Angiotensin II Reduces Mitochondrial Content in Skeletal Muscle and Affects Glycemic Control

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OBJECTIVE—Blockade of angiotensin (Ang) II has been shown to prevent new-onset type 2 diabetes. We focused on the effects of AngII on muscle mitochondria, especially on their biogenesis, as an underlining mechanism of type 2 diabetes.

RESEARCH DESIGN AND METHODS—C2C12 cells and C57bl/6 mice were used to examine roles for AngII in the regulation of muscle mitochondria and to explore whether the effect was mediated by type 1 AngII receptor (AT1R) or type 2 receptor (AT2R).

RESULTS—C2C12 cells treated with 10^{-8}–10^{-6} mol/l AngII reduced the mitochondrial content associated with downregulation of the genes involved in mitochondrial biogenesis. The action of AngII was diminished by blockade of AT2R but not AT1R, whereas overexpression of AT2R augmented the effect. AngII increased mitochondrial ROS and decreased mitochondrial membrane potential, and these effects of AngII were significantly suppressed by blockade of either AT1R or AT2R. Chronic AngII infusion in mice also reduced muscle mitochondrial content in association with increased intramuscular triglyceride and deteriorated glycemic control. The AngII-induced reduction in muscle mitochondria in mice was partially, but significantly, reversed by blockade of either AT1R or AT2R, associated with increased fat oxidation, decreased muscle triglyceride, and improved glucose tolerance. Genes involved in mitochondrial biogenesis were decreased via AT2R but not AT1R under these in vivo conditions.

CONCLUSIONS—Taken together, these findings imply the novel roles for AngII in the regulation of muscle mitochondria and lipid metabolism. AngII reduces mitochondrial content possibly through AT1R-dependent augmentation of their degradation and AT2R-dependent direct suppression of their biogenesis. *Diabetes* 58:710–717, 2009

Recent studies have shown that mitochondrial content and function are significantly reduced in the skeletal muscle of patients with type 2 diabetes (1,2). Percutaneous biopsy of vastus lateralis muscle has revealed that subsarcolemmal mitochondria, which are believed to be crucial for glucose transport and fatty acid oxidation, were decreased in type 2 diabetic patients, compared with body weight–matched nondiabetic patients (2). Moreover, reduced mitochondrial content and function in muscle have been also observed in pre-diabetic subjects with a family history of type 2 diabetes (3). Recent microarray analyses have revealed that expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation is coordinately decreased in the skeletal muscle of patients with type 2 diabetes (4,5), for example, peroxisome proliferator–activator receptor γ co-activator 1α (PGC1α), a representative transcriptional cofactor for the determination of mitochondrial content and function, and nuclear respiratory factor 1 (NRF1). Furthermore, it has been demonstrated that mitochondrial function evaluated by the rate of ATP synthesis is diminished in the skeletal muscle of diabetic patients and family history–positive pre-diabetic patients (6,7). These findings imply that reduced mitochondrial content in the skeletal muscle is likely to contribute to the development of insulin resistance and type 2 diabetes (8,9).

Angiotensin (Ang) II, which is composed of eight amino acids, is one of the most important molecules in the renin-angiotensin system. It provokes sodium reabsorption, vasoconstriction, and elevation of blood pressure and also plays a critical role in the physiological regulation of electrolytes and water homeostasis. However, an excess of AngII may lead to tissue damage, such as atherosclerosis, cardiomegaly, and heart and renal failure. AngII is known to exert its biological effects via two functional receptors, type 1 and type 2 angiotensin II receptors (AT1R and AT2R, respectively). To date, most of the known cardiovascular effects of AngII are believed to be attributable to AT1R (10). Recent large-scale clinical trials, including HOPE (Heart Outcomes Prevention Evaluation), LIFE (Losartan Intervention for Endpoint), CHARM (Candesartan in Heart Failure—Assessment of Mortality and Morbidity), and VALUE (Valsartan Antihypertensive Long-term Use Evaluation), have demonstrated that ACE inhibitors or angiotensin II receptor blockers (ARB) prevent new onset of type 2 diabetes via their ability to attenuate AngII signaling (11). As a result of these findings, the significance of AngII for the development of insulin resistance and regulation of energy metabolism has been attracting considerable attention (12–14).

It has been demonstrated that AngII provokes insulin resistance in the skeletal muscle through multiple mechanisms. AngII treatment was found to augment reactive oxygen species (ROS) production by stimulating NADPH oxidase in cultured skeletal muscle cells, thus activating multiple redox-sensitive signaling including nuclear factor-κB (NF-κB) and increasing proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), which impair insulin action (15,16). Inhibition of insulin signaling by AngII at multiple levels including insulin receptor, insulin receptor substrate 1, and phosphatidylinositol 3-kinase has been demonstrated in aortic smooth muscle cells (17). Stimulation of primary cultured human preadipocytes by AngII was found to inhibit differentiation to mature adipocytes, suggesting that the effect of AngII on adipose tissue

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.
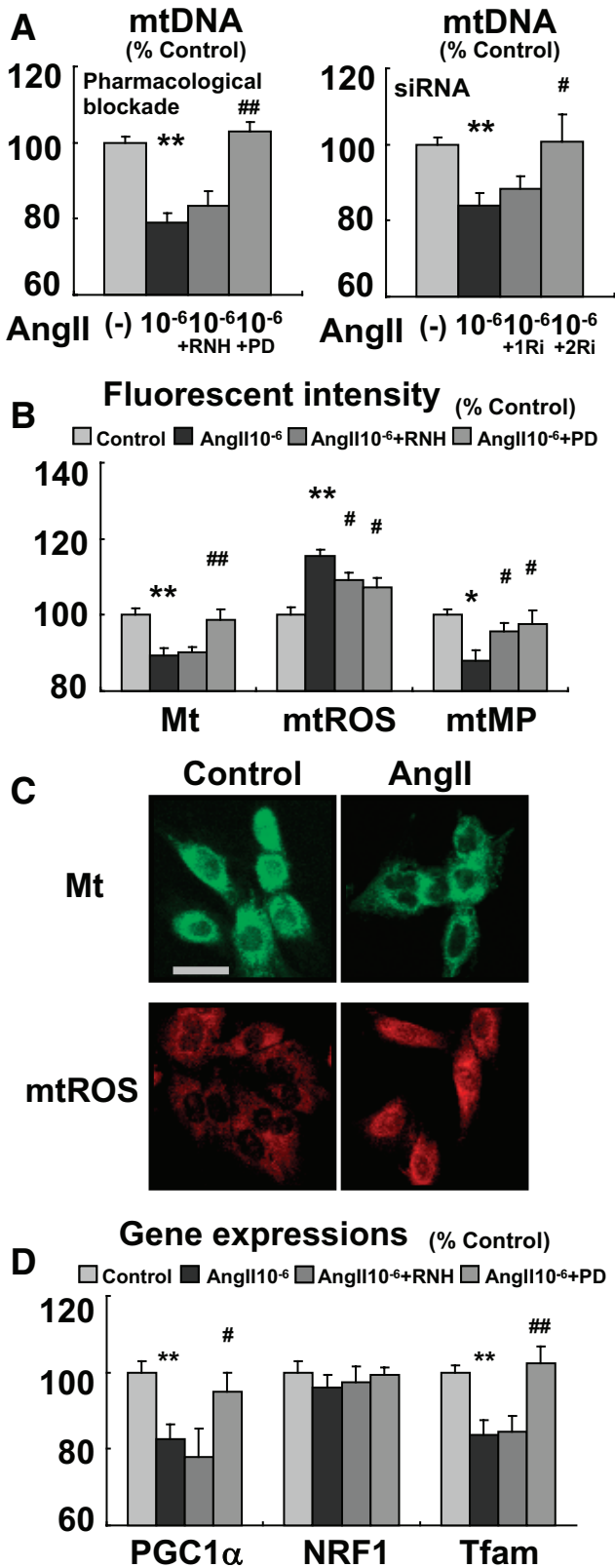
Animal experiments. All animal experiments were performed in accordance with the animal care and use guidelines of Keio University. Male C57bl/6 mice (Nihon Clea, Tokyo, Japan) were maintained under specific pathogen-free conditions, and exogenous AngII was infused at a dose of 0.1 μg/g body
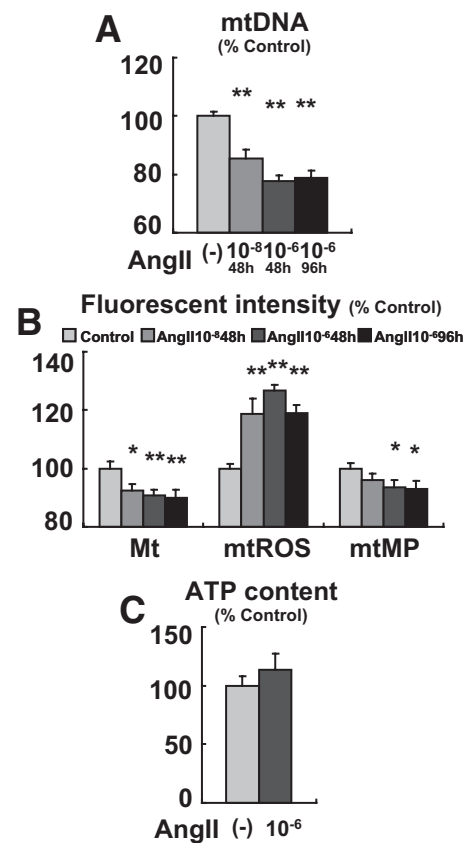
FIG. 1. AngII reduces mitochondrial content in association with an increase in mitochondrial ROS production in C2C12 cells. C2C12 cells were incubated with or without AngII (at the concentrations indicated: 10^{-8} or 10^{-6} mol/l) for 48 or 96 h. *A*: Mitochondrial DNA copy number (mtDNA) estimated 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.

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 (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.

A

mtDNA
(% Control)

mtDNA
(% Control)

B

Fluorescent intensity (% Control)

□ Control ■ AngII10⁻⁶ ■ AngII10⁻⁶+RNH ■ AngII10⁻⁶+PD

C

Control   AngII

Mt

mtROS

D

Gene expressions (% Control)

□ Control ■ AngII10⁻⁶ ■ AngII10⁻⁶+RNH ■ AngII10⁻⁶+PD

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

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. 1A) 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. 1B), in association with a significant increase in mitochondrial ROS (mtROS) (27% increase, Fig. 1B). Mitochondrial membrane potential (mtMP) was lowered (6% decrease, Fig. 1B) by AngII, whereas cellular ATP content was not significantly changed (Fig. 1C).

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. 2A 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. 2A). 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. 2B). Using a confocal microscope, we were able to confirm that the fluorescent probes were distributed specifically in mitochondria (Fig. 2C).

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. 2D); however, expression of NRF1 was not affected by AngII. Consistent with its effects on mitochon-

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.)
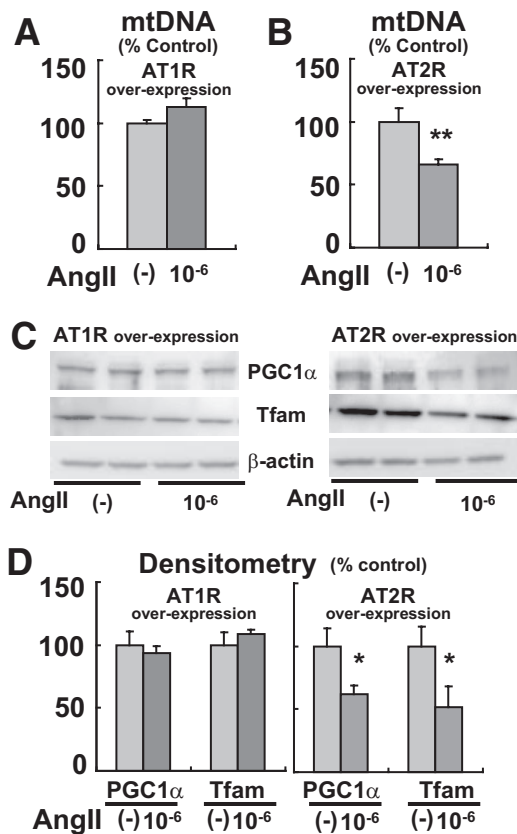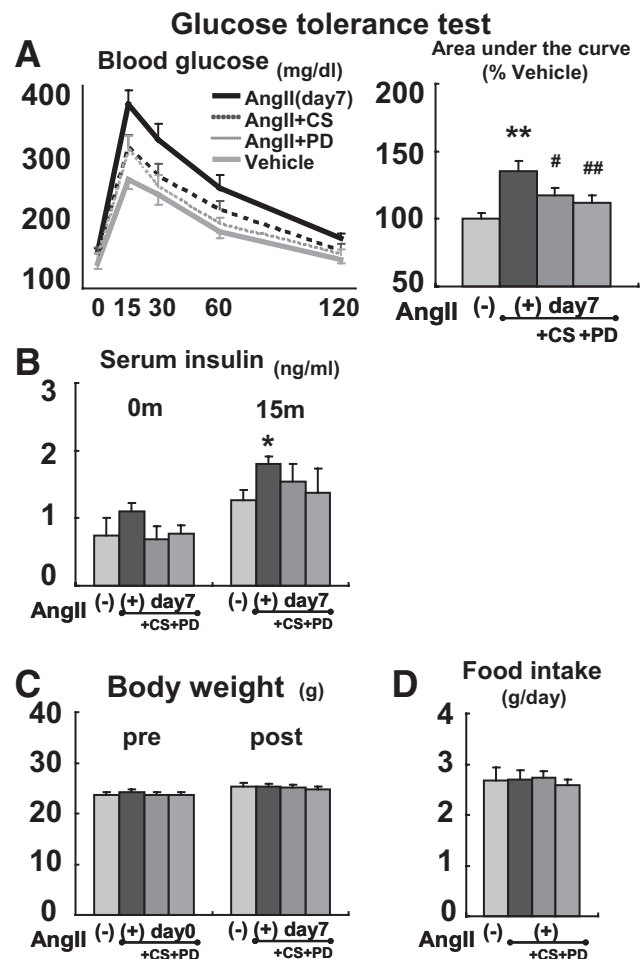
FIG. 3. Overexpression of AT2R in C2C12 cells augments AngII-induced reduction in mitochondrial biogenesis. Angiotensin II receptors (AT1R or AT2R) were overexpressed in C2C12 cells by CAG promoter–driven expression vectors previous to the treatment with AngII (10^{-6} mol/l) for 48 h (A–D). Mitochondrial 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.

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.

drial content (Fig. 2A 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. 2D).

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. 3A–D). In the AT1R overexpression group, mtDNA was not significantly changed as a result of treatment with AngII (Fig. 3A); 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. 3B). Western blot analysis confirmed that PGC1α and Tfam protein levels in the AT2R overexpression group were significantly diminished by AngII (Fig. 3C and D). These results were also compatible with those for

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

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. 4A), 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.
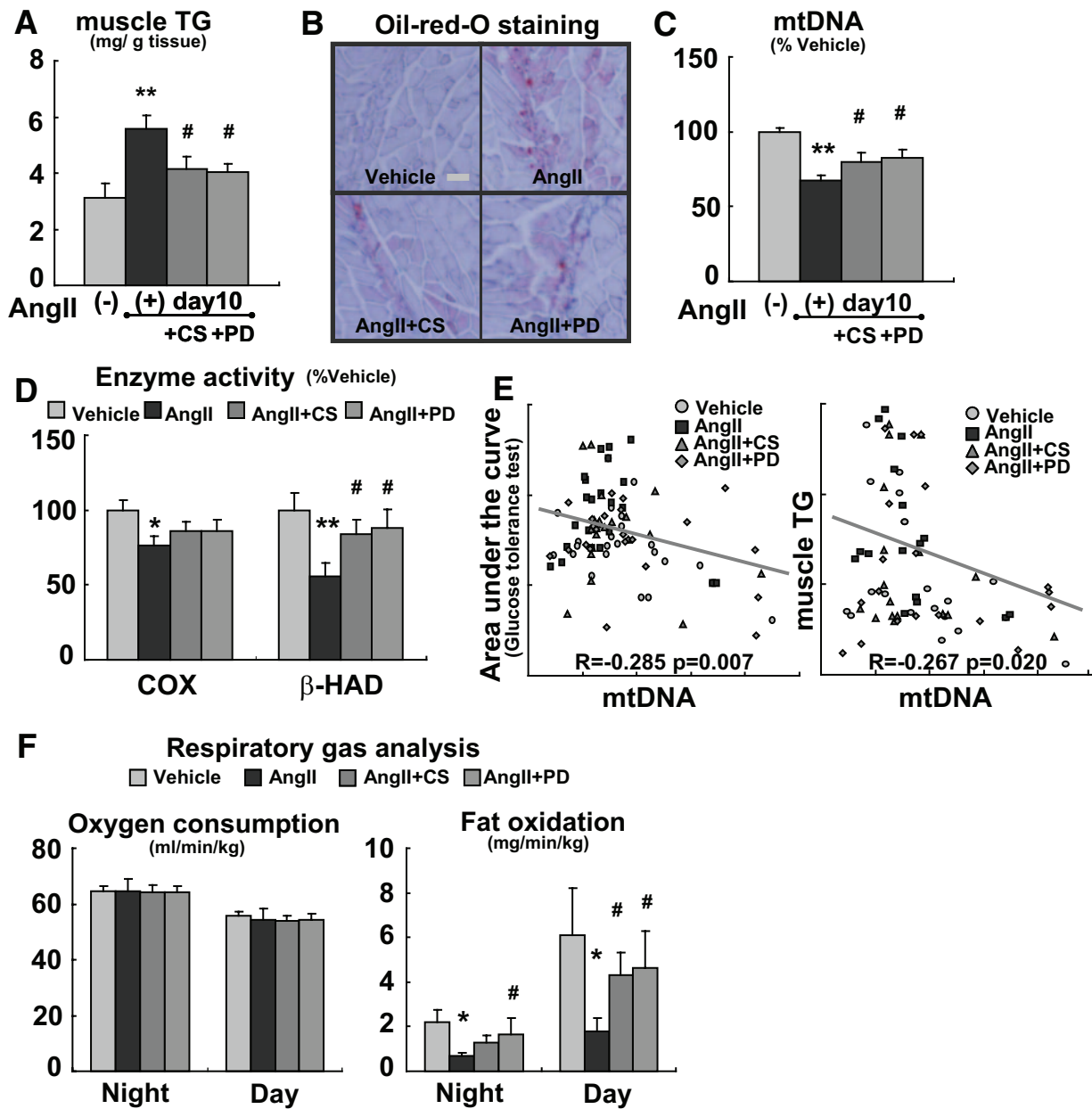
FIG. 5. AngII reduces mitochondrial content and increases triglyceride (TG) content in the skeletal muscle in both AT1R- and AT2R-dependent manners. *A*: Muscle triglyceride content (muscle triglyceride) measured in the quadriceps (*n* = 8 per group). *B*: Histological analysis of AngII-induced lipid 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

A Gene expressions (%Vehicle)

B

C Densitometry (%Vehicle)
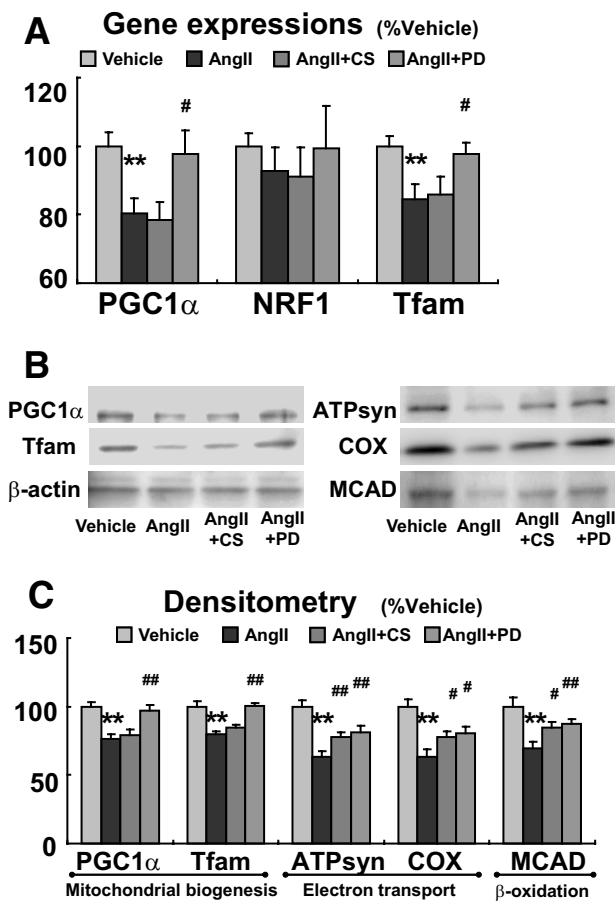
FIG. 6. Blockade of AT2R but not AT1R in AngII-infused mice reverses reduction in expression and protein levels of molecules involved in mitochondrial biogenesis. *A*: Expression of genes involved in mitochondrial biogenesis in the skeletal muscle of the mice ($n = 12$ per group). *B*: Western blots 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 densitom-

etry 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.

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