Angiotensin receptor-mediated oxidative stress is associated with impaired cardiac redox signaling and mitochondrial function in insulin-resistant rats

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METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of Kagawa Medical University, Japan, and the University of California, Merced.
Table 1. Primer sequences used for qualitative real-time PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>NOX2-F1</td>
<td>CCAATCAGTTGGCTGAGAC</td>
</tr>
<tr>
<td>NOX2-R1</td>
<td>GCTGCTGCTGATTCACATCCAG</td>
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<tr>
<td>NOX4-F1</td>
<td>GTGCTGCTGCTGATTCACATCCAG</td>
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<tr>
<td>NOX4-R1</td>
<td>GGTGCTGCTGCTGATTCACATCCAG</td>
</tr>
<tr>
<td>Nrf2 FW</td>
<td>AGCATCACGAGCACAGACCC</td>
</tr>
<tr>
<td>Nrf2 RV</td>
<td>CCAAGAGGATCATCGAGTGAC</td>
</tr>
<tr>
<td>aActinFW1</td>
<td>TCCGAGGCTCTCTCTCTGAC</td>
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<tr>
<td>aActinRV1</td>
<td>TTCCGAGGCTCTCTCTCTGAC</td>
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Animals. Male, 9-wk-old, lean (256 ± 6 g), strain-control Long Evans Tokushima Otsuka (LETO) and age-matched, obese (351 ± 2 g), Otsuka Long Evans Tokushima Fatty (OLETF) rats (Otsuka Pharmaceutical, Tokushima, Japan) were randomly assigned to one of the following groups (n = 8/group): 1) LETO, 2) untreated OLETF, and 3) OLETF + ARB (ARB; 10 mg olmesartan·kg⁻¹·day⁻¹). Our decision to exclude LETO + ARB group a priori was based on the following: 1) LETOs are normotensive, and ARB treatment in this group does not reduce their SBP significantly less than normal and thus would not allow us to decipher the hypertension component beyond that in the OLETF + ARB group; 2) although we have shown that ARB treatment does significantly reduce body mass (BM) and retro and epi fat compared with untreated animals in the OLETF strain (44), ARB treatment in LETO does not significantly reduce BM or fat mass (unpublished observations), which would not provide any further information on the contribution of the obesity-related increase in SBP; and 3) because plasma Ang II in LETO is already relatively low, dampening the AT1-mediated signaling even more with ARB treatment did not seem very fruitful. Thus the LETO and LETO + ARB are essentially the same phenotype and thus would not provide new information that justified the additional use of these animals. Animals were maintained at the Kagawa Medical University vivarium and housed in groups of three or four in a specific, pathogen-free facility under controlled temperature (23°C) and humidity (55%) with a 12-h light and dark cycle for 6 wk. By 15 wk of age, OLETF rats had developed insulin resistance, hypertension, and hyperlipidemia (37, 44). Animals were given free access to water and fed ad libitum standard laboratory rat chow (MF, Oriental Yeast, Tokyo, Japan). The ARB was mixed in the food and added according to initial food consumption rates to attain the desired dosage (44).

Blood pressure and tissue collection. Systolic blood pressure (SBP) was measured before and after 6 wk of treatment in conscious rats by tail-cuff plethysmography (BP-98A; Sofron, Tokyo, Japan) and has been reported elsewhere (44). After the treatment, animals were fasted overnight before being scarified the subsequent morning. The hearts were harvested, weighed, and snap-frozen in liquid nitrogen. Frozen samples were kept at −80°C until analyzed.

Western blots. Frozen tissue samples were homogenized in RIPA buffer (crude extracts) or using the Pierce NE-PER nuclear protein extraction kit. Mitochondrial protein fractions were prepared using a mitochondria isolation kit (Pierce, Rockford, IL). Protease and phosphatase inhibitors (Pierce) were added to extraction buffers. Total protein content was measured in crude extracts, nuclear, and mitochondrial fractions by Bradford assay (Bio-Rad, Hercules, CA). Content of the proteins of interest was semi-quantified by standard Western blot technique as described previously (37, 44, 55). Primary antibodies were purchased from Abcam (TBP, catalog no. ab818; COX IV, catalog no. ab16056; Cambridge, MA). Epitomics (Nrx4, catalog no. 3174-1, Burlington, CA), Millipore (Nox2, catalog no. 07-024, Temecula, CA), Novus Biologicals (UCP2, catalog no. NB 100-59742, Littleton, CO), and Santa Cruz Biotechnology (AMPK, catalog no. sc-128; p-AMPK Thr 172, catalog no. sc-33524; Akt2, catalog no. sc-7127; p-Akt Ser 473, catalog no. sc-7985; Nrf2, catalog no. sc-13032; Santa Cruz, CA).

Cardiac enzyme activities, oxidative damage, and succinate content. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) activities were measured in crude extracts using commercial kits (Cayman Chemical, Ann Arbor, MI). Aconitase activity was measured in mitochondrial protein fractions prepared by differential centrifugation using a homogenization buffer containing sodium citrate to avoid aconitase ex vivo oxidation as described previously (37). Succinate-coenzyme Q reductase (complex II; SDH) activity was measured in heart mitochondria as previously described (37). Citrate synthase (CS) activity was measured in heart mitochondria using a commercial kit (ScienCell, Carlsbad, CA). Cardiac 4-hydroxynonenal (4-HNE) and nitrotyrosine (NT) concentrations were measured in crude extracts using ELA kits (Cell BioLabs, San Diego, CA). Succinate content was measured as previously described (54).

Nuclear factor erythroid-2-related factor 2 transcriptional activity. Binding of activated Nrf2 to the electrophile-responsive element (EpRE) was measured in nuclear extracts using a TransAM Nrf2 Transcription Factor kit (Active Motif, Carlsbad, CA). Fifteen micrograms of nuclear protein were diluted in lysis buffer supplemented with protease and phosphatase inhibitors and incubated with immobilized oligonucleotides containing the EpRE consensus binding site (5'-GTCACAGTACTCAGAAGTA-3'). The assay was performed following manufacturer's instructions.

Real-time quantitative PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Genomic DNA was eliminated by digestion with DNase I (Roche, Indianapolis, IN). First-strand cDNAs were reverse-transcribed from total DNA-free RNA (1 μg) using the QuantiTec Reverse Transcription kit (Qiagen, Valencia, CA) and oligo-dT. Real-time PCR reactions were performed for Nox2, Nox4, and Nrf2, and normalized by the mRNA expression of actin using 1 μl of cDNA for each gene. Primer sequences used are shown in Table 2.

Table 2. SBP, BM, and absolute relative heart masses from lean LETO, obese, insulin-resistant OLETF, and OLETF + ARB rats after 6 wk of treatment

<table>
<thead>
<tr>
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<th>LETO</th>
<th>OLETF</th>
<th>OLETF + ARB</th>
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<tr>
<td>SBP, mmHg</td>
<td>113 ± 4</td>
<td>146 ± 4*</td>
<td>107 ± 2†</td>
</tr>
<tr>
<td>BM, g</td>
<td>385 ± 8</td>
<td>489 ± 6*</td>
<td>437 ± 6†</td>
</tr>
<tr>
<td>Absolute heart mass, g</td>
<td>1.13 ± 0.03</td>
<td>1.38 ± 0.02*</td>
<td>1.18 ± 0.02†</td>
</tr>
<tr>
<td>Relative heart mass, g/100 g BM</td>
<td>0.294 ± 0.003</td>
<td>0.281 ± 0.005*</td>
<td>0.270 ± 0.004†</td>
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Values are means ± SE. Systolic blood pressure (SBP) and body mass (BM) taken from Ref. 39. LETO, Long Evans Tokushima Otsuka rats; OLETF, Otsuka Long Evans Tokushima Fatty rats; ARB, angiotensin receptor type 1 blocker. *Significant difference vs. LETO (P < 0.05). †Significant difference vs. OLETF (P < 0.05).
RESULTS

SBP, BM, and heart mass. Data demonstrating the development of hypertension and obesity in OLETF rats and improvement with ARB treatment have been previously published (44) but are briefly included here to substantiate the characterization of the model. SBP increased 33% in OLETF compared with LETO and was normalized by ARB treatment (Table 2). A strain effect on food intake was observed, but ARB treatment did not significantly alter intake (44). This strain effect on food intake was associated with a 27% increase in mean BM. ARB treatment decreased BM by 11% compared with OLETF (Table 2). Absolute heart mass increased 22% in OLETF compared with LETO and returned to control levels with ARB treatment (Table 2). Relative heart mass decreased 5% in OLETF compared with LETO, whereas ARB treatment reduced it an additional 4% (Table 2).

NADPH oxidase mRNA and protein expression. Transcript and protein expression of Nox2 and Nox4 were measured to assess the effects of AT1 activation on Nox-derived oxidant production and signaling in the heart of obese, insulin-resistant animals. Mean Nox2 mRNA and protein expression increased 40% in OLETF compared with LETO (Fig. 1, A and B). ARB treatment did not alter mean mRNA expression (Fig. 1B); however, mean protein content was normalized (Fig. 1B). Mean Nox4 mRNA levels increased nearly 2.5-fold in OLETF compared with LETO and were increased by an additional 75% with ARB treatment (Fig. 1D). The 25% increase in mean Nox4 protein expression was not significant (P < 0.10), but the 36% increase with ARB treatment was significant (Fig. 1E) compared with LETO. These results suggest that, although ARB treatment protects from potentially damaging oxidant
production by decreasing Nox2, it does not interfere with oxidant signaling derived from Nox4 in the heart of obese, insulin-resistant rats.

**Cardiac oxidative damage: 4-hydroxynonenal and nitrotyrosine.** Levels of 4-HNE and nitrotyrosine (NT) were measured to assess the contribution of AT1 signaling on the redox-regulated cellular antioxidant defense in the heart of obese, insulin-resistant rats. Mean Nrf2 mRNA expression increased 43% in OLETF compared with LETO, and ARB treatment further increased expression levels an additional 47% (Fig. 3D). The increase in mRNA expression did not translate into a significant increase in nuclear protein content in OLETF rats, but the increase in expression in ARB-treated rats did coincide with a 30% increase in nuclear protein content (Fig. 3, A and B). In the same way, there was no difference in Nrf2 activity between LETO and OLETF, but the increase in nuclear Nrf2 protein was associated with a 7% increase in activity in ARB-treated rats (Fig. 3C).

**Nuclear factor erythroid-2-related factor 2 and antioxidant enzyme activities.** Nuclear factor erythroid-2 related factor 2 (Nrf2) mRNA, nuclear protein expression, and activity levels were measured to assess the contributions of AT1 signaling on the redox-regulated cellular antioxidant defense in the heart of obese, insulin-resistant rats. Mean Nrf2 mRNA expression increased 43% in OLETF compared with LETO, and ARB treatment further increased expression levels an additional 47% (Fig. 3D). The increase in mRNA expression did not translate into a significant increase in nuclear protein content in OLETF rats, but the increase in expression in ARB-treated rats did coincide with a 30% increase in nuclear protein content (Fig. 3, A and B). In the same way, there was no difference in Nrf2 activity between LETO and OLETF, but the increase in nuclear Nrf2 protein was associated with a 7% increase in activity in ARB-treated rats (Fig. 3C).

**Fig. 3.** A: representative Western blot of nuclear Nrf2 protein content. Means ± SE values of cardiac nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA expression (B), nuclear Nrf2 protein expression (C), and Nrf2 activity (D) from LETO, OLETF, and OLETF + ARB rats. *Significant difference from LETO (P < 0.05). †Significant difference from OLETF (P < 0.05).

**Fig. 4.** Mean ± SE values of cardiac catalase (A), superoxide dismutase (B), and glutathione peroxidase activities (C) from LETO, OLETF, and OLETF + ARB rats. *Significant difference from LETO (P < 0.05). †Significant difference from OLETF (P < 0.05).
Activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) were measured to assess the effects of the AT1-mediated oxidative stress on antioxidant capacity in the heart of obese, insulin-resistant rats. A strain effect on mean SOD activity was not detected, but ARB treatment increased activity levels 57% (Fig. 4A). Mean catalase activity decreased 66% in OLETF compared with LETO, and ARB treatment completely recovered activity levels with an additional 57% increase (Fig. 4B). Similarly, mean GPx activity decreased 63% in OLETF compared with LETO, and ARB treatment partially recovered activity levels to 35% of LETO (Fig. 4C). These changes in antioxidant activities suggest that AT1 activation diminishes the redox-mediated, antioxidant scavenging capacity in the heart of obese, insulin-resistant rats.

Mitochondrial enzyme activities, succinate content, and uncoupling protein 2 expression. Mitochondrial citrate synthase (CS), aconitase and succinate dehydrogenase (SDH) activities, heart succinate levels, and uncoupling protein 2 (UCP2) protein expression were measured to assess the contributions of AT1 activation on mitochondrial function in the heart of obese, insulin-resistant rats. Mean activity of CS decreased 41% in OLETF compared with LETO, and was restored to LETO levels with ARB treatment (Fig. 5A). Mean aconitase activity decreased 50% in OLETF compared with LETO, and ARB treatment increased mean levels twofold compared with LETO (Fig. 5B). Mean SDH activity decreased 76% in OLETF compared with LETO, and ARB treatment completely restored activity (Fig. 5C). Mean heart succinate levels decreased 33% in OLETF compared with LETO, and again ARB treatment completely restored levels (Fig. 5D). Mean UCP2 protein expression decreased 68% in OLETF compared with LETO and were partially recovered by ARB treatment (Fig. 5, E and F). Overall, these results suggest that AT1 activation impairs mitochondrial function by targeting TCA cycle activity and uncoupling protein 2 in the heart of obese, insulin-resistant animals.

5'-Adenosine monophosphate protein kinase and Akt. The phosphorylation levels of 5'-adenosine monophosphate protein kinase (AMPK) and Akt were measured to assess the effects of ARB treatment on pathways that regulate mitochondrial function in the heart of obese, insulin-resistant rats. Mean AMPK phosphorylation levels were increased 45% in OLETF compared with LETO, and completely restored by ARB treatment (Fig. 6A). Mean Akt phosphorylation levels were decreased 33% in OLETF compared with LETO, and completely restored by ARB treatment (Fig. 6B).

Fig. 5. Mean ± SE values of cardiac citrate synthase (A), mitochondrial aconitase (B) and succinate dehydrogenase activities (C), succinate levels (D), and cardiac uncoupling protein 2 (UCP2) protein expression (E) from LETO, OLETF, and OLETF + ARB rats. F: representative Western blot for mitochondrial UCP2 expression in LETO, OLETF, and OLETF + ARB rats. *Significant difference from LETO (P < 0.05). †Significant difference from OLETF (P < 0.05).
kinase (AMPK) and protein kinase B (Akt) were measured as indicators of the contribution of AT1 activation on insulin signaling and cellular energy homeostasis in the heart of obese, insulin-resistant rats. The mean ratio of p-AMPK to AMPK was not different between LETO and OLETF, but phosphorylation levels increased 60% with ARB treatment (Fig. 6, A and B). The mean ratio of p-Akt to Akt increased 41% in OLETF compared with LETO, and ARB treatment normalized phosphorylation levels (Fig. 6, A and C). Collectively, these data suggest that AT1 activation impairs cellular energy balance, which is associated with the upregulation of insulin signaling in the heart of obese, insulin-resistant rats.

**DISCUSSION**

Oxidative stress derived from increased Nox2 is a common consequence of AT1 activation during cardiovascular and metabolic disease (25, 49, 50, 63). However, the effects of chronic AT1 activation on mitochondrial function, antioxidant capacity, and redox signaling in the heart of obese, insulin-resistant animals are not fully understood. In the present study, we demonstrated that, in addition to stimulating Nox2 and increasing oxidative damage, AT1 activation decreases cardiac redox signaling and antioxidant enzyme activities, and impairs mitochondrial function and overall cellular energy balance during insulin resistance.

Ang II signaling downstream of AT1 promotes the phosphorylation of the p47phox and p67phox cytosolic complexes required for Nox2 activation (12). Therefore, AT1 antagonism is an effective way to decrease Nox2-derived oxidative stress during glucose-induced cardiac dysfunction (41). The ability of ARB treatment to reduce Nox2 expression and recover the levels of oxidative damage in the heart suggests that the AT1-mediated oxidative damage in the heart is associated with upregulation of cardiac Nox2 in a model of metabolic syndrome. The results also demonstrated that the expression of cardiac Nox4 are not entirely associated with AT1 activation in the heart of insulin-resistant rats, despite the observed increases in the mRNA and protein expression (not statistically significant) of Nox4 in the OLETF group. These increases are substantiated by previously reported increases in mice (1, 14).

In contrast to Nox2, Nox4 is localized to intracellular membranes (2, 7, 35, 61), does not require cytosolic activators (35), is constitutively active (39, 46, 56), and produces H2O2 instead of superoxide (45, 53). Therefore, increased Nox4 expression may be beneficial during cardiac remodeling, pressure overload, or myocardial infarction (42, 45, 62), since intracellular H2O2 can activate the Nrf2-mediated antioxidant response (9, 45). This Nrf2-mediated antioxidant response efficiently counteracts Nox2-derived oxidant generation and likely contributes to the prevention of oxidative damage. However, Nox4 overexpression may also result in overproduction of H2O2, which is associated with tissue hypertrophy and pro-inflammatory responses and may contribute to cardiac impairment in insulin resistance, metabolic dysfunction, and heart failure (8, 13, 64). Therefore, the fact that AT1 blockade further increased Nox4 expression may indicate incomplete cardiac protection in this condition.

The observed relationship between increased Nox4 expression, Nrf2, and antioxidant enzyme activities in ARB-treated animals suggests that cardiac AT1 activation reduces antioxidant enzyme activities and impairs the redox-mediated cellular antioxidant defense during metabolic syndrome. Another important finding of the present study is that AT1 activation also contributes to impaired mitochondrial function as indicated by the ability of the ARB treatment to normalize the reduced activities of CS, SDH, aconitase, and succinate levels and UCP2 expression in obese OLETF rats. This is important because it demonstrates that AT1-mediated oxidative stress via upregulation of Nox2 is associated with impaired mitochondrial function, antioxidant enzyme activities, and redox signaling in the heart of insulin-resistant animals. Mitochondrial dysfunction is well documented during diabetes (4, 47) and may contribute to increased oxidant generation during metabolic syndrome due to increases in glucose oxidation in the TCA cycle. This increase in glucose oxidation can eventually result in increased superoxide production (10) or NOS uncoupling (51). Moreover, AT1 activation induces mitochondrial dysfunction by activating Nox2 via a PKC pathway (18, 59), stimulating mitochondrial ROS formation and therefore contributing to mitochondrial dysfunction (16, 21, 48, 58). The improvement of mitochondrial function, the normalization of Akt phosphorylation, and the increase in AMPK activity with ARB treatment suggest that AT1 activation contributes

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Fig. 6. A: representative Western blots of phosphorylated AMPK and Akt. Mean ± SE phosphorylation levels of cardiac 5'-adenosine monophosphate protein kinase (AMPK; B) and protein kinase B (Akt; C) from LETO, OLETF, and OLETF + ARB rats. *Significant difference from LETO (P < 0.05). †Significant difference from OLETF (P < 0.05).
to impaired insulin signaling and overall cellular energy balance in the heart during metabolic syndrome by targeting these two intracellular signaling pathways. Increased activation of AMPK with AT1 antagonism are consistent with results from in vitro models of cardiac injury (28), as well as with results from in vivo models of aging in diabetic mice (30), salt-sensitive hypertensive rats (60), and animals fed with a high-fructose (33) or high-fat diet (57). Collectively, this suite of data highlights the contribution of AT1 signaling in the heart to the manifestation of cardiovascular and metabolic disease (19, 29, 65). Thus this suite of conditions helps to define the pathological phenotype of the diabetic heart induced by AT1-mediated oxidative stress.

In summary, results of the present study suggest that AT1 activation increases Nox2-mediated oxidative damage and impairs redox signaling and mitochondrial function while altering insulin signaling and the overall energy balance in the heart during metabolic syndrome. Therefore, aside from decreasing blood pressure, AT1 antagonism may also improve cardiac function in the heart of obese, insulin-resistant animals by stimulating a redox-sensitive protective mechanism that includes the upregulation of Nrf2 and an increase in antioxidant enzyme activities.

Perspectives

The present study reveals the impact of AT1 activation in the heart of insulin-resistant rats on Nox2-derived oxidative stress and the parallel impairment of mitochondrial function, redox signaling, and cellular energy balance. The ability of ARB treatment, for the most part, to ameliorate these detriments implicates AT1 activation as a potent contributing factor in the development of metabolic derangements in the heart during the manifestation of frank diabetes. In the scope of this study, ARB treatment provides the added benefits of improved cardiac cellular metabolism, which may translate into improved cardiac function during insulin resistance. Importantly, instead of reducing the expression of Nox4, which appears to play a role in the adaptive response to oxidative stress by activating Nrf2 (9, 45), AT1 antagonism increased Nox4, providing an alternative perspective of Nox4 function that deviates from its traditional role in promoting oxidative stress. Although a mechanistic explanation of the effects of AT1 signaling on Nox4 expression is still lacking and warrants further investigation, a compensatory mechanism derived from the down-regulation of Nox2 during AT1 blockade in the insulin-resistant heart may explain the observed response. In the same way, and despite the observed associations of Nox2- and mitochondrial-derived oxidant production, insulin signaling, and cellular energetic balance, the specific mechanisms driving the impacts of cardiac AT1 activation on each of those variables during metabolic disease remain elusive. Nonetheless, this suite of measures can provide a first estimation of the representative heart phenotype of an obese, insulin-resistant animal.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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