Evidence for the Importance of Adiponectin in the Cardioprotective Effects of Pioglitazone

Ping Li, Rei Shibata, Kazumasa Unno, Masayuki Shimano, Mayuko Furukawa, Taiki Ohashi, Xianwu Cheng, Kohzo Nagata, Noriyuki Ouchi, Toyoaki Murohara

Abstract—The favorable effects of the peroxisome proliferator-activated receptor-γ ligand pioglitazone on glucose metabolism are associated with an increase in the fat-derived hormone adiponectin in the bloodstream. A recent clinical trial, Prospective Pioglitazone Clinical Trial in Macrovacular Events, demonstrated that pioglitazone improved cardiovascular outcomes in patients with type 2 diabetes mellitus. However, the functional role of adiponectin in cardioprotection by pioglitazone has not been examined experimentally. Here we investigated the effect of pioglitazone on angiotensin II (Ang II)–induced cardiac hypertrophy and assessed the potential contribution of adiponectin to the action of pioglitazone on the heart. Wild-type or adiponectin-deficient mice were treated with pioglitazone as food admixture at a concentration of 0.01% for 1 week followed by 2 weeks of infusion with Ang II at 3.2 mg/kg per day. Ang II infusion in wild-type mice resulted in exacerbated myocyte hypertrophy and increased interstitial fibrosis, which were accompanied by elevated phosphorylation of extracellular signal-regulated kinase and expression of transforming growth factor-β1 in the heart. Treatment of wild-type mice with pioglitazone attenuated cardiac hypertrophy and fibrosis, extracellular signal-regulated kinase phosphorylation, and transforming growth factor-β1 expression in response to Ang II. Pioglitazone also increased the plasma adiponectin level and phosphorylation of cardiac AMP-activated protein kinase in wild-type mice in the presence of Ang II. The suppressive effects of pioglitazone on Ang II–induced cardiac hypertrophy and fibrosis were diminished in adiponectin-deficient mice. Furthermore, pioglitazone had no effects on the phosphorylation of extracellular signal-regulated kinase and AMP-activated protein kinase in the Ang II–infused heart of adiponectin-deficient mice. These data provide direct evidence that pioglitazone protects against Ang II–induced pathological cardiac remodeling via an adiponectin-dependent mechanism. (Hypertension. 2010;55:69-75.)

Key Words: pioglitazone ■ adiponectin ■ cardiac hypertrophy ■ fibrosis ■ angiotensin II

The renin-angiotensin system plays a key role in cardiovascular homeostasis by regulating blood pressure (BP) and cardiac function. Angiotensin II (Ang II) is an important component of the renin-angiotensin system and functions as a crucial regulator of myocyte hypertrophy, inflammation, and fibrosis, as well as vascular tone. Clinically, pharmacological blockade of the renin-angiotensin system reduces the risk for the development of type 2 diabetes mellitus. It has been shown that the Ang II receptor antagonist improves insulin resistance in obese diabetic rats. Furthermore, Ang II is reported to antagonize insulin signaling in liver and skeletal muscle. Thus, Ang II contributes to the genesis of myocardial remodeling and insulin resistance.

Adiponectin is an adipose-derived hormone for which the concentration is downregulated in subjects with obesity-related disorders. Low adiponectin levels are associated with the increased prevalence of type 2 diabetes mellitus.
hypertension, and ischemic heart disease. Although adiponectin-deficient (APN-KO) mice are apparently normal at the basal nonstressed state, they develop exacerbation of insulin resistance on a high-fat/high-sucrose diet and enhancement of cardiac hypertrophy caused by pressure overload or Ang II treatment. Experimental studies also indicate that adiponectin displays protective actions on the cardiovascular systems by directly acting on the component cells in the heart and blood vessels.

Pioglitazone has been shown to increase the expression and secretion of adiponectin by activating peroxisome proliferator-activated receptor-γ in adipocytes, leading to elevated levels of circulating adiponectin. The favorable effects of pioglitazone on glucose metabolism in patients with type 2 diabetes mellitus are associated with an increase in the plasma concentration of adiponectin. In this regard, the pioglitazone-induced improvement of insulin resistance is partly dependent on the ability of adiponectin to improve insulin sensitivity. On the basis of the above-mentioned observations, we hypothesized that pioglitazone protects against the development of myocardial hypertrophy caused by Ang II through the regulation of adiponectin levels. Here we investigated the effect of pioglitazone on Ang II–induced cardiac remodeling and assessed the possible involvement of adiponectin in the cardioprotective action of pioglitazone.

Methods
Methods in detail are described in the online Data Supplement. Please see the supplemental Methods section at http://hyper.ahajournals.org.

Animals and Experimental Protocol
Male wild-type (WT) mice and APN-KO mice in a C57BL/6J background at the age of 8 to 10 weeks were used in the present study. The study protocol was approved by the Nagoya University School of Medicine Institutional Animal Care and Use Committee. WT and APN-KO mice were divided into 4 groups, as follows: mice treated with pioglitazone; mice treated without pioglitazone; Ang II–infused mice treated with pioglitazone; and Ang II–infused mice treated without pioglitazone. Treatment with pioglitazone was initiated 1 week before Ang II infusion and continued for 3 weeks as food admixture at a concentration of 0.01%. Ang II was administered subcutaneously with osmotic minipumps (Durect Corporation) at a dose of 3.2 mg/kg per day. At 14 days after Ang II infusion, heart rate and systolic BP were determined using a tail-cuff pressure analysis system (BP-98A; Softron) with mice in the conscious state. All of the mice survived during the observation period.

Results
Effect of Pioglitazone on Ang II–Induced Cardiac Hypertrophy
Systolic BP was significantly increased in response to Ang II infusion in WT mice at 14 days. Treatment with pioglitazone did not affect the Ang II–induced increased systolic BP (Table S1, available in the online Data Supplement). Ang II infusion for 14 days increased the ratio of heart weight to body weight (BW), and treatment with pioglitazone attenuated the increased HW/BW ratio in response to Ang II infusion in WT mice (Table S1). There were no significant differences in BP, HW/BW, or heart rate between pioglitazone-treated mice and nontreated mice without the Ang II infusion. Ang II infusion and pioglitazone treatment had no effects on glucose, insulin, and homeostasis model assessment-insulin resistance levels in WT mice. Echocardiographic analysis revealed that the interventricular septum thickness and left ventricular posterior wall thickness were significantly increased in response to Ang II infusion in WT mice (Figure 1A and Table S2). Treatment with pioglitazone attenuated the increase in interventricular septum thickness and left ventricular posterior wall thickness. Pioglitazone treatment did not affect the interventricular septum thickness and left ventricular posterior wall thickness in WT mice without the Ang II infusion. There were no significant differences in BP, HW/BW, or heart rate between pioglitazone-treated mice and nontreated mice without the Ang II infusion. Microscopic analysis revealed that the cross-sectional area of cardiac myocytes was increased 60% under Ang II infusion (Figure 1B and 1C).

![Image](https://hyper.ahajournals.org/)

Figure 1. Pioglitazone attenuated Ang II–induced cardiac hypertrophy in WT mice. A, Representative M-mode echocardiogram for WT mice treated with or without pioglitazone at 14 days after Ang II infusion. B, Hematoxylin-eosin staining of the heart tissues from WT mice treated with or without pioglitazone at 14 days after Ang II infusion. C, Quantitative analysis of cardiac myocyte cross-sectional area (µm²) in WT mice treated with or without pioglitazone at 14 days after Ang II infusion. D, Phosphorylation of ERK in heart tissues from WT mice treated with or without pioglitazone at 7 days after Ang II infusion. Phosphorylation of ERK was normalized to the GAPDH signal and was expressed as a percentage of the signal intensity of WT mice.
Treatment with pioglitazone significantly attenuated the Ang II–induced increase in myocyte cross-sectional area by 15%. The activation of extracellular signal-regulated kinase (ERK) is an important mediator of myocyte hypertrophy both in vitro and in vivo. Thus, we assessed the phosphorylation of ERK by Western blot analysis. Ang II infusion resulted in a significant increase in ERK phosphorylation in the WT hearts, whereas pioglitazone treatment significantly attenuated Ang II–induced ERK phosphorylation (Figure 1D).

Effect of Pioglitazone on Ang II–Induced Cardiac Fibrosis
Masson trichrome staining revealed that interstitial fibrosis in the left ventricular myocardium of WT mice was increased in response to Ang II infusion. This increase in the extent of cardiac fibrosis was significantly inhibited by treatment with pioglitazone (Figure 2A and 2B). We next measured mRNA levels of genes related to cardiac fibrosis by real-time RT-PCR analysis. The mRNA levels of collagen I and III in the left ventricular tissues of WT mice were increased after Ang II infusion. Pioglitazone treatment significantly reduced collagen I and III mRNA expression of transforming growth factor-β (TGF-β1) in the myocardium of WT mice, which was suppressed by treatment with pioglitazone (Figure 2E).

Effect of Pioglitazone on Plasma Adiponectin Levels and Cardiac AMP-Activated Protein Kinase
Pioglitazone has been shown to increase plasma levels of adiponectin. In addition, we have shown that adiponectin modulates cardiac remodeling in response to Ang II infusion. These findings led us to hypothesize that the increase in circulating adiponectin levels contributes to improved cardiac hypertrophy and fibrosis in the pioglitazone-treated animals. We assessed plasma adiponectin levels in each group. Ang II infusion did not affect the plasma concentration of adiponectin in WT mice. Treatment with pioglitazone resulted in a 1.6-fold increase in this parameter independent of Ang II infusion (Figure 3A). Ang II did not affect protein expression of adiponectin in the WT hearts (Figure S1). Pioglitazone treatment significantly increased adiponectin protein levels in the Ang II–infused hearts but not in control hearts (Figure S1).

Because adiponectin functions as an AMP-activated protein kinase (AMPK) activator in the heart, the phosphorylation of AMPK was assessed by Western blotting. Ang II infusion significantly increased AMPK phosphorylation levels in the WT hearts (Figure 3B). Ang II–stimulated AMPK phosphorylation in the hearts of WT mice was further enhanced by pioglitazone treatment, whereas pioglitazone did not affect the phosphorylation of AMPK without Ang II infusion.

Adiponectin Is Essential for Cardioprotective Effects of Pioglitazone
To examine the involvement of adiponectin in the cardioprotective effects of pioglitazone, we investigated the effect of pioglitazone on Ang II infusion–induced cardiac hypertrophy and fibrosis in APN-KO mice. The increase in BP after Ang II infusion was similar in WT and APN-KO mice. Direct measurement of BP by Millar catheter also revealed that BP...
at day 14 after Ang II infusion did not differ between the 2 strains (129.0±2.1 mm Hg in WT versus 133.0±3.5 mm Hg in APN-KO mice). Treatment of APN-KO mice with pioglitazone did not affect the Ang II–induced increase in BP (Table S1). APN-KO mice subjected to Ang II infusion exhibited increased HW/BW compared with WT mice, and the increased HW/BW in APN-KO mice was not attenuated by treatment with pioglitazone (Table S1). Glucose, insulin, and homeostasis model assessment-insulin resistance levels in APN-KO mice did not differ among the 4 different treatment groups. Echocardiographic measurement at 14 days after Ang II infusion showed increased intraventricular septal thickness and left ventricular posterior wall thickness in APN-KO mice as compared with WT mice. Treatment with pioglitazone did not affect the increase in intraventricular septal thickness and left ventricular posterior wall thickness in response to Ang II infusion in APN-KO mice (Table S2 and Figure 4A). Left ventricular end diastolic dimension, left ventricular end systolic dimension, and fractional shortening in APN-KO mice in the absence of Ang II were not affected by treatment with pioglitazone (Table S2). A greater increase in the myocyte cross-sectional area after Ang II infusion was
dependent mechanism. The cardioprotective actions via adiponectin
of pioglitazone on ERK activation and TGF-
phosphorylation in the myocardium was attenuated in APN-KO mice
after Ang II infusion. Ang II–induced ERK phosphorylation
(Figure 5C).

cardiac AMPK activation in Ang II–treated APN-KO mice
pioglitazone-treated WT mice, pioglitazone had no effects on
induced AMPK phosphorylation was enhanced in
APN-KO mice (Figure 5A and 5B). Although Ang II–
stimulated ERK activation and TGF-
expression were enhanced in APN-KO mice as
compared with the results for WT mice. AMPK phosphorylation
in the myocardium was attenuated in APN-KO mice
compared with WT mice. In contrast to the inhibitory effects of
pioglitazone on ERK activation and TGF-
expression in Ang II–treated WT mice, pioglitazone did not influence Ang
II–stimulated ERK activation and TGF-
expression in APN-KO mice (Figure 5A and 5B). Although Ang II–
induced AMPK phosphorylation was enhanced in
pioglitazone-treated WT mice, pioglitazone had no effects on
cardiac AMPK activation in Ang II–treated APN-KO mice
(Figure 5C).

Discussion
The present study provides evidence that pioglitazone protects against Ang II–induced pathological cardiac remodeling in a mouse model. Treatment of WT mice with pioglitazone attenuated Ang II–induced cardiac hypertrophy and fibrosis without lowering BP, which was accompanied by upregulation of adiponectin. The beneficial action of pioglitazone on cardiac remodeling was abolished under conditions of ablation of adiponectin. Because overexpression of adiponectin attenuates Ang II–induced cardiac hypertrophy, pioglitazone exerts the cardioprotective actions via an adiponectin-dependent mechanism.

We and other groups have shown that adiponectin is protective against the development of cardiac remodeling under various pathological conditions. Ablation of adiponectin causes severe concentric cardiac hypertrophy in response to pressure overload caused by aortic constriction. Supplementation of adiponectin attenuates cardiac hypertrophy and fibrosis caused by Ang II infusion in APN-KO and WT mice. Adiponectin deficiency also contributes to impaired cardiac function after ischemia-reperfusion injury. Furthermore, APN-KO mice develop exacerbated left ventricular dilation and contractile dysfunction after myocardial infarction, which was accompanied by myocyte hypertrophy and increased interstitial fibrosis. Pioglitazone has been shown to attenuate detrimental cardiac remodeling after pressure overload and myocardial infarction. Our observations here show that pioglitazone significantly increased plasma adiponectin levels and that the protective actions of pioglitazone in the heart were abolished in APN-KO mice. Thus, the upregulation of adiponectin by pioglitazone treatment could represent a common mechanism in the cardioprotection by this reagent.

Our previous work has shown that adiponectin transcript levels in an injured heart are lower by a factor of 33 000 compared with those in adipose tissue and that adiponectin accumulates in ischemic injured hearts mainly through leakage from the vessel compartment, as determined by immunohistochemical and Western blot analyses. Although we could not detect the appreciable increase in adiponectin protein in Ang II–infused heart tissue of WT mice by Western blotting, it has been reported that accumulation of adiponectin protein is observed in the myocardium of WT mice after Ang II infusion by immunohistochemistry. Furthermore, it was shown that adiponectin colocalizes with myocardial collagen type III, a major collagen in the cardiac extracellular matrix. Thus, it is conceivable that adiponectin accumulates in Ang II–infused hearts by binding collagens. In the present study, pioglitazone treatment increased the expression of adiponec-
tin protein in Ang II–infused hearts of WT mice. In contrast, pioglitazone did not affect mRNA levels of adiponectin in the myocardium of WT hearts after Ang II treatment (data not shown). These data suggest that pioglitazone enhances the affinity of adiponectin to damaged hearts, thereby contributing to the favorable effects on the heart.

ERK activation is implicated in the hypertrophic response of cardiomyocytes to Ang II. Recently, we have reported that pioglitazone attenuates cardiac hypertrophy and ERK activation and promotes AMPK phosphorylation in the heart in hypertensive rats. We have also shown that adiponectin stimulates the phosphorylation of AMPK and suppresses agonist-stimulated ERK activation and hypertrophic response in cultured cardiac myocytes through its ability to activate AMPK signaling. Consistent with these observations, the current studies show that treatment of WT mice with pioglitazone stimulated AMPK activation and attenuated ERK phosphorylation in the presence of Ang II, which was accompanied by elevated adiponectin levels. Of note, the abilities of pioglitazone to modulate myocardial signals were abrogated in APN-KO mice. Taken together, these data supported the antihypertrophic effect of pioglitazone is attributed to reduced ERK activation that is involved in the adiponectin-AMPK regulatory axis.

TGF-β1 is a key regulator of extracellular matrix synthesis. Blockade of TGF-β1 reduces myocardial fibrosis induced by aortic constriction and suppresses inflammatory responses in the heart and vessels caused by chronic inhibition of NO synthesis. Recently, it was reported that pioglitazone attenuates high-salt diet-induced cardiac fibrosis and TGF-β1 expression in rat models. Pioglitazone is also shown to attenuate liver fibrosis caused by carbon tetrachloride in WT mice and renal fibrosis in the rat model of type 2 diabetes mellitus. In the current study, treatment with pioglitazone attenuated Ang II–induced interstitial fibrosis and expression of TGF-β1 and collagen I and III in WT mice, and the antifibrotic action of pioglitazone was diminished in mice lacking adiponectin. It is reported that adiponectin supplementation attenuates cardiac fibrosis after Ang II infusion and permanent coronary ligation. Ablation of adiponectin also causes extensive liver fibrosis with an enhanced expression of TGF-β1 after carbon tetrachloride–induced hepatic injury. Furthermore, adiponectin deficiency contributes to exacerbated fibrosis and an elevated level of TGF-β1 in the kidney after subtotal renal resection. Collectively, pioglitazone-mediated increase in adiponectin may be implicated in the suppression of fibrosis in various tissues.

Perspectives

Our observations show that pioglitazone could modulate cardiac remodeling in response to Ang II through stimulation of adiponectin levels, suggesting that adiponectin plays an essential role in the cardioprotective effects of pioglitazone. Therapeutic approaches aimed at increasing adiponectin production using pioglitazone could be beneficial for the treatment of cardiovascular disease.

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Disclosures

None.

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Evidence for the importance of adiponectin in the cardioprotective effects of pioglitazone

Short Running Title: Pioglitazone and cardiac remodeling

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E-mail: nouchi@bu.edu
Materials and Methods

Materials
Ang II was purchased from Sigma Chemical Co, St (Louis, Missouri, USA). Phospho-AMPK (Thr172), pan-α-AMPK and phospho-p42/44ERK (Thr202/Tyr 204) and total ERK antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody was purchased from Biogenesis Inc. Pioglitazone was provided as a generous gift by Takeda Pharmaceutical Company Limited (Tokyo, Japan).

Echocardiographic analysis
To measure left ventricular (LV) wall thickness and chamber dimensions, echocardiography was performed with an Apio SSA-700A machine using a 15-Mhz probe (Toshiba, Tochigi, Japan). After a good quality 2-dimensional image was obtained, M-mode images of the LV dimension and wall thickness were measured.

Histological analysis
Heart tissues were obtained at 14 days after Ang II infusion. Tissue samples were embedded in OCT compound (Miles, Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. 5µm tissue slices were prepared and stained either with hematoxylin-eosin for evaluation of myocyte cross-sectional area or with Masson trichrome for evaluation of the extent of tissue fibrosis. The shortest transverse myocyte cross sectional area was measured in 200 nucleated transverse sections of myocytes in each tissue section. To quantify the percent fibrosis area, the blue pixel content of digitized images was measured relative to total tissue area using the image analyzer Win ROOF (Mitani Corp.). Blood vessels and perivascular interstitial tissues were excluded from fibrosis quantification.

Measurement of plasma parameters
Blood samples were collected from mice after an overnight fast at 14 days after Ang II infusion. Plasma adiponectin levels were determined using ELISA kits (Otsuka Pharmaceutical Co Ltd, Tokyo, Japan). Glucose levels were measured with enzymatic kits (Wako Chemicals, Richmond, Virginia, USA). Insulin levels were measured with EIA kit (Wako Chemicals, Richmond, Virginia, USA). HOMA-IR (homeostasis model assessment insulin resistance index) values were calculated as described¹.
Western blot analysis
Tissue samples obtained at 7 days after Ang II infusion were homogenized in lysis buffer containing 20mM tris-HCl (pH 8.0), 1% NP-40, 150mM NaCl, 0.5% deoxycholic acid, 1mM sodium orthovanadate, and protease inhibitor cocktail (Sigma Chemical Co, St. Louis, Missouri, USA). Identical amounts of protein were separated with denaturing SDS 10% polyacrylamide gels. The membranes were immunoblotted with the primary antibodies at a 1:1000 dilution followed by secondary antibody at a 1:5000 dilution. Bands were visualized using ECL Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Real-time reverse transcriptase-polymerase chain reaction
Total RNA from heart was isolated with the use of guanidium isothiocyanatephenol chloroform solution (TRIzol reagent, Invitrogen Life Technologies). The cDNA was produced using oligo-dT primer and superscript II reverse transcriptase (superscript II, Invitrogen Life Technologies). Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) was performed using 1µg cDNA on Mx3000P Real-Time PCR System (Stratagene) using SYBR Green I as a double-stranded DNA-specific dye according to manufacture’s instruction (Applied Biosystem). Primers were as follows: forward 5’-GTCCCAACCCCAAGAC-3’ and reverse 5’-CAGCTTCTGAGTTTGATGAT-3’ for mice collagen I; forward 5’-TGGTTTCTTCTCACCCTTCTT-3’ and reverse 5’-TGCATCCCAATTCACTTACGT-3’ for mice collagen III; forward 5’-CACCGGAGAGCCTGGATA-3’ and reverse 5’-TTCCAACCCAGGTCTT CCT-3’ for mice TGF-β1.

Statistical analysis
Data are presented as means±SEM. All of the data were subjected to one-way ANOVA followed by Scheff’s analysis for comparison between any two means. Statistical significance was also evaluated using ANOVA for comparison among four or eight groups. P values <0.05 were considered to be statistically significant.
References

Table S1: Characteristics in WT and APN-KO mice at 14 days after Ang II infusion

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<th>APN-KO</th>
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<td></td>
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<tr>
<td>HR (bpm)</td>
<td>542±22</td>
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<td>SBP (mmHg)</td>
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<td>132.2±3.1*</td>
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<td>HW/BW (mg/g)</td>
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<td>Glucose (mg/dl)</td>
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<td>Insulin (ng/ml)</td>
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<td>5.65±0.69</td>
<td>6.03±0.38</td>
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Ang II, angiotensin II; WT, wild type; APN-KO, adiponectin knockout mice; Pio, pioglitazone; BW, body weight; HR, heart rate; SBP, systolic blood pressure; HW, heart weight; HOMA-IR, homeostasis model assessment insulin resistance index; Results are presented as mean ± SEM. *p<0.001 vs. WT Ang II (-) Pio (-); †p<0.05 vs. WT Ang II (+) Pio (-); ‡p<0.001 vs. APN-KO Ang II (-) Pio (-); §p<0.01 vs. WT Ang II (+) Pio (+).
Table S2: Echocardiographic measurements in WT and APN-KO mice at 14 days after Ang II infusion

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<th>APN-KO</th>
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<tr>
<td></td>
<td>Ang II (-)</td>
<td>Ang II (+)</td>
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<tr>
<td>IVS (mm)</td>
<td>Pio(-) (n=5) 0.72±0.02</td>
<td>Pio(+) (n=5) 0.73±0.02</td>
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<tr>
<td>LVPW (mm)</td>
<td>0.76±0.03</td>
<td>0.73±0.02</td>
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<td></td>
<td>2.86±0.07</td>
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<td></td>
<td>1.24±0.08</td>
<td>1.21±0.10</td>
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<td></td>
<td>56.6±2.56</td>
<td>57.1±3.25</td>
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<tr>
<td>FS (%)</td>
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<td>57.1±3.22</td>
<td>57.5±2.65</td>
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Ang II, angiotensin II; WT, wild type; APN-KO, adiponectin knockout mice; Pio, pioglitazone; BW, body weight; HR, heart rate; SBP, systolic blood pressure; HW, heart weight; IVS, interventricular septum; LVPW, left ventricular posterior wall; LVDd, left ventricular end diastolic dimension; LVDs, left ventricular end systolic dimension; FS, fractional shortening. FS was calculated as (LVED-LVES)/LVEDx100 and expressed as a percentage. Results are presented as mean ± SEM. *p<0.001 vs. WT Ang II (-) Pio (-); †p<0.05 vs. WT Ang II (+) Pio (-); ‡p<0.001 vs. APN-KO Ang II (-) Pio (-); §p<0.01 vs. WT Ang II (+) Pio (+)
Figure S1. Adiponectin in heart tissues from WT mice treated with or without pioglitazone at 7 days after Ang II infusion. Expression of adiponectin was normalized to the GAPDH signal and expressed as percentage of the signal intensity of WT mice (n=4).