Mineralocorticoid Receptor Blockade Improves Insulin Sensitivity in the Rat Heart and a Possible Molecular Mechanism

Mei Wang  Yongjun Li  Kun Zhou  Guoru Zhang  Yaling Wang  Tao Liu  Yang Zhang  Anjun Guo  Yu An

Department of Cardiology, the Second Hospital of Hebei Medical University, Shijiazhuang, China

Key Words
Cardiomyocytes • Mineralocorticoid receptor • Insulin resistance • P38 mitogen-activated protein kinase • Peroxisome proliferator-activated receptor α

Abstract
Background/Aims: Extensive research has explored the role of aldosterone in insulin resistance. Recent evidence suggests that the mineralocorticoid receptor (MR) mediates aldosterone-induced dysregulation of cytokines, and most of this research has focused on adjustments in fat tissue and adipocytes. However, the direct effect of MR blockade on insulin resistance in cardiomyocytes remains largely unknown. In the present study, we investigated whether MR blockade improves insulin-sensitizing factors in insulin-resistant rats and attenuates the dysregulation of the aldosterone-related transport of adiponectin and glucose in cardiomyocytes and examined the underlying mechanisms. Methods: The effects of aldosterone, MR inhibitors (e.g., eplerenone), a peroxisome proliferator-activated receptor (PPAR) α agonist, and a p38 mitogen-activated protein kinase (MAPK) inhibitor on adiponectin and glucose transport were studied at the mRNA and protein levels in vitro and in vivo. Results: Our data revealed that aldosterone reduced the expression of adiponectin and inhibited the transport of glucose in cardiomyocytes and that MR blockade reversed these effects. In vivo, MR blockade improved insulin-sensitive parameters and increased adiponectin expression in the myocardia of high-fat diet rats. Furthermore, aldosterone promoted p38MAPK expression but negatively affected PPARα expression, and the downregulation of adiponectin by aldosterone was reversed by MR blockade, a PPARα agonist, and a p38 MAPK inhibitor. Conclusion: The above results suggested that aldosterone promoted insulin resistance in the heart and that this effect could be partly reversed by MR blockade through signal transduction in the P38 MAPK pathway and PPAR α.

Introduction

Chronic low-grade inflammation in adipose tissue leads to insulin resistance and various pathological responses [1-3]. The anti-inflammatory adipocytokine adiponectin has
been found to be closely associated with insulin resistance and cardiovascular disease [4-7]. Recent studies have suggested that the suppression of inflammation and upregulation of adiponectin may attenuate metabolic syndrome and insulin resistance [5, 7].

P38 mitogen-activated protein kinase (MAPK) plays an important role in activating immune responses. In conditions of insulin resistance, ischemia, and hyperosmolar, inflammatory cytokines have been demonstrated to stimulate p38 MAPK activity [8]. Peroxisome proliferator-activated receptors (PPARs) α and γ are members of the nuclear hormone receptor superfamily that regulates the metabolism of glucose and lipids [9]. PPARα and γ can prevent inflammation in white adipose tissue and enhance the expression of adiponectin [10]. PPARα is a downstream effector of p38 kinase-dependent stress-activated signaling in the heart, and the activation of PPARα can ameliorate the development of insulin resistance [11].

Aldosterone is known to play a classic role in the balance of water and electrolytes and is thus recognized as an important risk factor for cardiovascular diseases. Aldosterone not only exists in the circulation, where it is driven by renal renin, but can also be detected in many tissues and cells. Recent findings suggest a pivotal role of aldosterone in the pathogeneses of inflammation, insulin resistance and metabolic syndrome [12, 13]. Evidence indicates that a high level of aldosterone leads to a greater prevalence of hyperglycemia and induces a high rate of cardiovascular events [14]. Moreover, recent studies have revealed that aldosterone treatment increases the mRNA levels of tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) and inhibits adiponectin mRNA expression; however, the possible mechanisms remain unclear. Recent studies have demonstrated the effects of mineralocorticoid receptor (MR) blockade on inflammation, adiponectin, and insulin resistance. MR blockade with eplerenone (an MR antagonist) has been demonstrated to rectify the reductions in the mRNA expressions of adiponectin and PPARγ that are induced by aldosterone in adipocytes. However, contrasting results have demonstrated that pretreatment with eplerenone does not affect the reduction in the adiponectin mRNA level that is induced by aldosterone [15, 16].

In this study, we sought to test the hypothesis that MR blockade attenuates the dysregulation of the aldosterone-related transport of adiponectin and glucose in cardiomyocytes and MR blockade upregulates insulin-sensitizing factors in insulin-resistant rats.

Materials and Methods

Cell culture

All experimental procedures were performed in accordance with the Guide of the Chinese Ministry of Public Health for the Care and Use of Laboratory Animals and were approved by the Health Science Center Institutional Animal Care and the Use Committee of Hebei Medical University. Neonatal rat cardiomyocytes were obtained from the ventricles of 3-day-old Sprague-Dawley rats as described previously [17]. These ventricular myocytes were incubated in culture medium with 10% fetal calf serum at 37°C in humidified air with 5% CO₂, 5-bromo-2-deoxyuridine (BrdU) 0.1 mmol/L was routinely applied for 48 hours to inhibit the growth of cardiac fibroblasts. Forty-eight hours after seeding, the cardiomyocytes were serum-starved for 24 hours in serum-free medium to achieve cell cycle synchronization. Next, 30 minutes before aldosterone administrations involving different concentrations and different durations, the cardiomyocytes were pretreated with the MR inhibitor eplerenone, the PPARα specific agonist WY-14643, and the p38 MAPK specific inhibitor SB203580 (all drugs were from Sigma Chemicals, St. Louis, MO, USA). Twenty-four hours later, the cardiomyocytes were harvested for further detection. The cell viabilities exceeded 95% as assessed with trypan blue.

Animals

The study was approved by the Ethics Review Committee for Animal Experimentation of Hebei Medical University (Shijiazhuang, China). Male Sprague–Dawley (SD) rats (160-180 g) were purchased from...
the Laboratory Animal Centre of Hebei Medical University. The insulin-resistant rat model was created as described in a previous study [1] with a few modifications. The rats were singly housed and maintained on a 12-h light/dark cycle in a temperature-controlled room (22°C). After acclimatization, the rats were fed either a normal diet (Nd, 18% fat, 25% protein, and 57% carbohydrate) or a high-fat diet (Hfd, 40% fat, 13% protein, and 47% carbohydrate) for 8 weeks. From weeks 8 to 16, both diet groups were treated with either the MR antagonist eplerenone (Sigma Chemicals, St. Louis, MO, USA) at 0.6 mg/g of diet [17] (eplerenone group; Epl) or no compound (control group; Con). The groups were as follows: Nd Con, Nd+Epl, Hfd Con and Hfd+Epl. At week 16, the body weights were measured, the systolic blood pressures of the conscious animals were monitored via tail-cuff micro-photoelectric plethysmography, and the plasma was collected for the detection of adiponectin (via an ELISA kit, Huadong Company, Nanjing, China), plasma insulin (FINS), plasma blood glucose (FBG), interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), aldosterone (all with radioimmunoassay kits; North Institute of Biotechnology, Beijing, China), potassium (Ortho Clinical Vitros 250 Chemistry System, Johnson & Johnson Company, American). The homeostatic model assessment (HOMA) index was calculated according to the following formula: FBG×FINS/22.5, and hyperinsulinemic-euglycemic clamp techniques were applied to assess insulin sensitivity in vivo. Next, the rats were sacrificed, and the hearts were harvested. A piece of tissue from the left ventricle was snap-frozen in liquid nitrogen and stored at -80°C until processing for protein extraction.

**Hyperinsulinemic-euglycemic clamp techniques**

As previously described [18, 19], following fasting for 12-14 h, the rats were anesthetized with pentobarbital sodium (48 mg/kg; ip). The right jugular vein was cannulated for the infusion of glucose and insulin (using separate catheters), and the left carotid artery was used for blood sampling. The glucose and insulin solutions were stored in two digital syringe pumps that were joined by a “Y” connector to the jugular catheter. Insulin (100 mU/ml; Novolin R, Novo Nordisk Pharmaceuticals, Bagsvaerd, Denmark) was infused at a speed of 11 mU/kg/min. Meanwhile, blood glucose was serially detected. The infusion of 5% glucose into the right jugular vein was initiated when the blood glucose was found to be within euglycemic levels (5.0 ± 0.5 mmol/L). To maintain the blood glucose within the euglycemic range, we adjusted the glucose infusion rate (GIR) every 5-10 min. Steady state was achieved over 60 min and maintained for another 30 min. The calculation of the mean GIR was based on the GIR readings from the last six samplings.

**RNA isolation and RT-PCR analysis**

Total RNA was extracted from the cardiomyocytes with Trizol reagent (SBS, China). The purity of the RNA was assessed by measuring the absorbance at 260 nm. The purity of the isolated RNA was determined by ultraviolet spectrometry. The primer sequences were as follows: adiponectin (forward: 5'-CAGGAGATGCCTGGAATGA- 3’; reverse: 5'-GATACGTGCTAGGTGAAG- 3’), amplified product: 358 bp; and β-actin (forward: 5'-AGGGAAATCGTGCGTGAC- 3’; reverse: 5'-CTGGAAAGTGGACAGTGAG- 3’), amplified product: 460 bp. The RT-PCR reaction was run for 30 cycles under the following conditions: DNA template denaturing at 94°C for 60 seconds; specific annealing at 52°C for 60 seconds and 72°C for 60 seconds; and a final extension step at 72°C for 10 minutes. The amplification was linear under these conditions and was performed on a Biometra T-gradient Thermoblock PCR System (Santa Cruz Biotechnology). All RT-PCRs were performed at the same time and with the same batch of Taq polymerase to reduce variation in the RT-PCR efficiency. The band densities were quantified using a scanning densitometer coupled to the scanning software Gel-Pro Analyzer 3.1.

**Western blot analyses**

The adiponectin, P38MAPK and PPARα proteins from the cultured cardiomyocytes and cardiac tissues were analyzed via quantitative Western blotting as previously described [20, 21]. Briefly, the cardiomyocytes or cardiac tissues were lysed, and the proteins were extracted and measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Approximately 30 µg of each sample was loaded. Each sample was separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The polyvinylidene fluoride membranes were then blocked with 5% skim milk (Becton, Dickinson and Company) in 20 mM Tris·Cl, 150 mM NaCl, 0.05% Tween 20 Tris-buffered solution (TBS) and Tween 20 (TBS-T) at pH 7.4 for 1 hour at room temperature. The membranes were immunoblotted with 1:5000 anti-adiponectin antibody.
Measurement of glucose transport

The rate of glucose transport was determined by measuring the uptake of 2-deoxy-[3H]D-glucose as previously described [22] with slight modifications. Briefly, 24 or 48 hours after treatment, the cells were washed three times with 20 mmol/L HEPES buffer (pH 7.4, 140 mmol/L NaCl, 1 mmol/L CaCl₂, 5 mmol/L KCl, 2.5 mmol/L MgCl₂, 20 mmol/L HEPES, and 0.1% BSA). Subsequently, the cells were incubated at 37°C for 30 minutes in the culture medium mixed with 1 ml HEPES buffer containing 1 μCi/ml 2-deoxy-[3H]D-glucose (Boston, MA, USA). Next, the cells were solubilized with 1 mol/L NaOH, and the radioactivity was determined by liquid scintillation spectrometry.

Statistical analysis

The results are expressed as the means ± the SEs. Student’s t-tests were used for the statistical comparisons. P-values below 0.05 were considered statistically significant.

Results

Aldosterone reduces adiponectin mRNA expression in a dose- and time-dependent manner and inhibits glucose transport in cardiomyocytes

To evaluate the effects of aldosterone on adiponectin expression and glucose transport in the cardiomyocytes, we treated the cardiomyocytes with different concentrations of aldosterone for different durations. When exposed to 10⁻⁷-10⁻⁵ mol/L aldosterone for 24 hours, significant and dose-dependent decreases in adiponectin mRNA expression compared with the 0 mol/L aldosterone treatment (P < 0.05) were observed, and the adiponectin mRNA expression was significantly decreased at the maximum concentration of aldosterone of 10⁻⁵ mol/L (Fig. 1A). We also observed a time-dependent effect on adiponectin mRNA expression. The maximal decreases were observed at the 24 h and 48 h time points (P < 0.05), and no difference was observed between these time points following the 10⁻⁵ mol/L aldosterone treatment (Fig. 1B). Aldosterone induced a significant decrease in glucose uptake at the 10⁻⁶ and 10⁻⁵ mol/L concentrations. Glucose transport was markedly decreased following the 10⁻⁵ mol/L aldosterone treatment at 24 h and 48 h compared with the values of the control group (P < 0.05), but there was no significant difference between the 24 h and 48 h time points following treatment with 10⁻⁵ mol/L aldosterone (Fig. 1C).

Mineralocorticoid receptor blockade reverses the decreases in adiponectin and glucose transport induced by aldosterone in cardiomyocytes

Cardiomyocytes express the mineralocorticoid receptor [23]. Therefore, we tested whether treatment with the MR blocker eplerenone elicited a beneficial effect in cardiomyocytes. As expected, aldosterone treatment alone reduced the adiponectin mRNA levels and glucose transport in dose-dependent manners, and pretreatment with different concentrations of eplerenone reversed these effects (P < 0.05, Fig. 2A, B, C). Treatment with 10⁻⁵ mol/L eplerenone alone had no significant effect on adiponectin mRNA or protein expression or on glucose transport (Fig. 2A, B, C).

Mineralocorticoid receptor blockade increases insulin sensitivity in rats

To prove that MR blockade increased insulin sensitivity in vivo, we tested whether treatment with the MR blocker increased insulin sensitivity in Hfd rats. Hfd treatment for
4 weeks significantly increased the body weight and the concentrations of plasma glucose, insulin, HOMA, aldosterone, IL-6, and TNF-α and decreased the levels of adiponectin and GIR ($P < 0.05$). In contrast, the Hfd rats that were administered eplerenone did not exhibit significant changes in body weight, systolic blood pressure, or plasma glucose, but the plasma insulin, HOMA, aldosterone, IL-6 and TNF-α values were decreased. However, the levels of adiponectin and GIR were increased ($P < 0.05$, Table 1).

Mineralocorticoid receptor blockade increases adiponectin and PPARα protein expression and decreases p38 MAPK protein expression in the heart tissue

We determined the expressions of the adiponectin, PPARα, and p38 MAPK proteins in the heart tissue. The protein levels of adiponectin and PPARα protein markedly decreased
in the heart tissues of the Hfd rats compared with the levels in the Con rats ($P < 0.05$). In contrast, p38 MAPK protein expression in Hfd rats was significantly increased compared with that in the Con rats. MR blockade with eplerenone in the Hfd rats improved the protein expressions of both adiponectin and PPARα but suppressed the expression of the p38 MAPK protein ($P < 0.05$, Fig. 3).

**The P38 MAPK pathway and PPARα are involved in the effect of the mineralocorticoid receptor on adiponectin protein expression**

The P38 MAPK pathway and PPARα are associated with the glucose metabolism. Therefore, we investigated whether the p38 MAPK pathway and PPARα are involved in the regulation of adiponectin by MR. Compared with the control cells, aldosterone treatment resulted in a significant increase in p38 MAPK protein expression ($P < 0.05$). However, after pretreatment with $10^{-5}$ mol/L eplerenone, this change in p38 MAPK protein expression was reversed ($P < 0.05$, Fig. 4A). Aldosterone decreased adiponectin expression in the cardiomyocytes, but the addition of the p38 MAPK inhibitor SB203580 ($10^{-5}$ mol/L) led to a significant increase in aldosterone-induced adiponectin protein expression ($P < 0.05$, Fig. 4B). Interestingly, aldosterone reduced the level of PPARα protein, but pretreatment with eplerenone reversed this effect ($P < 0.05$, Fig. 4C). After pretreatment with the PPARα agonist WY-14643 ($3 \times 10^{-5}$ mol/L), the expression of adiponectin protein in the myocytes was increased ($P < 0.05$, Fig. 4D).
Table 1. Characteristics of different group rats at 16 weeks. \(^*P<0.05, \text{vs Con rats.}\) \(^{1}P<0.05, \text{Hfd+Epl rats vs Hfd rats.}\)

<table>
<thead>
<tr>
<th></th>
<th>Con (n=10)</th>
<th>Epl (n=10)</th>
<th>Hfd (n=10)</th>
<th>Hfd+Epl (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>205.8±1.08</td>
<td>205.7±1.01</td>
<td>237.4±1.03</td>
<td>236.8±1.77</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117.2±1.93</td>
<td>117.2±1.93</td>
<td>117.6±5.6</td>
<td>118.4±7.03</td>
</tr>
<tr>
<td>Blood measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.51±0.65</td>
<td>4.52±0.64</td>
<td>9.24±1.54</td>
<td>9.0±1.30</td>
</tr>
<tr>
<td>Insulin, mU/L</td>
<td>10.88±0.83</td>
<td>10.78±1.14</td>
<td>25.34±2.98</td>
<td>17.57±2.92</td>
</tr>
<tr>
<td>HOMA2</td>
<td>2.18±0.31</td>
<td>2.15±0.31</td>
<td>10.41±2.16</td>
<td>6.9±1.01</td>
</tr>
<tr>
<td>GIR, mg/Kg/min</td>
<td>27.6±6.09</td>
<td>20.6±0.97</td>
<td>17.6±0.52</td>
<td>22.9±0.93</td>
</tr>
<tr>
<td>Adiponectin, ug/ml</td>
<td>1.7±0.17</td>
<td>1.72±0.12</td>
<td>1.10±0.09</td>
<td>1.30±0.07</td>
</tr>
<tr>
<td>Potassium, ug/ml</td>
<td>5.51±0.15</td>
<td>5.53±0.11</td>
<td>5.56±0.12</td>
<td>5.59±0.14</td>
</tr>
<tr>
<td>Akkosterone, pg/ml</td>
<td>60.16±3.66</td>
<td>69.20±1.50</td>
<td>113.0±8.63</td>
<td>86.8±3.06</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>4.9±0.72</td>
<td>4.9±0.72</td>
<td>23.2±1.12</td>
<td>13.8±1.03</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>22.9±1.72</td>
<td>22.2±1.70</td>
<td>41.3±2.74</td>
<td>28.8±1.79</td>
</tr>
</tbody>
</table>

Fig. 3. The effects of eplerenone (Epl) on adiponectin, p38 MAPK, PPARα protein expression in heart tissue. (A) Adiponectin protein expression. (B) p38 MAPK protein expression. (C) PPARα protein expression. \(^*P < 0.05\) vs Con group. \(^{†}P < 0.05\) vs Hfd group.

Discussion

The most important findings of the present study were as follows: 1) aldosterone can markedly decrease adiponectin mRNA and protein expression and inhibit glucose transport in cardiomyocytes in time- and dose-dependent manners; 2) MR blockade reverses the reductions of adiponectin protein expression and glucose transport that are induced by aldosterone in cardiomyocytes; 3) MR blockade improves insulin sensitivity in insulin-resistant rats; and 4) aldosterone reduces PPARα protein expression and promotes p38 MAPK protein expression, while and MR blockade, a PPARα agonist and a p38 MAPK inhibitor reversed the down-regulating effect of aldosterone on adiponectin protein expression.
Recently, the direct effect of aldosterone on adipocytes had been investigated. Aldosterone reduces the amount of insulin receptor substrate protein and inhibits insulin-induced glucose uptake in 3T3-L1 adipocytes [24]. Adiponectin is one of the most important insulin-sensitizing hormones and exhibits direct anti-inflammatory, antidiabetic and antiatherogenic properties. Aldosterone treatment results in the dysregulation of proinflammatory cytokines and adiponectin mRNA levels in preadipocytes and differentiated 3T3-L1 adipocytes [25]. However, there are few studies that have examined the direct effect of aldosterone on cardiomyocytes. Our study demonstrated that aldosterone downregulates adiponectin expression and inhibits glucose uptake in dose- and time-dependent manners in cardiomyocytes. Compared with adipocytes, there are relatively higher concentrations ($10^{-7}$-$10^{-5}$ mol/L) of aldosterone in cardiomyocytes that regulate adiponectin. More than $10^{-6}$ mol/L aldosterone was needed to effectively suppress adiponectin expression and
glucose uptake. In vivo, 8 weeks of treatment with Hfd significantly increased the levels of plasma aldosterone, glucose, insulin, HOMA, IL-6, and TNF-α while decreasing the levels of adiponectin and GIR. Together, the above results indicate that aldosterone increases insulin resistance in vivo and in vitro.

The glucocorticoid receptor (GR) or MR that mediates aldosterone-induced dysregulation of adiponectin is a subject that is in a state of intense debate. A recent report on human adipocytes demonstrated that aldosterone impairs basal and insulin-stimulated glucose uptake through GR activation [24]. Another study also proved that the effects of aldosterone on adiponectin expression in adipocytes appear to be mediated through GRs and not MRs [26]. In contrast, one study demonstrated that aldosterone treatment results in the dysregulation of proinflammatory cytokines and adiponectin through MRs in adipocytes [24]. Furthermore, MR antagonism effectively reverses the dysregulation of cytokine expression levels [27]. In our observations, aldosterone decreased adiponectin and glucose uptake in cardiomyocytes, but the MR antagonist prevented these effects. These findings support the perspective that MRs mediate the aldosterone-induced dysregulation of adiponectin. In vivo, compared with the Hfd rats, those that received the 4-week treatment with eplerenone exhibited decreased plasma insulin, HOMA, GIR, aldosterone, IL-6, and TNF-α levels but the level of adiponectin was increased. These findings supported the notion that MR activation is a key factor in the regulation of insulin-sensitizing factors, and MR blockade improves insulin resistance. To our knowledge, this is the first report to demonstrate that MR blockade increases adiponectin expression in cardiomyocytes, which suggests that cardiomyocytes and adipocytes exhibit similar responses to aldosterone in terms of insulin resistance.

Although growing evidence indicates that MR mediates aldosterone-induced dysregulation of adiponectin, the molecular mechanism remains unknown. In the present study, we proposed a mechanism by which MR blockade improves adiponectin expression. The activation of the AMPK/p38 MAPK signaling cascade stimulates glucose uptake in adult cardiomyocytes, and the inhibition of the p38 MAPK signaling pathway abolishes the stimulation of glucose uptake in response to hypoxia [28, 29]. In muscle cells, adiponectin activates AMPK and p38MAPK, which sequentially stimulates PPARα activity [30]. PPARα activation upregulates adiponectin receptor mRNA expression in mice, ameliorates insulin resistance, and reduces obesity-related inflammation in adipose tissue [31]. In our study, we found that aldosterone inhibited PPARα and adiponectin protein expression and promoted inflammation and insulin resistance in cardiomyocytes; however, in contrast to muscle cells, p38MAPK protein expression was increased in the cardiomyocytes. MR blockade with eplerenone reversed these effects, and this finding accords with those of recent studies that demonstrated that aldosterone stimulates the p38MAPK signaling pathway in rat podocytes [32] and that the inhibition of PPARα increases IL-18 expression in cardiomyocytes [33]. Therefore, it is possible that p38MAPK and PPARα are both involved in MR-induced adiponectin expression in cardiomyocytes. However, the relationship between p38MAPK and PPARα is unknown. A negative feedback mechanism may exist between p38MAPK and PPARα. Further studies are needed to fully elucidate this mechanism.

Conclusions

Our studies suggest that MR activation plays important roles in the cardiac expression of adiponectin and insulin resistance. MR blockade inhibited inflammation and insulin resistance by promoting heart adiponectin and PPARα protein expression in addition to downregulating p38MAPK protein expression.

Acknowledgments

We are grateful to all the staff of the Laboratory Animal Center of the Fourth Hospital of Hebei Medical University for providing excellent assistance with the project.
Disclosure Statement

None.

References


