Valsartan Protects Pancreatic Islets and Adipose Tissue From the Inflammatory and Metabolic Consequences of a High-Fat Diet in Mice

Banumathi K. Cole, Susanna R. Keller, Runpei Wu, Jeffrey D. Carter, Jerry L. Nadler, Craig S. Nunemaker

Abstract—Obesity, hypertension, cardiovascular disease, and inflammation are closely associated with the rising incidence of diabetes mellitus. One pharmacological target that may have significant potential to lower the risk of obesity-related diseases is the angiotensin type 1 receptor (AT1R). We examined the hypothesis that the AT1R blocker valsartan reduces the metabolic consequences and inflammatory effects of a high-fat (Western) diet in mice. C57BL/6J mice were treated by oral gavage with 10 mg/kg per day of valsartan or vehicle and placed on either a standard chow or Western diet for 12 weeks. Western diet-fed mice given valsartan had improved glucose tolerance, reduced fasting blood glucose levels, and reduced serum insulin levels compared with mice fed a Western diet alone. Valsartan treatment also blocked Western diet–induced increases in serum levels of the proinflammatory cytokines interferon-γ and monocyte chemotactic protein 1. In the pancreatic islets, valsartan enhanced mitochondrial function and prevented Western diet–induced decreases in glucose-stimulated insulin secretion. In adipose tissue, valsartan reduced Western diet–induced macrophage infiltration and expression of macrophage-derived monocyte chemotactic protein 1. In isolated adipocytes, valsartan treatment blocked or attenuated Western diet–induced changes in expression of several key inflammatory signals: interleukin 12p40, interleukin 12p35, tumor necrosis factor-α, interferon-γ, adiponectin, platelet 12-lipoxygenase, collagen 6, inducible NO synthase, and AT1R. Our findings indicate that AT1R blockade with valsartan attenuated several deleterious effects of the Western diet at the systemic and local levels in islets and adipose tissue. This study suggests that AT1R blockers provide additional therapeutic benefits in the metabolic syndrome and other obesity-related disorders beyond lowering blood pressure. (Hypertension. 2010;55:715-721.)

Key Words: valsartan ■ angiotensin type 1 receptor ■ inflammation ■ adipose tissue ■ macrophages ■ islets

Obesity is a metabolic disorder characterized by chronic inflammation and dyslipidemia and is a strong predictor for the development of hypertension, diabetes mellitus, and cardiovascular disease. One potential pharmacological target for treating obesity-related metabolic disorders is angiotensin II, a regulator of cardiovascular homeostasis (reviewed in Reference 1). Dysregulated angiotensin activity leads to hypertension and other cardiovascular complications. Specific inhibitors, such as the angiotensin type 1 receptor (AT1R) blockers (ARBs) and angiotensin-converting enzyme (ACE) inhibitors, have implicated this pathway also in the development of metabolic diseases, like obesity and type 2 diabetes mellitus. A meta-analysis of clinical trials with these inhibitors revealed an overall 25% reduction in new-onset diabetes mellitus.2 In addition, ARB treatment decreases fat cell volume and fat accumulation and improves differentiation of adipocytes, which is associated with a more insulin-sensitive phenotype.3–6 Thus, targeting angiotensin II may be a viable strategy in the treatment of the metabolic syndrome, type 2 diabetes mellitus, and other obesity-related disorders.

Angiotensin II is part of the renin-angiotensin system (RAS) and acts as a vasoconstrictor of blood vessels, thereby regulating blood pressure, vascular tone, and fluid and electrolyte balance. Angiotensin II is also proinflammatory in blood vessels, causing increased reactive oxygen species release, increased nuclear factor-κB nuclear translocation and subsequent proinflammatory cytokine transcription, and reduced endothelial NO synthase activity (reviewed in Reference 7). These proinflammatory pathways can be blocked by ARBs.8

In spite of clinical support for ARB and ACE inhibitor treatment in reducing diabetes mellitus onset, the protection offered to pancreatic islets and adipocytes remains unclear. Local RAS have been identified in both pancreatic and
adipose tissues and may play roles in the progression of the metabolic syndrome (reviewed in References 9 and 10). To concurrently address the in vivo effects of ARB administration on islets and adipose tissue, we orally administered valsartan to C57BL/6J mice fed either a chow or high-fat (Western) diet. We evaluated the drug’s effects on metabolism, pancreatic islet and adipocyte function, and macrophage infiltration into adipose tissue and ensuing systemic inflammation. The results suggest that valsartan ameliorates the damaging effects of high-fat feeding on pancreatic β-cell function and adipose tissue inflammation. Our study is the first to address the potential of ARB protection of both islets and adipose tissue in vivo in a high-fat diet mouse model.

Methods
Male C57BL/6J mice were treated daily by oral gavage with 10 mg/kg per day of valsartan or vehicle and placed on either a standard chow or Western diet for 12 weeks beginning at 6 to 8 weeks of age, as reported previously. Measurements of glucose and insulin tolerance, body weight, and fasting blood glucose (BG) were made before termination. Pancreas, fat, and serum were then collected for additional studies. Methodologic details are provided in the online Data Supplement (available at http://hyper.ahajournals.org). All of the experiments were performed in accordance with an animal study protocol approved by the University of Virginia Institutional Animal Care and Use Committee.

Results
Effects of Valsartan and Western Diet on Body Weight and BG
C57BL/6J mice were placed either on a chow or Western (west) diet for 12 weeks and treated orally with 10 mg/kg per day of valsartan (V) or water vehicle. Body weight and BG were measured weekly. As shown in Figure 1A, the Western diet caused significantly greater weight gain (including epididymal fat pad weight gain; see Figure 5B) by the end of the 12-week trial for both valsartan-treated and vehicle-treated mice. Neither body weights nor weight gains differed between west and west+V groups. Valsartan, thus, does not impact diet-induced weight gain or enlargement of adipose tissue.

Weekly BG measurements did not differ among treatment groups at any point throughout the 12-week trial and remained <250 mg/dL in all of the treatment groups, indicating no incidence of diabetes mellitus (mean BG at last reading: chow, 168±4; chow+V, 169±7; west, 169±6; west+V, 174±5 mg/dL). However, as shown in Figure 1B, overnight fasting BG in mice at 10 weeks on the diet showed significant increases in the west group as compared with the chow-fed groups. Nonfasted insulin levels obtained at the termination of the study were substantially increased by the high-fat diet (Figure 1C). This effect was attenuated by valsartan. Consistent with increased and attenuated insulin secretion, respectively, islet hyperplasia was present in the west group but was absent in the west+V group (Figure S1). Furthermore, the proportion of insulin-stained β-cells in the islets was significantly decreased in the west group and preserved with valsartan treatment (Figure S1).

Glucose and Insulin Tolerance
Mice were subjected to a glucose tolerance test and insulin tolerance test at weeks 10 and 11, respectively. As shown in Figure 2, chow and chow+V groups displayed similar responses to the intraperitoneal glucose challenge. The west group had higher fasting BG before the glucose bolus (P<0.05; see also Figure 1B), did not respond as quickly, and glucose remained elevated when compared with chow-fed groups (P<0.01), suggesting that the west group has impaired glucose tolerance (Figure 2). The west+V group showed a significant improvement in glucose tolerance compared with the west group but did not fully normalize to the level of controls (Figure 2). The insulin tolerance test indi-

Figure 1. Valsartan reduces fasting BG and nonfasting serum insulin in Western diet–fed mice. A, Mean body weights at the start (■) and end (○) of the 12-week diet for various treatment groups. B, Overnight fasting BG (mg/dl) and (C) nonfasting serum insulin levels (ng/ml) for each treatment group. *P<0.05 vs chow; †P<0.05 vs west. n=13 to 14 mice per group.

Figure 2. Valsartan improves glucose tolerance. BG levels were measured at the indicated time points after an intraperitoneal glucose injection. §P<0.01 by 2-way ANOVA. n=13 to 14 mice per group.
Valsartan Reduces Levels of Serum Cytokines
We obtained blood serum measurements for 32 circulating cytokines at the termination of the 12-week trial using a Luminex system (see online Data Supplement for list of cytokines, at http://hyper.ahajournals.org). As shown in Table 1, interferon (IFN-γ) -γ and monocyte chemotactic protein (MCP) 1, both key products of obesity-mediated inflammation, were significantly elevated in the west group compared with the chow group. This effect was reversed in the west+V group. In addition, macrophage inflammatory protein 1β and regulated on activation, normal T expressed and secreted (RANTES) is also called chemokine C-C motif ligand 5 (CCL5); MIP, macrophage inflammatory protein; n=7 to 8 per treatment group per cytokine. *P<0.05 vs chow; †P<0.05 vs west; ‡P<0.10 vs west.

Table 1. Effects of Valsartan on Serum Cytokine Levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Chow, pg/mL</th>
<th>Chow+V, pg/mL</th>
<th>West, pg/mL</th>
<th>West+V, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>Not detected</td>
<td>2.34±2.34</td>
<td>7.61±2.32</td>
<td>1.51±1.51</td>
</tr>
<tr>
<td>MCP-1</td>
<td>25.15±9.95</td>
<td>43.67±9.08</td>
<td>68.86±11.01</td>
<td>36.11±10.31</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>92.79±32.63</td>
<td>90.65±11.29</td>
<td>67.60±22.19</td>
<td>17.24±17.24</td>
</tr>
<tr>
<td>RANTES</td>
<td>97.81±13.73</td>
<td>93.63±7.21</td>
<td>72.28±17.36</td>
<td>53.84±11.93</td>
</tr>
</tbody>
</table>

RANTES indicates regulated on activation, normal T expressed and secreted, and RANTES is also called chemokine C-C motif ligand 5 (CCL5); MIP, macrophage inflammatory protein; n=7 to 8 per treatment group per cytokine. *P<0.05 vs chow; †P<0.05 vs west; ‡P<0.10 vs west.

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Valsartan Improves Glucose-Stimulated Insulin Secretion
Isolated islets were assessed for physiological function by measuring basal insulin secretion (3 mmol/L of glucose) followed by glucose-stimulated insulin secretion (28 mmol/L of glucose). As shown in Figure 3, islets from both west and west+V mice showed an increase in basal and glucose-stimulated insulin secretion (28 mmol/L) compared with chow-fed groups. Mice in the west+V group also showed enhanced insulin secretion in 28 mmol/L of glucose compared with mice fed a western diet alone (Figure 3). The ratio of stimulated:basal glucose (glucose stimulation index) was marginally reduced for the west group compared with the other treatment groups. These data suggest that valsartan tends to enhance glucose-stimulated insulin secretion in the context of a high-fat diet.

Valsartan Enhances Islet Mitochondrial Activity
We next examined islet mitochondrial activity because the angiotensin system may affect cell metabolism (reviewed in Reference 12). As shown in Figure 4A, increases in mitochondrial membrane potential were observed among islets from mice treated with valsartan. This result was supported by separate measurements of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Figure 4B), a known product of mitochondrial activity. Thus, valsartan clearly has a stimulatory effect on the mitochondrial gradient that drives ATP production and increases the byproducts of mitochondrial activity.

Islet Gene Expression
We examined islet expression of several key proinflammatory genes (IFN-γ, tumor necrosis factor [TNF]-α, MCP-1,
IL-12p35, and IL-12p40). The 12-week Western diet caused an increase only in IFN-γ expression (6.13-fold) that approached significance (P=0.07) and that was reduced by valsartan treatment (0.58-fold; P=0.06).

Valsartan Reduces Macrophage Infiltration Into Adipose Tissue
To investigate anti-inflammatory effects of valsartan, adipose tissue (epididymal fat pads) was collected and processed to isolate stromal vascular cells for fluorescence-activated cell sorter analysis. As shown in Figure 5A, macrophage content detected by both F4/80 and CD11b antibodies in the CD45-positive gate was nearly double in the west group compared with the chow group. However, macrophage numbers were significantly lower in the west+V group compared with the west group. As shown in Figure 5B, fat pad weights were much greater for all of the mice fed the western diet, and valsartan treatment did not reduce fat pad weight. Adipocyte size was significantly increased with Western diet, and this effect was ameliorated by valsartan (Figures 5C and S3). These findings suggest that valsartan reduces the inflammatory response caused by Western diet–induced changes in adipose tissue. The ~2-fold reduction in adipocyte size in the presence of dramatically increased fat pad weight in the west+V group suggests that valsartan increases total adipocyte number in adipose tissue.

Effects of Valsartan on Gene Expression in Visceral Adipose Tissue and Isolated Adipocytes
We also investigated the possible effects of valsartan on the expression of key proinflammatory genes in visceral fat. As shown in Table 2, a substantial increase in MCP-1 expression, a key component in macrophage signaling, was seen in epididymal fat tissue from the west group. The increase was attenuated by valsartan treatment. TNF-α and IL-6 showed a similar trend as MCP-1, but changes were not significant (Table 2). Adiponectin, an anti-inflammatory adipocyte-derived factor, showed reduced expression levels among mice on the Western diet (Table 2); valsartan did not affect adiponectin expression. Increased expression of IL-12p40 was observed in the west group when compared with the chow group (Table 2). Valsartan increased IL-12p40 in the chow and west groups to similar levels.

We also examined differences in gene expression in isolated adipocytes. Although some findings were similar to those reported for whole fat tissue, some of the differences were more pronounced, and new changes were seen in isolated adipocytes. As shown in Table 3, the west group showed significant upregulation of IL-12p35 and IL-12p40, whereas valsartan downregulated these proinflammatory cytokines in the chow and west groups. The Western diet also upregulated TNF-α, IFN-γ, and the anti-inflammatory enzyme platelet 12-LO, implicated in angiotensin regulation (Table 3; see discussion for 12-lypoxgenase [12-LO]); these effects were prevented in the west+V group. Adiponectin was significantly downregulated in the west group (Table 3); however, unlike in whole fat tissue, valsartan reversed the effects of the Western diet in isolated adipocytes. The Western diet did not significantly upregulate toll-like receptor 4, MCP-1, or IL-6 (Table 3), although valsartan treatment reduced expression for each of these genes compared with chow controls to some degree. In addition, expression of the extracellular matrix protein collagen 6 and inducible NO synthase, both upregulated in obese rodent models in an inflammation-dependent manner,13,14 are induced by the Western diet and decreased with valsartan treatment (Table 3). Finally, the AT1R was significantly upregulated by the Western diet, and the effect was abrogated with valsartan.

Table 2. Effects of Valsartan on Gene Expression in Adipose Tissue

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow</th>
<th>Chow+V</th>
<th>West</th>
<th>West+V</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>1.05±0.12</td>
<td>1.10±0.15</td>
<td>23.98±8.00*</td>
<td>5.70±1.99†</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>1.08±0.14</td>
<td>2.86±0.76*</td>
<td>1.91±0.29*</td>
<td>2.92±1.07</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.04±0.11</td>
<td>0.87±0.09</td>
<td>2.29±0.86</td>
<td>1.21±0.38</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.07±0.15</td>
<td>1.21±0.24</td>
<td>33.20±21.33</td>
<td>15.33±9.86</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1.09±0.23</td>
<td>1.31±0.21</td>
<td>0.36±0.06*</td>
<td>0.50±0.11*</td>
</tr>
</tbody>
</table>

Data are normalized to total actin and are presented as the fold change in expression compared with the chow-fed control group for each gene; n=9 to 12 per treatment group per gene.

*P<0.05 vs chow.
†P<0.05 vs west.

Figure 5. Adipose tissue macrophage counts, adipose tissue weight, and adipocyte size. A, Valsartan reduces macrophage infiltration into visceral fat as determined by fluorescence-activated cell sorter analysis; n=4 mice per group. The macrophage count was normalized to gram of starting epididymal fat tissue. B, Weights from epididymal fat pads used for measuring macrophage infiltration; n=4 mice per group. C, Average area of adipocytes from mice; n=3 to 4 mice per group. †P<0.05 vs chow; †P<0.05 vs west.
Table 3. Effects of Valsartan on Gene Expression in Isolated Adipocytes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chow</th>
<th>Chow + V</th>
<th>West</th>
<th>West + V</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p35</td>
<td>1.01±0.08</td>
<td>0.67±0.06*</td>
<td>1.93±0.16*</td>
<td>0.60±0.03‡</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>1.01±0.07</td>
<td>0.67±0.08*</td>
<td>1.31±0.12†</td>
<td>0.92±0.11§</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.03±0.15</td>
<td>0.83±0.12</td>
<td>2.35±0.38*</td>
<td>1.39±0.33</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.02±0.15</td>
<td>0.79±0.16</td>
<td>2.49±0.56†</td>
<td>0.93±0.32§</td>
</tr>
<tr>
<td>Platelet 12-L0</td>
<td>1.11±0.32</td>
<td>0.69±0.08</td>
<td>1.76±0.25</td>
<td>0.91±0.16‡</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1.02±0.12</td>
<td>1.05±0.11</td>
<td>0.55±0.08*</td>
<td>0.91±0.07‡</td>
</tr>
<tr>
<td>TLR4</td>
<td>1.01±0.05</td>
<td>0.65±0.08*</td>
<td>0.98±0.04</td>
<td>0.81±0.06§</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.02±0.12</td>
<td>0.76±0.10</td>
<td>0.60±0.04*</td>
<td>0.55±0.05*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.02±0.13</td>
<td>0.58±0.09†</td>
<td>0.65±0.10‡</td>
<td>0.37±0.05§</td>
</tr>
<tr>
<td>Collagen 6</td>
<td>1.01±0.10</td>
<td>0.03±0.03*</td>
<td>2.98±0.19*</td>
<td>1.54±0.20††</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.01±0.09</td>
<td>0.85±0.13</td>
<td>3.64±0.27*</td>
<td>1.57±0.32‡‡</td>
</tr>
<tr>
<td>AT1R</td>
<td>1.05±0.19</td>
<td>0.78±0.08</td>
<td>1.86±0.11*</td>
<td>1.11±0.21‡‡</td>
</tr>
</tbody>
</table>

Data are normalized to total actin and are presented as the fold change in expression compared with the chow-fed control group for each gene; n=4 per treatment group per gene. iNOS indicates inducible NO synthase; TLR, toll-like receptor.

÷P<0.05 vs chow.
†P<0.10 vs chow.
‡P<0.05 vs west.
§P<0.10 vs west.

Discussion

Our study provides evidence that treatment of Western diet–fed mice with the AT1R blocker valsartan significantly reduces the deleterious effects of a high-fat intake. Noticeably, Western diet–fed mice treated with valsartan exhibited improved glucose tolerance, improved insulin sensitivity (as indicated by lower serum insulin), reduced signs of visceral adipose tissue inflammation and macrophage infiltration, and improved pancreatic islet function compared with mice fed a Western diet alone. These data suggest that the RAS plays a key role in modulating metabolism and the inflammatory response in response to a high-fat diet (see Figure S4).

When challenged with a high-fat diet, free fatty acids and inflammatory mediators are secreted by adipocytes and stromal inflammatory cells in the adipose tissue. These factors are released into the circulation to exert systemic effects. Elevated MCP-1 is associated with obesity, diabetes mellitus, and cardiovascular disease. MCP-1, the strongest known chemotactic factor for monocytes, is responsible for macrophage infiltration into the adipose tissue, leading to further cytokine expression, such as TNF-α, and thereby promoting inflammation. MCP-1 expression is increased by angiotensin II and reduced by RAS blockade. Consistent with these earlier findings, we observed that, in the context of a high-fat diet, valsartan reduced systemic and local adipose tissue MCP-1 levels and macrophage infiltration into the visceral adipose tissue. This may be one key mechanism by which valsartan reduces adipose tissue inflammation. We observed that MCP-1 was upregulated by a Western diet in total adipose tissue and not in isolated adipocytes, whereas proinflammatory cytokines were upregulated in isolated adipocytes and not in total adipose tissue. This is consistent with reported observations that angiotensin II induces MCP-1 expression in preadipocytes and the macrophage-containing stromal vascular pool and not in adipocytes, whereas MCP-1 induces the expression of proinflammatory cytokines in adipocytes.

Central obesity correlates with an increased risk of developing type 2 diabetes mellitus. Adipose tissue dysfunction leads to increased release of proinflammatory mediators, which, in turn, impair insulin signaling and pancreatic β-cell function. For example, MCP-1, IFN-γ, IL-6, IL-12, and inducible NO synthase act systemically to impair whole body insulin sensitivity, and acute expression of a dominant-negative MCP-1 in wild-type C57BL/6J mice fed a high-fat diet or db/db mice can reverse insulin resistance. Indeed, in our study, the mice fed a Western diet exhibited increased MCP-1, IFN-γ, IL-12, and inducible NO synthase expression in serum and adipose tissue and impaired glucose tolerance and insulin sensitivity, and these changes were normalized by valsartan treatment. Adiponectin is an adipocyte-specific adipokine that improves insulin sensitivity by increasing energy expenditure and fatty acid oxidation. However, MCP-1 activation can decrease adiponectin expression.

Type 2 diabetics often exhibit decreased levels of adiponectin, and insulin sensitivity is improved by adiponectin therapy. Treatment of hypertensive patients with ARBs leads to increased serum adiponectin levels. We observed that treatment of Western diet–fed mice with valsartan ameliorated the diet-induced decrease in adiponectin in adipocytes, providing further support for a therapeutic role of AT1R blockade in restoring insulin sensitivity.

Angiotensin II primarily acts via the G protein-coupled transmembrane receptor AT1R (reviewed in References 1 and 7). In our study, we observed that AT1R expression in adipocytes, which express all of the components of the RAS, was significantly upregulated when mice were fed a Western diet, and this effect was reversed with valsartan administration. This is the first report that a high-fat diet leads to increased AT1R expression in adipocytes and that this can be prevented by ARB treatment. High-fat diet–induced adipocyte hypertrophy leads to fibrosis of adipose tissue, which promotes shear stress and inflammation. Consistent with this, we observed that collagen 6 is upregulated in adipocytes from Western diet–fed mice, and this is ameliorated by valsartan treatment. Studies using valsartan have demonstrated decreases in adipocyte size and total visceral fat mass in rodents. We observed a decrease in adipocyte size when Western diet–fed mice are treated with valsartan. This is consistent with the idea that AT1R blockade improves adipocyte function by promoting the development of smaller and more metabolically efficient adipocytes. No changes were observed in body weight or epididymal fat pad weight between the west and west + V groups in our study. This may be explained by differences in the dosage and duration of valsartan treatment in the previous studies, suggesting that longer valsartan treatment in our mice
may eventually decrease adipose tissue weight. This is of clinical relevance, because short-term valsartan administration in humans did not improve β-cell function and insulin responsiveness. Thus, longer therapeutic ARB treatment in humans may be needed for metabolic improvement.29

The existence of a local RAS in the pancreatic islets is gaining increasing recognition, and this RAS is induced by physiological and pathophysiological stimuli (reviewed in Reference 9). The pancreatic islet RAS regulates endocrine and exocrine functions relevant to the metabolic syndrome, such as local islet blood flow, islet β-cell proinsulin biogenesis, glucose-stimulated insulin release, mitochondrial function, and pancreatic duct and acinar cell secretion (reviewed in References 9 and 12). Angiotensin II impairs islet blood flow and can directly impair glucose-mediated insulin release.30 It also promotes mitochondrial oxidant release leading to decreased energy metabolism in the islets. Dysfunctional mitochondria contribute to the pathophysiology of diseases, such as hypertension, cardiovascular disease, and diabetes mellitus (reviewed in Reference 12). In support of a role for the islet RAS in mediating β-cell function, we observed that valsartan decreased islet hyperplasia, enhanced mitochondrial activity, decreased IFN-γ expression, and mildly enhanced insulin secretion in islets. Similarly, islets from the obesity-induced type 2 diabetes db/db mouse model exhibited upregulation of RAS components and decreased β-cell function, and treatment with the ARB losartan improved glucose-induced insulin release from these islets.31 This improvement may occur in part through reduction of the local islet inflammatory response in the presence of ARB.32,33 However, it is possible that ARB effects in islets may be secondary to reduced inflammation in visceral adipose tissue and reduced systemic inflammation. This is consistent with the lack of altered gene expression of components of the pancreatic RAS (data not shown).

Finally, our data indicate that ARB administration may target members of the fatty-acid metabolizing 12-LO family, because we observed decreased expression of the platelet form of 12-LO in adipocytes of valsartan-treated mice. We reported recently that 12-LO products promote an inflammatory response and impair insulin signaling in adipocytes.34 Moreover, 12-LO activation plays a significant role in promoting β-cell toxicity, inflammation, and atherosclerosis;13 (reviewed in Reference 35). The major arachidonic acid metabolite of 12-LO, (S)-hydroxyeicosatetraenoic acid, enhances AT1R expression in type 2 diabetic rat glomeruli36 and is secreted in patient urine during the early diabetic process (reviewed in Reference 35). Angiotensin II increases expression of 12(S)-hydroxyeicosatetraenoic acid in porcine vascular smooth muscle cells and fails to elicit vascular responses in 12-LO–deficient mice (reviewed in References 35 and 37). The ARB drugs valsartan and losartan decrease 12(S)-hydroxyeicosatetraenoic acid expression in streptozotocin-induced diabetic mice and 12-LO expression in obese Zucker rat renal cortical tissue, respectively.38,39 Thus, AT1R may be a regulator of 12-LO activity and is also regulated by 12-LO activity. Establishing the role that 12-LO plays in mediating RAS activity and ensuing complications will require additional studies.

**Perspectives**

In summary, AT1R blockade with valsartan significantly ameliorates several detrimental effects of a high-fat diet in mice: it reduces the inflammatory response, improves glucose tolerance, and offers protection for islet and adipose tissue (see Figure S4). Our study is unique in that we concurrently examined 2 key tissues targeted in the progression of the metabolic syndrome: type 2 diabetes mellitus and other obesity-related disorders. Our results indicate that angiotensin II blockade may be an effective therapeutic strategy for the treatment of these diseases.

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**Disclosures**

None.

**References**


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[Title]
VALSATARN PROTECTS PANCREATIC ISLETS AND ADIPOSE TISSUE FROM THE INFLAMMATORY AND METABOLIC CONSEQUENCES OF A HIGH-FAT DIET IN MICE

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Methods

Animals and treatments

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free facility at the University of Virginia (UVA) for one week prior to treatments. Mice were weighed and divided into four treatment groups of equal numbers: chow diet (chow), chow diet + valsartan (chow+V), western diet (west), or western diet + valsartan (west+V). Treatment began at 6-8 weeks of age as previously reported (1). Valsartan, or water vehicle alone, was delivered daily by oral gavage at a dose of 10 mg/kg/day using 12.5 µL of 0.8 mg/ml of valsartan in water per gram of body weight. Valsartan doses were revised once per week to adjust for weight gain. Treatments (valsartan and diet) were continued for 12 weeks for all mice. Two trials were performed under identical experimental conditions with each trial containing 6-8 mice per treatment group. All experiments were performed in accordance with an animal study protocol approved by the UVA Institutional Animal Care and Use Committee.

The chow and western diet foods were purchased from Harlan Teklad (Madison, WI); the western diet consisted of 42% of calories from fat, 15.3% of calories from protein, and 42.7% of calories from carbohydrate, primarily sucrose (TD#88137). During treatment period blood glucose and body weights were measured weekly before 10 AM under random-fed (ad libidum) conditions to not interrupt normal eating behavior. Blood glucose was measured using Accu-Chek Advantage Glucose Monitors (Roche Applied Science, Indianapolis, IN) in tail vein blood samples. Body weight was measured by placing the mice on a scale using the same scale for each set of measurements.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

The GTT and ITT were performed by the UVA Animal Characterization Core following their approved protocols and as described by others (1-2). For testing glucose tolerance, mice were fasted overnight and then injected intraperitoneally (IP) with glucose (2 g/kg). Blood glucose measurements were performed with an UltraTouch glucometer using blood samples taken from cut tail tips at baseline and at 10, 20, 30, 60, 90, and 120 minutes after the injection of glucose. The ITT was performed in random-fed mice by IP injection of insulin (0.75 U/kg) in 0.9% NaCl beginning at 2 PM. A tail vein blood sample was taken immediately prior to and 15, 30, 45, and 60 minutes after the injection for determination of blood glucose levels. GTT and ITT were performed on the same set of mice eight days apart.

Serum cytokine measurements

Blood samples from random-fed mice were obtained by cardiac puncture following euthanization. The samples were centrifuged to isolate serum, frozen, and shipped to Millipore (Billerica, MA). Cytokine concentrations were determined using a mouse cytokine/chemokine LINCOplex mouse 32-plex cytokine array, according to the manufacturer’s protocol. Cytokines measured included Eotaxin, G-CSF, GM-CSF, INF-γ, IL-1α, M-CSF, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-13, IL-15, IL-17, IP-10, MIP-2, KC, LIF, LIX, MCP-1, MIP-1α, MIP-1β, MIG, RANTES, TNF-α, IL-12p70, and VEGF.
**Islet isolation**

Mouse pancreatic islets were isolated following euthanization as described previously (3). Briefly, the pancreas was perfused through the common bile duct with 5 mL of 1.4 mg/mL collagenase P (Roche Applied Science), then removed and incubated at 37°C for 8-11 minutes in 1 mL Hank’s buffered salt solution. Following incubation, pancreatic tissue was centrifuged, resuspended in Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), and centrifuged again to separate islets from acinar tissue. All islets were incubated overnight in RPMI-1640 medium to allow sufficient recovery time from collagenase digestion before any experiments were performed.

**Glucose-stimulated insulin secretion**

Islets were tested for insulin secretion as described previously (1). Briefly, islets were preincubated at 37°C and 5% CO2 for 1 hour in Krebs Ringer HEPES Bicarbonate (KRHB) buffer (120 mmol/L NaCl, 4 mmol/L KH2PO4, 1 mmol/L MgSO4, 1 mmol/L CaCl2, 10 mmol/L NaHCO3, and 30 mmol/L HEPES, pH 7.4), then washed and incubated in KRHB supplemented with 3 mmol/L glucose for 1 hour followed by a 1 hour treatment with KRHB containing 28 mmol/L glucose. The supernatant was collected after each treatment, and insulin concentration in the supernatant was measured by an EIA method (Mercodia, Uppsala, Sweden) with a mouse insulin standard. Intra-assay variation was <5% and interassay variability <10%.

**Islet mitochondrial activity**

To measure changes in mitochondrial membrane potential (as described in (4)), islets were loaded with 5 µmol/L rhodamine 123 for 15-20 minutes and then imaged with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Japan) attached to an Olympus BX51WI fluorescence microscope (Olympus, Tokyo, Japan) using 488 nm excitation and 510 nm emission light. During each recording, islets were perifused in a small volume chamber (Warner Instruments, Hamden, CT) with KRHB buffer using a peristaltic pump (Gilson, France) at ~35°C by an in-line heater (Warner Instruments). Data were analyzed with IP Lab software Version 4.0 (Scanalytics, Rockville, MD).

To assess mitochondrial metabolism, islets were washed and maintained in freshly made KRHB. The culture medium was then replaced with KRHB containing 0.1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) with or without 28 mmol/L D-glucose (Sigma-Aldrich) for 1 hour. After removal of medium and washing of islets, insoluble MTT metabolites within cells were extracted by isopropanol (Sigma-Aldrich). The absorbance of the extract was measured as arbitrary OD units at 590 nm by a spectrophotometer, SpectraMAXplus (Molecular Devices, Sunnyvale, CA).

**Islet and adipocyte morphological analysis**

Pancreata and epididymal visceral fat tissues were isolated and fixed with 4% paraformaldehyde for 24-30 hours at room temperature and embedded in paraffin. 5-µm-thick paraffin-embedded tissue sections were then deparaffinized and rehydrated in graduated alcohol in distilled water.

For pancreas sections, antigens were retrieved using a high-temperature antigen-unmasking technique (Antigen Unmasking Solution; Vector
Laboratories, Burlingame, CA). The sections were then incubated for 30 minutes at room temperature with diluted normal blocking serum (Vector Laboratories) and stained at 4°C overnight with anti-insulin (Abcam, Cambridge, MA) followed by Cy-3-conjugated goat anti-guinea pig (Jackson Immunoresearch Laboratories, West Grove, PA) antibodies. Two-dimensional images were acquired using a Zeiss Plan-Apochromat 10X, 0.45 numerical aperature dry objective lens on a Zeiss Axio Observer.Z1 microscope with AxioVision Rel. 4.7 software and a Zeiss AxioCamMR3 camera (Carl Zeiss, Oberkochen, Germany). All insulin-stained islets from each section were measured for islet area. The percentage of insulin-secreting beta-cell area per islet area was determined from 6 islets of similar size per mouse pancreas and 3 mice per treatment group. Islet area and insulin-staining were quantified using ImageJ (NIH website: http://rsb.info.nih.gov/ij/download.html)

Visceral fat tissue sections were counterstained with hematoxylin and eosin and two representative images were acquired per section, three sections per sample, using a Zeiss Plan-Apochromat 20X, 0.8 numerical aperature dry objective lens. Adipocyte area was quantified using ImageJ.

Final images were prepared with Adobe Photoshop CS3 Extended, version 10.0.1.

**Isolation of adipocytes for RNA preparation and stromal vascular cells (SVCs) for flow cytometry**

Samples of adipose tissue were digested with collagenase for isolation of adipocytes and SVCs as described in (5-6) with minor modifications. Adipose tissue was isolated and minced into fine pieces in KRHB buffer containing 3 mmol/L glucose, 20 mmol/L adenosine, and 10 mg/ml bovine serum and collagenase (1 mg/ml). Collagenase digestion was performed at 37°C for 1 hour in a shaking water bath. Once digestion of adipose tissue was complete, the cell suspension was filtered through a 0.4 mm Nitex nylon mesh (Sefar America Inc., Kansas City, MO) and adipocytes allowed to float and processed for RNA extraction.

The infranatant was removed and centrifuged at 500 g for 5 minutes to pellet the SVCs. The pellet was resuspended in erythrocyte lysis buffer and incubated for 5 minutes. The erythrocyte-depleted SVCs were centrifuged at 500 g for 5 minutes and the pellet resuspended in fluorescence-activated cell sorting (FACS) buffer for subsequent FACS analysis. Counted SVCs were incubated in the dark with fluorophore-conjugated primary antibodies for 30 minutes at room temperature. Antibodies used in these studies included: CD-11b-FITC (Serotec, Bavaria, Germany), CD45-PerCP (BD Pharmingen, San Jose, CA), and F4/80-APC (Serotec). Cells were washed two times with 2 ml FACS buffer and analyzed on a FACSCalibur using CellQuest software. The macrophage content was defined by both F4/80 and CD11b positive in CD45 positive gate. The macrophages were determined per gram of adipose tissue used as starting material.

**RNA extraction and real-time PCR**

The isolated adipocytes were washed four times with the buffer in which digestion was performed and separated after each wash by flotation with the exception of the last wash after which cells were separated by centrifugation at 200 g for 2 minutes. TRIzol (Invitrogen, Carlsbad, CA) was added to the isolated adipocytes and lysates prepared by pulling the cells in TRIzol ten times through an 18 gauge needle attached to a
syringe. RNA was subsequently extracted according to manufacturer’s instructions. RNA from islets and fat tissue was prepared using the RNeasy kit (Qiagen, Valencia, CA) and RipoPure kit (Ambion, Austin, TX), respectively. Generation of cDNA from RNA and quantitative measurement of PCR products derived from cDNA was performed as described previously (7).

CDNA was made from 5 µg of total RNA using MMLV reverse transcriptase (Invitrogen) in 20 µL reaction volume using random hexamers (Invitrogen). For quantitative measurement of PCR products, 3 µL of the cDNA reaction (five-fold diluted) was used as template for PCR with Jump Start Taq-Polymerase (Sigma-Aldrich) in a reaction volume of 25 µL for PCR. Taqman probes for all genes, except collagen 6, were purchased from Applied Biosystems (Carlsbad, CA) and real-time PCR was performed according to manufacturer’s instructions. Primers specific for collagen 6 alpha 3 were used: F: 3’-GATGAGGCTGAAGTGAGGA-5’, R: 3’-CAGCACGAAGAGGATGTCAA-5’ and performed at an annealing temperature of 60ºC. All thermal cycling was performed using the CFX96 Thermal Cycler (Bio-Rad, Hercules, CA). All reactions were performed in triplicate and the data was normalized to the actin or GAPDH housekeeping gene and evaluated using the 2^ΔΔCT method. Expression levels are presented as fold induction/downregulation of transcripts of respective genes relative to control.

Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). All data are presented as mean ± SEM, unless otherwise stated. GTTs and ITTs were assessed by two-way ANOVA for time- and treatment-dependent differences between groups. One-way ANOVA followed by a Newman-Keuls post-test was used for comparing all groups unless otherwise stated. A P-value of <0.05 was considered to indicate statistically significant differences.
References


Figure S1. Islet area and insulin content. Consistent with effects of western diet, islet hyperplasia and reduced insulin content were observed in the west group but not in the west+V group. (A) Mean islet areas estimated by measuring all insulin-positive islets from pancreatic sections. #P=0.07. N=3-4 sections per group. (B) Percentage of the insulin-stained area of the total islet area is given. ***P<0.001 vs chow; ††P<0.001 vs west. N=18 islets per treatment group. (C-F) Representative images of insulin-stained islets from each experimental group: (C) chow, (D) chow+V, (E) west, (F) west+V. Scale bar = 25µm.
Figure S2. Insulin tolerance. Blood glucose levels were measured at the indicated time points following an insulin injection. Differences were observed in insulin tolerance tests between chow and chow+V, *P<0.05 by two-way ANOVA, but not between west and west+V mice. Although non-fasting serum levels were higher in the west compared to the west+V group, indicating better insulin sensitivity in the west+V mice, insulin concentrations achieved in the insulin tolerance test are supraphysiological and may overcome the insulin resistance present in the west group. N=13-14 mice per group.
Figure S3. Valsartan reduces western diet-induced increase in adipocyte size. Representative images of hematoxylin and eosin stained epididymal adipose tissue sections from each experimental group: (A) chow, (B) chow+V, (C) west, (D) west+V. Scale bar = 25µm.
Figure S4. A schematic representing the anti-inflammatory action of valsartan. High-fat diet-stimulation of the renin-angiotensin system (RAS) leads to angiotensin type 1 receptor (AT1R) activation, subsequent expression of pro-inflammatory cytokines, and ensuing islet and adipose tissue dysfunction. Valsartan inhibits AT1R activity and subsequent inflammatory and metabolic consequences induced by a high-fat diet.