week pulse treatment on cardiovascular hypertrophy and

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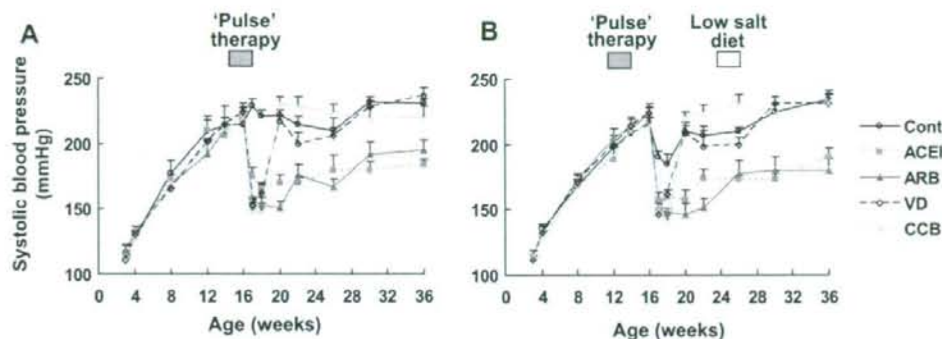

Figure 1. Effects of pulse treatment with antihypertensive agents, with and without a low-salt diet, on systolic blood pressure in SHRs. A, Results without a low-salt diet. B, Results with a low-salt diet. Cont indicates control; ACEI, treated with pulse enalapril; ARB, treated with pulse candesartan; VD, treated with pulse hydralazine; and CCB, treated with pulse nifedipine from age 16 to 18 weeks. Systolic blood pressures in the ACEI and ARB groups were significantly ($P<0.01$) decreased vs the Cont groups at all time points from age 20 weeks onward in both A and B (symbols have been omitted for the sake of clarity).

parameters of renal function in 4 groups of rats: normotensive WKY rats, control SHRs, and SHRs treated with either ARB or CCB for 2 weeks, and data were obtained immediately at the end of the pulse treatment (age 18 weeks). Pulse treatment with ARB was associated with a small decrease in heart weight:body weight ratios compared with CCB, but the results did not attain statistical significance. Similarly, aortic media:lumen ratios were not significantly changed in the rats treated with ARB or CCB. In contrast, the media:lumen ratios in the small (30 to 100 μm) arterioles were markedly reduced by the ARB pulse treatment but not by the CCB (Figure 2). Of interest, the decreases in media:lumen ratios were found to be specific for renal small arterioles and were not found in larger renal arterioles (100 to 300 μm) or arterioles from other tissues, namely, the mesentery, heart, and brain (Table S1). Urine albumin excretion was significantly decreased in the SHRs treated with ARB (WKY: 7.2±2.6 mg/d; SHR: 9.2±3.0 mg/d; SHR+ARB: 0.8±0.4 mg/d [$P<0.05$ vs SHR]; SHR+CCB: 3.6±1.2 mg/d), but no significant differences in blood urea nitrogen, plasma creatinine, or plasma lipid peroxides were found in the different groups.

Microarray Analysis of Differences in Renal Gene Expressions in SHRs Treated With Pulse ARB or CCB for 2 Weeks

The differences in expression of a total of 28 000 genes in the kidneys of SHRs treated with ARB or CCB were examined using the Affymetrix rat 230 2.0 gene expression array. A total of 1345 genes were elevated in the ARB-treated rats compared with the CCB-treated rats, whereas 5671 were reduced. Several extracellular matrix–related genes, including type IV procollagen and MMP-15, were elevated in the ARB-treated rats, whereas MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-2, and TIMP-3 gene expressions

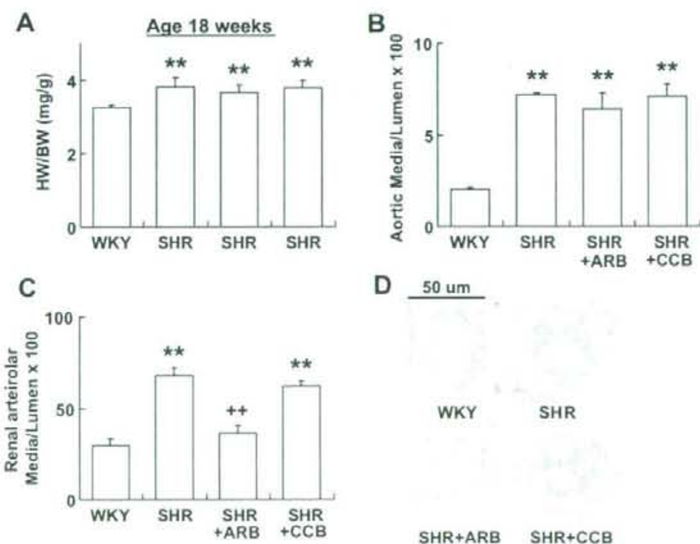

Figure 2. Short-term effects of pulse treatment with ARB or CCB on cardiovascular hypertrophy at age 18 weeks in SHRs. A, Heart weight:body weight ratios. B, Aortic media:lumen ratios. C, Media:lumen ratios of renal small arterioles (30 to 100 μm). D, Representative photomicrographs of renal arterioles in the different groups. WKY indicates untreated WKY rats; SHR, untreated SHRs; SHR+ARB, SHRs treated with pulse candesartan; SHR+CCB, SHRs treated with pulse nifedipine. **$P<0.01$ vs WKY rats; ++$P<0.01$ vs SHRs.

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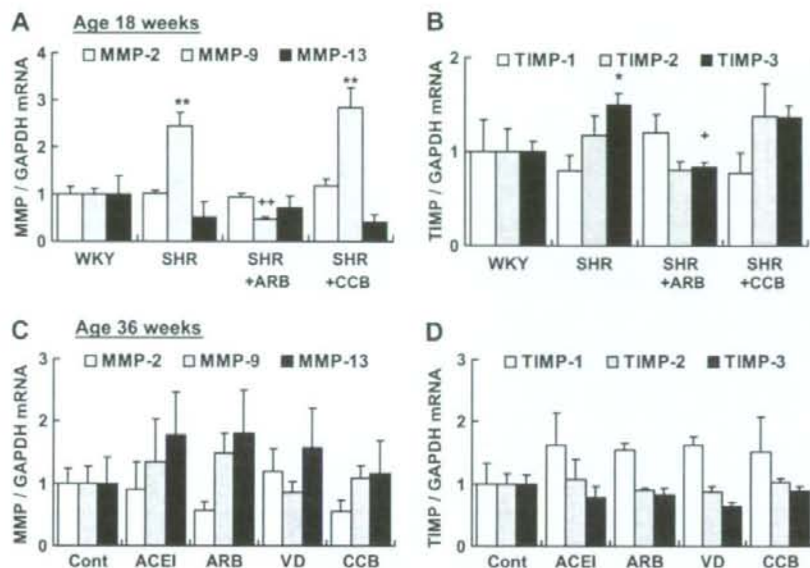

Figure 3. Effects of pulse treatment with ARB or CCB on renal gene expressions in SHRs. A, MMP-2, MMP-9, and MMP-13 mRNA at age 18 weeks. B, TIMP-1, TIMP-2, and TIMP-3 mRNA at age 18 weeks. C, MMP-2, MMP-9, and MMP-13 mRNA at age 36 weeks. D, TIMP-1, TIMP-2, and TIMP-3 mRNA at age 36 weeks. Abbreviations of groups as in Figures 1 and 2. *$P<0.05$, **$P<0.01$ vs WKY rats; +$P<0.05$, ++$P<0.01$ vs SHRs.

were decreased in the ARB-treated group (Table S2). Among the genes of the RAS, only renin mRNA was increased in the ARB-treated group, which was expected as a feedback response to inhibition of the RAS.

Real-Time RT-PCR Analysis of the Short-Term Effects of Pulse Treatment With ARB or CCB on Renal Gene Expressions at Age 18 Weeks in WKY Rats and SHRs

To confirm the results of the microarray analysis, the differences in the gene expression of MMP-2, MMP-9, MMP-13, TIMP-1, TIMP-2, and TIMP-3 were assessed by real-time RT-PCR. As shown in Figure 3, pulse treatment of SHRs with ARB caused a significant decrease in MMP-9 and TIMP-3 mRNA expression, whereas no significant effect was seen with CCB, findings that were consistent with the results of the microarray analysis. Values of MMP-13 mRNA expression were somewhat higher in the ARB-treated rats compared with CCB at the end of the pulse treatment (18 weeks), but the results did not attain statistical significance. No significant differences were found in any of these genes between the different groups at age 36 weeks.

Short-Term Effects of Pulse Treatment With ARB or CCB on Vascular MMP Activity and Expression at Age 18 Weeks in WKY Rats and SHRs

High-resolution, high-sensitivity in situ zymography was performed to examine the activity of MMPs in the renal microvasculature. As shown in Figure 4, degradation of type I collagen (type I collagenolytic activity) in the renal arterioles was found to be clearly increased in the SHRs treated with ARB but not with CCB. Parallel experiments were performed in the presence of an MMP-13 inhibitor, because MMP-13 is known to be the predominant MMP involved in degradation of type I collagen in the rat, which lacks the MMP-1 gene. The type I collagenolytic activity in the vasculature was inhibited by the MMP-13 inhibitor, confirming that the changes seen reflected MMP-13 activity. In the case of type IV collagenolytic activity, MMP-9–dependent degradation of type IV collagen was decreased in ARB-treated rats but not in the CCB-treated rats. Both type I and type IV collagenolytic activities were completely inhibited by the broad-spectrum MMP inhibitor 1-10 phenanthrolene (data not shown). Examination of MMP-13, MMP-2, and MMP-9 expression by immunofluorescence staining showed a similar trend to the results of in situ zymography (Figure 5).

Discussion

The main findings of this study were as follows: (1) pulse treatments with ARB and ACEI (with or without concomitant low-salt diet treatment) were equally effective in causing a long-term reduction in blood pressure; (2) the reductions in blood pressure were accompanied by long-term reductions in cardiac and vascular hypertrophy; (3) the pulse treatment caused a remarkable regression of renal arteriolar hypertrophy in the course of just 2 weeks; and (4) these changes were associated with changes in expression and activity of vascular MMPs in the kidney.

The fact that long-term reductions in blood pressure were seen with an ARB and ACEI but not with the CCB or vasodilator is of interest in view of the widespread use of these agents for the treatment of hypertension. Concerning the dose of ARB or ACEI required to obtain regression, we are performing a companion study to examine the effects of

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