Insulin decreases myocardial adiponectin receptor 1 expression via PI3K/Akt and FoxO1 pathway

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Aims
Adiponectin is considered an important adipokine protecting against diabetes, atherosclerosis, and cardiovascular disease. Because adiponectin receptors (AdipoRs) are critical components in the adiponectin signalling cascade, we investigated the effect of insulin on the expression of myocardial AdipoRs and explored the possible molecular mechanism.

Methods and results
The hyperinsulinaemia rat model was induced by infusion of insulin (1 U/day) for 28 days: serum and myocardial adiponectin levels were increased, and skeletal muscle and myocardial AdipoR1 expression and AMP-activated protein kinase (AMPK) phosphorylation were decreased. In primary cultured neonatal rat ventricular myocytes (NRVMs), insulin decreased AdipoR1 but not AdipoR2 expression and AMPK phosphorylation; high glucose had no affect on AdipoRs expression. Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation was increased in insulin-treated hearts and in NRVMs. PI3K inhibitor LY294002 and Akt1/2 kinase inhibitor but not the ERK1/2 kinase (MEK) inhibitors PD98059 and U0126 blocked the insulin-induced reduction in AdipoR1 expression and AMPK phosphorylation. Insulin induced forkhead/winged helix box gene group O-1 (FoxO1) phosphorylation and translocation from the nucleus to the cytosol, and this was blocked by LY294002. FoxO1 small interfering RNA reduced AdipoR1 expression and AMPK phosphorylation. In electrophoretic mobility shift assay and chromatin immunoprecipitation, FoxO1 bound to the putative site from -167 to -157 bp of the AdipoR1 promoter both in vitro and in living cells; insulin suppressed this binding, which was blocked by LY294002.

Conclusion
Insulin inhibits myocardial AdipoR1 expression via PI3K/Akt and FoxO1 pathways, and FoxO1 mediates AdipoR1 transcription by binding to its promoter directly.

Keywords
Adiponectin receptor • Cardiomyocyte • Insulin • FoxO1

1. Introduction
Adiponectin is an adipocyte-specific adipokine that plays an important role in energy metabolism, regulation, and cardiovascular protection. The circulating adiponectin level is decreased in obesity, type 2 diabetes, and coronary artery disease.1,2 Adiponectin-deficient mice exhibit insulin resistance, diabetes, and concentric cardiac hypertrophy.3,4 Administration of adiponectin reduces blood glucose level, ameliorates insulin resistance, and protects against cardiac remodeling in adiponectin-knockout and db/db mice.5,6 Adiponectin also protects against myocardial ischaemia-reperfusion injury and improves systolic dysfunction.7,8 Therefore, adiponectin is an important endogenous adipokine protecting against diabetes, atherosclerosis, and cardiovascular disease.

Two types of adiponectin receptors (AdipoRs) mediate the activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-α, as well as fatty acid oxidation and glucose uptake by adiponectin.9,10 AdipoR1 is ubiquitously expressed, with a relatively high level in skeletal muscle, whereas AdipoR2 is predominantly expressed in liver.11 AdipoRs expression is significantly decreased in adipose tissue, liver, and muscle of obese diabetic animal and individuals and in infarcted mouse hearts.12,13 AdipoRs are critical components in the adiponectin signalling cascade, with the decrease in plasma adiponectin level and down-regulation of...
AdipoR expression being involved in insulin resistance, type 2 diabetes, and coronary artery disease. However, AdipoR mRNA levels in human skeletal muscle have been found to be positively associated with obesity and insulin resistance\(^{14}\) and to be up-regulated in skeletal muscle from obese mice induced by a high-fat diet.\(^{15}\) Therefore, the relationship between the expression of AdipoRs and insulin resistance and type 2 diabetes still needs further investigation.

Both AdipoR1 and AdipoR2 are expressed in cardiomyocytes.\(^{11}\) Recently, myocardial AdipoR1 expression was found to be decreased in rats with high-fat and high-sugar diets, possibly associated with decreased heart function.\(^{16}\) However, the regulatory mechanisms involved in myocardial AdipoR1 expression remain unclear. Also, myocardial AdipoR1 expression was found to be increased in streptozotocin-induced diabetic rats,\(^{17}\) which suggests that the myocardial AdipoR1 level may be negatively correlated with the serum insulin level.

Because hyperinsulinemia is usually associated with obesity, type 2 diabetes, and cardiovascular diseases, and insulin enhances while adiponectin inhibits angiotensin-induced cardiac hypertrophy,\(^{18,19}\) we hypothesized that insulin might interfere with the myocardial adiponectin signalling cascade by regulating the expression of AdipoRs. We examined the effect of insulin on AdipoR expression in a rat model of continuous insulin infusion and in cultured cardiomyocytes. Moreover, we investigated the molecular mechanism by which insulin regulates AdipoR expression.

2. Methods

All experimental procedures were approved by the Ethics Committee of Animal Research, Peking University Health Science Center, and the investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1 Materials

Antibodies for phospho- and total AMPK, Akt, p38 mitogen-activated protein kinase (p38MAPK), c-Jun N-terminal kinase (JNK), and fork-head/winged helix box gene group O-1 (FoxO1) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies for AdipoR1, AdipoR2, β-tubulin, and phospho- and total extracellular signal-regulated kinase 1/2 (ERK1/2) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Insulin, LY294002, Akt1/2 kinase inhibitor, PD98059, and U0126 were from Sigma-Aldrich Co. (St Louis, MO, USA).

2.2 In vivo rat model of continuous insulin infusion

Male Sprague-Dawley rats weighing 250−280 g were randomly divided into control and insulin groups. An osmotic mini-pump (model 2004, Durect Corp., Cupertino, CA, USA) was subcutaneously embedded in rats under anaesthesia with sodium pentobarbital (50 mg/kg, i.p.). Insulin (1 U/day) or normal saline was infused constantly for 28 days, then blood samples were collected from abdominal aorta, and hearts were excised under anaesthesia as above. No neuromuscular blocking or paralytic agents were used. The adequacy of anaesthesia was determined by monitoring blood pressure and heart rate by electrocardiography.

2.3 Measurement of blood glucose and serum insulin

The blood glucose level was measured by use of Accu-Chek Active Blood Glucose Test Strips (Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin content was measured by use of an insulin radioimmunoassay kit (Linco Research, St. Charles, MO, USA).

2.4 Measurement of serum and heart adiponectin

Frozen heart tissues were homogenized in lysis buffer and centrifuged as described.\(^{17}\) The supernatants were collected and the protein content was measured by the Bradford method. Serum and heart adiponectin levels were measured by use of a commercially available ELISA kit (Adipo Biotech, Beijing).

2.5 Primary culture of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVMs) were prepared as described previously.\(^{19}\) Briefly, ventricles of 1- to 3-day-old Sprague-Dawley rats were minced and digested in phosphate-buffered saline containing 0.1% trypsin and 0.05% type I collagenase for 8−10 cycles, then cells were centrifuged and suspended in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) containing 15% foetal bovine serum (FBS) and antibiotics (1% penicillin and streptomycin). Myocytes that did not attach to culture dishes were plated at 1 × 10^5 cells/cm^2 in the same medium and supplemented with 0.1 mM bромodeoxyuridine to inhibit the proliferation of fibroblasts and obtain higher purity of cardiomyocytes. Cells were placed in a serum-free medium for 24 h before experiments. The identity of NRVMs was confirmed by morphological examination and by staining with anti-sarcomeric α-actin antibody; most (>95%) of the cells were identified as NRVMs.

2.6 RNA isolation and RT–PCR

Total RNA of myocardium and NRVMs was isolated by use of Trizol reagent (Invitrogen). cDNA was generated from total RNA by use of the ReverTra First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada) and amplified by PCR with the gene-specific primers for AdipoR1, which were not within the mRNA coding sequence, but able to evaluate the AdipoR1 mRNA levels. forward, 5′-AGGCAACTGCTGTGTCCTCTAC-3′, reverse, 5′-TGCCCAACCGGTCTGTAATTGC-3′; AdipoR2, forward, 5′-AACCCACACTTGTCTTCATC-3′, reverse, 5′-TCAGGCCGCTTCCCTCTAGT-3′; FoxO1 forward, 5′-GGTGAAACACATTGCTCAC-3′, reverse, 5′-GTGTCTTCAGGAAATGATGAGCTC-3′; and β-actin forward, 5′-TCCTCCCTGGGAAAGACTCTA-3′, reverse, 5′-TCAGGGAGGCAATGACTTGG-3′ as a housekeeping control. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The bands were visualized by Genegius Gel Imaging System (Syngene, Synoptics, Inc., Frederick, MD, USA) and processed by use of Genetools 3.06 (Syngene).

2.7 Western blot analysis

Myocardial tissues or NRVMs were lysed in a buffer containing 50 mM Tris–HCl, pH 7.2, 0.1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 40 mM NaF, 2.175 mM NaVO₄, 0.1% SDS, 0.1% aprotinin, and 1 mM PMSF. The lysates were centrifuged and the supernatants were collected. Equal amounts of protein (40 μg) were separated on 7.5% or 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies and probed with horseradish peroxidase-conjugated secondary antibodies. Bands were detected with an enhanced chemiluminescence kit (Amersham Biosciences Inc., Piscataway, NJ, USA). The densities of bands were quantified by use of the LI-COR LI-COR Image system (Leica, Mannheim, Germany).

2.8 Immunofluorescence

NRVMs were cultured on glass coverslips and fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with 1% bovine serum albumin for 30 min. Then NRVMs were incubated with anti-FoxO1 antibody (1:200) followed by FITC-labelled secondary antibody (1:400). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Fluorescence images were captured by the Leica TCS SP5 confocal system (Leica, Wetzlar, Germany).
2.9 Small interfering RNA (siRNA)
siRNA targeting FoxO1 and negative control siRNA were from Invitrogen. The sequence for FoxO1 was 5′-GGGAAAGACGCGCAGAATTT-3′ and for non-specific control 5′-UUUCCGCAAGGUGUCAGCATT-3′. NRVMs were transfected with the siRNA (100 nM for 48 h) by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

2.10 Electrophoretic mobility shift assay
Nuclear protein fractions were separated as described. Briefly, NRVMs were suspended in hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 1 mM NaVO₃, and 10 μg/mL aprotinin for 15 min. After adding NP-40 (10%), the mixture was centrifuged twice and supernatants were collected. The DNA sequence of AdipoR1 was obtained from the UCSC Genome Browser Database (http://www.genome.ucsc.edu). Putative binding sites for FoxO1 (142 to 152 bp and -167 to -157 bp) were identified by use of PROMO 3.0 with the database TRANSFAC 8.3. Electrophoretic mobility shift assay (EMSA) involved use of the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, USA). Briefly, nuclear protein (8 μg) was incubated with biotin-labelled DNA probes containing two putative binding sites (underlined sequence) for FoxO1, 5′-biotin-CACACTCGTGATCTTGTGATTGTTAGCTGTA-3′ (sense, 131 to 163 bp) and 5′-biotin-GGAGGGGGCGGAATATTACGTTGGACCTTT-3′ (sense, -178 to -146 bp). After reaction, the DNA–protein complexes underwent 6% natively PAGE and were transferred to a nitrocellulose membrane, then cross-linked to the membrane at 120 mJ/cm² by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

2.11 Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChiP) assay was performed as described. Briefly, NRVMs were cross-linked with 1% formaldehyde and collected into lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1, 1× protease inhibitor cocktail). After sonication, the lysates underwent immunoclearing and were immunoprecipitated with anti-FoxO1 antibody or normal rabbit IgG as a negative control. After incubation with protein A-Sepharose and salmon sperm DNA and a sequential washing, precipitates were heated at 65°C for 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified by use of the TIANquick Midi Purification kit (Tiangen Biotech, Beijing). PCR analysis involved the following primer sequences for putative FoxO1 binding sites: site 1 forward, 5′-TTGCCAGGTGGTGATCCG-3′ (–18 to –1 bp) and reverse, 5′-TCGGTCCACGGTCTTCTTCA-3′ (292 to 310 bp); site 2 forward, 5′-CAGCCAGGGCAGTAATGTCG-3′ (–291 to –273 bp) and reverse, 5′-TGTCCGATCCAAACTCG-3′ (–15 to 4 bp).

2.12 Statistical analysis
Data are presented as means±SD. Differences between groups were evaluated by Student’s t-test or one-way ANOVA followed by Tukey’s multiple comparison post hoc tests by use of GraphPad Prism 5.0 software. 

3. Results
3.1 Insulin decreases AdipoR1 expression and AMPK phosphorylation in vivo and in vitro
Compared with controls, insulin-infused rats showed significantly increased serum insulin level but no change in body weight, blood glucose, ratio of left ventricle to body weight, or cardiac myocyte cross-sectional area (Figure 1A–E). Insulin infusion increased serum and cardiac adiponectin levels (Figure 1F and G) but reduced myocardial AdipoR1 protein by 40% (P < 0.01) with no change in AdipoR2 (Figure 1H and I). Phosphorylation of AMPK-α at Thr-172, a downstream kinase of AdipoR1, was significantly decreased in the insulin-infused heart (Figure 1J). Moreover, the mRNA and protein expression of AdipoR1 in skeletal muscle was decreased by insulin infusion, but the AdipoR2 mRNA level was not affected (see Supplementary material online, Figure S1A–C).

To further explore the effect of insulin on AdipoR expression, NRVMs were incubated with 1–200 nmol/L insulin for 24 h. Insulin dose-dependently (Figure 2A and B) and time-dependently (Figure 2C and D) reduced the mRNA expression of AdipoR1 but not AdipoR2. We further verified that insulin decreased the protein level of AdipoR1 in a time- and dose-dependent manner (Figure 2E and F). H₂O₂, reported to inhibit AdipoR expression in NRVMs,13 was used as a positive control. Insulin also decreased AMPK phosphorylation in NRVMs (Figure 2G). These results suggest that insulin inhibited AdipoR1 expression and function in NRVMs.

Because hyperinsulinaemia and hyperglycaemia usually coexist in obesity and type 2 diabetes, and high glucose decreases AdipoR1 expression in L6 myoblasts,22 we further tested the effect of high glucose on AdipoR expression. NRVMs were incubated with constant high glucose (25.5 mmol/L) or intermittent high glucose (changing 5.5 and 25.5 mmol/L glucose medium every 12 h) for 24–96 h. The mRNA expression of AdipoR1 was not affected (see Supplementary material online, Figure S2A–C). Furthermore, when NRVMs were incubated with both 100 nmol/L insulin and 25.5 mmol/L glucose, the inhibitory effect on AdipoR1 protein expression did not differ from that with insulin incubation alone.

3.2 Insulin decreases AdipoR1 expression in NRVMs via the PI3K/Akt pathway
Phosphatidylinositol 3-kinase (PI3K) and Akt are the key molecules mediating the insulin signal pathway.23 We found the phosphorylation of Akt at Ser-473 significantly increased in insulin-treated rat hearts and NRVMs (Figure 3A and E). ERK1/2, p38MAPK, and JNK are major members of the MAPK family. We found that insulin significantly increased the phosphorylation of ERK1/2 without change in p38 MAPK (Figure 3F–H). To further ascertain the roles of Akt and ERK1/2 in insulin-reduced AdipoR1 expression, NRVMs were preincubated with the PI3K inhibitor LY294002 (10 μmol/L), Akt1/2 kinase inhibitor (1 μmol/L), or PD98059 (10 μmol/L) and U0126 (10 μmol/L), inhibitors of ERK1/2 kinase (MEK), before insulin treatment. LY294002 and Akt1/2 kinase inhibitor but not PD98059 or U0126 blocked insulin-induced AdipoR1 decrease in expression (Figure 3I). The effects of these inhibitors on phosphorylation of Akt and ERK1/2 were confirmed in NRVMs (see Supplementary material online, Figure S2A–D), and each of them alone had no effect on AdipoR1 expression. These results suggested that insulin decreased AdipoR1 expression via a PI3K/Akt pathway. Furthermore, when NRVMs were preincubated with LY294002 and Akt1/2 kinase inhibitor, the insulin-induced decrease in p-AMPK was reversed (Figure 3M and N).
3.3 Insulin induces FoxO1 phosphorylation and exclusion from the nucleus
FoxO1 is a critical nuclear transcription factor regulated by insulin-independent phosphorylation. Phosphorylation of FoxO1 at Ser-256 was significantly increased in insulin-infused hearts and insulin-treated NRVMs (Figure 4A and B). Under the basal condition, FoxO1 localized mainly in the nucleus (Figure 4C). After insulin treatment (100 nmol/L, 1 h), FoxO1 translocated from the nucleus to the cytosol. Preincubation with the PI3K inhibitor LY294002 blocked the insulin-induced FoxO1 translocation. These data suggest that insulin induced FoxO1 phosphorylation and exclusion from the nucleus via a PI3K/Akt pathway.

3.4 FoxO1 siRNA reduces FoxO1 levels and decreases AdipoR1 expression in NRVMs
To further verify the importance of FoxO1 in inhibiting AdipoR1 gene expression, NRVMs were transfected with control or FoxO1 siRNA. The mRNA and protein expression of FoxO1 was significantly...
Insulin decreases myocardial AdipoR1 expression

Figure 2 Expression of AdipoRs and AMPK phosphorylation in NRVMs. RT–PCR analysis of AdipoR1 (A and C) and AdipoR2 (B and D) mRNA in NRVMs treated with insulin for 24 h at indicated concentrations or with 100 nmol/L insulin for the indicated times. β-Actin was an endogenous control. Western blot analysis of the AdipoR1 protein level in NRVMs treated with insulin for 24 h at the indicated concentrations (E) or with 100 nmol/L insulin for the indicated times (F). β-Tubulin was an endogenous control. H₂O₂ (10 μmol/L, 24 h) was a positive control. (G) Western blot analysis of p-AMPK and t-AMPK in NRVMs treated with insulin (100 nmol/L, 24 h). Data are mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 vs. Con.
Signalling molecules mediating insulin-mediated AdipoR1 expression. Western blot analysis of Akt (A and E), ERK1/2 (B and F), JNK (C and G) and p38MAPK (D and H) phosphorylation in rat hearts and NRVMs, respectively. The phosphorylation levels were normalized to their total protein levels. Western blot analysis of AdipoR1 protein in NRVMs treated with insulin (100 nmol/L, 24 h) with or without preincubation with LY294002 (I), Akt1/2 kinase inhibitor (J), PD98059 (K), or U0126 (L). β-Tubulin was an endogenous control. Western blot analysis of AMPK phosphorylation in NRVMs treated with insulin (100 nmol/L, 24 h) with or without preincubation with LY294002 (M) or Akt1/2 kinase inhibitor (N). The phosphorylation level was normalized to t-AMPK. Data are mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 vs. Con. #P < 0.05 vs. insulin.
decreased (Figure 5A and B). Transfection with FoxO1 siRNA caused a 54% (P < 0.01) and 33% (P < 0.01) decrease in AdipoR1 mRNA and protein levels, respectively, with no change in AdipoR2 mRNA expression (Figure 5A and B). Moreover, FoxO1 siRNA inhibited AMPK phosphorylation by 40% (P < 0.01). These results suggested that AdipoR1 was a downstream target gene of FoxO1 in NRVMs.

3.5 FoxO1 is required for insulin-reduced AdipoR1 expression

To further reveal the mechanism of FoxO1-mediated AdipoR1 expression, we screened the rat AdipoR1 promoter sequence. We found two putative FoxO1 binding sites located from −2000 to 200 bp of the promoter sequence: site 1 between 142 and 152 bp and site 2 between −167 and −157 bp (Figure 6A). EMSA results showed the formation of a protein–DNA complex when nuclear extracts were incubated with only site 2 oligonucleotides, which could be competed out with 100-fold unlabelled oligonucleotides and suppressed by insulin incubation (Figure 6B). To determine whether FoxO1 bound to either of the two putative binding sites in intact NRVMs, ChIP assay was performed (Figure 6C). PCR analysis of input samples revealed a product for both sites, whereas analysis of FoxO1 antibody-immunoprecipitated samples revealed a product corresponding to site 2 under the same experimental conditions. Insulin suppressed the binding of FoxO1 to site 2, and LY294002 blocked the inhibitory effect of insulin. These data suggested that FoxO1 regulated AdipoR1 transcription by binding to its promoter region directly, and insulin inhibited FoxO1 binding via a PI3K/Akt pathway.

4. Discussion

In the present study, we demonstrated that insulin decreased the cardiac expression of AdipoR1 but not AdipoR2 in rats with continuous insulin infusion and in cultured NRVMs. Furthermore, FoxO1 mediated AdipoR1 transcription by binding to the AdipoR1 promoter region directly. The activation of PI3K/Akt and translocation of FoxO1 were involved in the inhibitory effect of insulin on AdipoR1 expression.

Insulin plays a crucial role in regulating glucose and lipid metabolism, protein synthesis, growth, and contractility in cardiomyocytes, and hyperinsulinemia is usually associated with obesity, type 2 diabetes, and cardiovascular disease. Previous studies showed that insulin decreases AdipoR1 but not AdipoR2 expression in C2C12 myoblasts and in mouse skeletal muscle. Insulin deficiency induced by streptozotocin increases and insulin replacement reduces...
AdipoR expression in skeletal muscle and liver. However, insulin does not alter the expression of AdipoRs in adipocytes, and AdipoR expression is reduced in liver and elevated in muscles in hyperinsulinaemic mice. Therefore, the exact role of insulin on AdipoR expression may depend on the model and tissue investigated and the cause of insulin resistance.

Here, we provide more evidence that insulin inhibited not only skeletal muscle but also myocardial AdipoR1 expression in chronic hyperinsulinaemia rats. Furthermore, insulin down-regulated AdipoR1 but not AdipoR2 levels in NRVMs. Our results also show similar plasma glucose levels in rats with insulin infusion and controls, as reported previously. Constant and intermittent high glucose had no effect on AdipoR expression in NRVMs. These data indicated that insulin selectively inhibited myocardial AdipoR1 but not AdipoR2 expression independent of change in the glucose level in vivo and in vitro. Myocardial insulin resistance is associated with myocardial dysfunction, and the restoration of insulin sensitivity in the failing heart improves myocardial function. AdipoRs play an important role in mediating the insulin-sensitizing effect of adiponectin. Our results suggest that insulin-reduced myocardial AdipoR1 expression may blunt myocardial insulin sensitivity and contribute to the insulin-induced cardiac dysfunction.

Although circulating adiponectin levels are usually decreased in animals and subjects with obesity, insulin resistance, and cardiovascular disease, a recent study reported increased plasma adiponectin level and decreased cardiac AdipoR1 expression in the early stage of type 1 diabetes and adiponectin level was gradually decreased during the progression of diabetes. In type 2 diabetic rats, cardiac hypertrophy and decreased heart function are associated with decreased plasma and myocardial adiponectin levels, myocardial AdipoR1 expression, and AMPK phosphorylation. These results suggest that adiponectin levels may vary by stage of disease and disease type. Samuelsson et al. reported that insulin infusion (2 U/day) for 7 weeks induced cardiac hypertrophy and reduced cardiac output. However, we observed no significant cardiac hypertrophy in our hyperinsulinaemia model (insulin 1 U/day, for 28 days). The increased serum and heart adiponectin levels might be explained at least in part by a compensatory change during the early stage of hyperinsulinaemia.

Adiponectin exerts its metabolic modulation and cardioprotective effects by the AMPK signalling pathway. Targeted disruption of AdipoR1 results in the abrogation of adiponectin-induced AMPK activation in mouse liver. We found myocardial AMPK phosphorylation decreased in insulin-treated hearts and NRVMs. Despite an increase in serum and myocardial adiponectin levels, the decreased AdipoR1 expression and AMPK phosphorylation suggested that adiponectin resistance might occur in the early stage of hyperinsulinaemia. These observations further support the possibility of impaired AdipoR1-mediated biological effects in insulin-treated cardiomyocytes, which may contribute in part to the occurrence and progression of insulin-induced cardiac dysfunction.

PI3K/Akt and MAPK pathways play crucial roles in the signal transduction of insulin. PI3K/Akt is considered the main player of the metabolic action of insulin, whereas the MAPK pathway is principally involved in cell growth and differentiation. In the present study, insulin induced Akt phosphorylation in vivo and in vitro, and LY294002 and Akt1/2 kinase inhibitor blocked the effect of insulin on AdipoR1 expression, which indicates that PI3K/Akt is an important signalling pathway mediating the insulin-inhibited AdipoR1 expression in cardiomyocytes. Insulin increased the phosphorylation of ERK1/2, but pretreatment with ERK1/2 kinase (MEK) inhibitor did not affect insulin-reduced AdipoR1 expression, which suggests that the MAPK family might not be involved in the signal transduction of insulin-inhibited AdipoR1 expression. The results agree with those of previous studies of C2C12 cells. A recent study showed that insulin activates a feed-forward inhibitory pathway via PI3K/Akt to cause insulin resistance in 3T3-L1 adipocytes. High-fat diet-induced insulin resistance/hyperinsulinaemia is associated with increased Akt phosphorylation, and blockade of the elevated Akt phosphorylation by LY294002 reversed the insulin sensitivity. Thus, insulin-induced PI3K/Akt activation may contribute to the development of insulin resistance.

FoxO1 is an important nuclear transcription factor that represses or activates target gene transcription after insulin-dependent phosphorylation. Akt-mediated FoxO1 phosphorylation at Ser-256 is crucial for subsequent nuclear exclusion. The AdipoR1 mRNA level is associated with FoxO1 mRNA expression.
in obese insulin-resistant subjects, and FoxO1 enhances transcription of the AdipoR1 promoter in HepG2 cells.37 We showed that insulin induced FoxO1 phosphorylation and translocation from the nucleus to the cytosol. The levels of AdipoR1 mRNA and protein were decreased by FoxO1 silencing, which suggests that FoxO1 acted positively on the expression of AdipoR1. Furthermore, LY294002 and Akt1/2 kinase inhibitor blocked the inhibitory effects of insulin on AMPK phosphorylation, and FoxO1 siRNA inhibited AMPK phosphorylation.

**Figure 6** Effect of FoxO1 on insulin-mediated AdipoR1 expression. (A) Two putative FoxO1 binding sites between 142 and 152 bp (site 1) and −167 and −157 bp (site 2) of the AdipoR1 promoter sequence. (B) Nuclear extracts of NRVMs treated with or without insulin (100 nmol/L, 6 h) were incubated with biotin-labeled probes corresponding to site 1 or site 2 before being applied to a non-denaturing gel. Competition were performed by preincubation with 100-fold of the corresponding unlabelled probe (cold). (C) NRVMs were preincubated with or without LY294002, then treated with insulin (100 nmol/L, 6 h) and underwent ChIP assay. PCR analysis of the input-, FoxO1-, and normal rabbit IgG-immunoprecipitated (negative) samples involved the primer sets corresponding to sites 1 and 2. All results are representative of three independent experiments.

AdipoR expression is mediated by various physiological and pathological factors. However, the precise transcription mechanism involved in AdipoR expression has not been fully evaluated. Recently, C2C12 cells were found to contain a novel insulin-responsive region in the AdipoR1 promoter with a strong repressor element, termed nuclear inhibitory protein, involved in the negative regulation of AdipoR1 promoter by insulin.34 In the present study, we found that the AdipoR1 promoter sequence from −167 to −157 bp contains a critical regulatory site for FoxO1. EMSA and ChIP assay provided the first evidence that FoxO1 bound directly to the AdipoR1 promoter both in vitro and in living cells. Moreover, we demonstrated
that insulin suppressed FoxO1 binding to the sequence via a PI3K/Akt pathway.

In summary, we demonstrate that insulin decreased myocardial AdipoR1 but not AdipoR2 expression in vivo and in NRVMs. Our results also indicate that insulin decreases AdipoR1 expression via the PI3K/Akt pathway and that FoxO1 plays an important role in insulin-mediated AdipoR1 transcription in cardiomyocytes by binding directly to the AdipoR1 promoter. These findings may improve our understanding of the molecular mechanism involved in insulin-associated myocardial dysfunction and provide new insights for future therapeutic targets.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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References