Tissue-Specific Impairment of Insulin Signaling in Vasculature and Skeletal Muscle of Fructose-Fed Rats

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The relation between insulin resistance/hyperinsulinemia and cardiovascular diseases has attracted much attention. Insulin affects not only glucose metabolism, but also protein synthesis and cell growth. Insulin stimulates both the phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways, but the relationship between cardiovascular disease and selective insulin signal pathways is unclear. We investigated the tissue specificity and intracellular signal transduction selectivity of insulin resistance in the vasculature and skeletal muscle of fructose-fed rats (FFR). Sprague-Dawley rats were fed either normal rat chow (control rats) or fructose-rich chow. Normal saline with or without 1,000 (μg/kg) insulin was injected, and then the thoracic aorta or soleus muscle was removed under anesthetization. Insulin-induced tyrosine phosphorylation of insulin receptor β subunit (IR β) and insulin receptor substrate-1 (IRS-1) and tyrosine/threonine phosphorylation of p44/42 MAPK (ERK-1/2) were evaluated. There were no significant differences in the degree of phosphorylation of IR β or ERK-1/2 in the thoracic aorta or in the soleus muscle between FFR and controls. However, tyrosine phosphorylation of IRS-1 in the soleus muscle of FFR was significantly reduced to 80% (p < 0.001) of that in controls. The results suggest that PI3-K pathway in skeletal muscle is selectively impaired in FFR, and this impairment may induce hyperinsulinemia, which in turn may stimulate the MAPK pathway and lead to atherosclerosis. Thus PI3-K pathway may be one of the factors underlying the onset of cardiovascular disease in patients with insulin resistance. (Hypertens Res 2003; 26: 169–176)

Key Words: insulin resistance, insulin receptor β subunit, insulin receptor substrate-1, extracellular-regulated protein kinase, atherosclerosis

Introduction

Insulin resistance and hyperinsulinemia are frequently found in patients with hypertension (1). These impairments in glucose metabolism are commonly associated with diabetes mellitus, hypertension and dyslipidemia, which are high risk factors of cardiovascular diseases, and recent evidence has indicated that they may play a role in the development of cardiovascular disease in particular (2–4).

Insulin affects not only aspects of glucose metabolism, such as glucose transport and glycogen synthesis, but also gene expression, protein synthesis, and cell growth. It is well known that insulin stimulates both the phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways (5–8). The binding of insulin to its specific receptor stimulates the receptor’s intrinsic tyrosine kinase activity, resulting in autophosphorylation of the insulin receptor β subunit (IR β), which activates tyrosine phosphorylation of insulin receptor substrates such as insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2), and Shc. Tyrosine-phosphorylated IRS-1 and IRS-2 bind to the SRC homology 2 (SH2) domains of intracellular proteins, including the p85 regulatory subunit of PI3-K, Grb2, the
SH2 domain containing protein tyrosine phosphatase (SHP2), and Nck. Activation of the PI3-K pathway by insulin is related to steps in aspects of the stages of glucose metabolism, such as glucose transporter 4 (GLUT 4) translocation to the plasma membrane. Tyrosine-phosphorylated Shc and IRS proteins bind to Grb2 and then activate the MAPK pathway, thereby stimulating gene expression and cell growth.

Both insulin resistance and hyperinsulinemia have been reported to be independent risk factors for cardiovascular diseases. Jiang et al. reported that there was selective insulin resistance to the PI3-K pathway but not to the MAPK pathway in the vascular tissues of obese Zucker rats (9). However, they examined selective insulin resistance only in the vasculature; they did not investigate tissue specificity. Since skeletal muscle plays an important role in glucose metabolism, we hypothesize that the PI3-K pathway, which is related to glucose metabolism, is selectively impaired in skeletal muscle and that this impairment induces hyperinsulinemia, which in turn stimulates the MAPK pathway in the vasculature, resulting in atherosclerosis. In this study, we used fructose-fed rats (FFR). FFR show an acquired form of insulin resistance and hypertension that is diet-induced, but they do not show obesity. The aim of this study was to elucidate the tissue specificity and intracellular signal transduction selectivity of insulin resistance in the vasculature and skeletal muscle of FFR in vivo.

Methods

Materials

Human recombinant insulin (Humalin R) was purchased from Eli Lilly Japan Corp. (Kobe, Japan). A polyclonal antibody against IRβ was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA), and a polyclonal antibody against IRS-1 was obtained from Upstate Biotechnology (Lake Placid, USA). A monoclonal antibody against phosphotyrosine (PY20) was obtained from Transduction Laboratories (Lexington, USA). A polyclonal antibody against p44/42 MAPK and phosphorylated MAPK were obtained from Cell Signaling Technology (Beverly, USA). Rabbit anti-mouse immunoglobulin antibody was obtained from DAKO Corp. (Kyoto, Japan). Donkey anti-rabbit immunoglobulin horseradish peroxidase-linked antibody and an enhanced chemiluminescence (ECL) kit were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, USA). Protein A-Sepharose 6MB was from Pharmacia Biotech AB (Uppsala, Sweden).

Animals

All procedures were in accordance with institutional guidelines for animal research. Six-week-old male Sprague-Dawley rats (Charles River Japan Inc., Yokohama, Japan) were used for the experiments. Before any manipulation, all rats were fed standard rat chow containing 60% vegetable starch, 5% fat, and 24% protein (Oriental Yeast Co., Tokyo, Japan). They were maintained on a 12-h light/dark cycle and were given water and chow ad libitum. The rats were acclimated to handling before randomization and then divided into two groups at the start of the study: those fed a standard chow (control rats) and those given a fructose-rich chow (containing 60% fructose, 5% fat, and 20% protein; No.78463; Teklad, Madison, USA) (FFR) for 6 weeks.

Blood Pressure and Heart Rate Measurements

Systolic blood pressure (SBP) and heart rate (HR) were measured in all conscious rats using the indirect tail-cuff method (BP-98A; Softran, Tokyo, Japan) on a 37°C preheated cloth jacket for about 10 min. The averages of five such recordings were taken as the individual SBP and individual HR.

Euglycemic Hyperinsulinemic Glucose Clamp Technique

At the end of the treatment period, each rat was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The right common carotid artery and the right jugular vein were exposed and then cannulated with a polyethylene tube (PE50; Becton Dickinson and Co., Sparks, USA) for collecting blood samples and administration of insulin and glucose. The technique used for the glucose clamp was the same as that described previously (10). Briefly, on the day after an overnight fast of approximately 12 h, each conscious rat was placed in a foam plastic jacket that allowed movement of all four limbs and forward vision. At the start of the glucose clamp, the fasting blood glucose concentration was measured, and the initial load of insulin (25 mU/kg of humalin R, U-40; Eli Lilly Japan Corp.) was infused by bolus injection, followed by an infusion of insulin at a rate of 4 mU·kg⁻¹·min⁻¹ for 150 min. During the glucose clamp, 12.5% glucose solution was infused as needed to maintain blood glucose at the basal level. Ten microliters of arterial blood was sampled at 7-min intervals for determination of blood glucose level. The average of the rate of glucose infusion for the last 35 min was taken as the index of insulin sensitivity (M value) of each rat.

Biochemical Measurements

Blood glucose levels were measured by the glucose oxidase method in an ExacTech 2A glucose analyzer (MediSense, Inc., Waltham, USA). Fasting Plasma insulin concentration was assayed using a rat insulin radioimmunoassay kit (Linco Research, Inc., St. Charles, USA).

Isolation of the Thoracic Aorta and Soleus Muscle

Food was withdrawn 14 h before experiments were per-
Table 1. Characteristics of Rats at 12 Weeks of Age in Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>SBP (mmHg)</th>
<th>HR (/min)</th>
<th>FBG (mmol/l)</th>
<th>FPI (pmol/l)</th>
<th>M value (mg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>386 ± 8</td>
<td>132 ± 2</td>
<td>319 ± 6</td>
<td>5.2 ± 0.2</td>
<td>117 ± 39</td>
<td>17.1 ± 1.2</td>
</tr>
<tr>
<td>FFR (n = 10)</td>
<td>394 ± 6</td>
<td>144 ± 3*</td>
<td>334 ± 7</td>
<td>4.8 ± 0.2</td>
<td>323 ± 51*</td>
<td>12.1 ± 0.8*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *p < 0.01 vs. control; †p < 0.001 vs. control. FFR, fructose-fed rats; BW, body weight; SBP, systolic blood pressure; HR, heart rate; FBG, fasting blood glucose; FPI, fasting plasma insulin. FFR showed significantly lower M value and higher fasting insulin level and SBP than in control rats.

Result

Animal Characteristics

There were no significant differences in body weights, heart rates or fasting blood glucose levels between the FFR and control rats. SBP was significantly higher in FFR. Fasting insulin level was also significantly higher in FFR than in control rats. The average rate of glucose infusion during the last 35 min of glucose clamp, which was used as an M value, was significantly lower in FFR than in control rats (Table 1).

Time Courses of Phosphorylation of MAPK in the Thoracic Aorta and IRS-1 in the Soleus Muscle Following Bolus Injection of Insulin

Each rat was injected into the right jugular vein with 1 ml of normal saline (0.9% NaCl) with or without 1,000 µg/kg insulin. At different time points, the thoracic aorta and soleus muscle were excised and placed on a PBS-precooled plate. The aorta tissue was dissected from the adherent fat and connective tissues on ice, and then frozen in liquid nitrogen.

Immuno precipitation of IRβ and IRS-1

The supernatant was precipitated with antibodies against IRβ and IRS-1 overnight and then incubated with 60 µl of protein A-Sepharose 6MB for 2 h at 4°C. The beads were washed 3 times with lysis buffer, then Laemmli buffer was added and the beads were heated in boiling water for 4 min. The protein extracts were used for Western immunoblotting.

Western Immunoblotting

Immunoprecipitated protein extracts were separated by SDS-PAGE, electrotransferred to PVDF membranes, and immunoblotted. To detect tyrosine phosphorylation of IRβ and IRS-1, the blots were first incubated with monoclonal anti-phosphotyrosine antibody (1 µg/ml) overnight, then with rabbit anti-mouse antibody (1:1,000 dilution) for 2 h, and finally with [125I]protein A for 1 h at room temperature. To detect protein levels of IRβ and IRS-1, blots were first incubated with polyclonal anti-IRβ and anti-IRS-1 antibodies, respectively, and then incubated with [125I]protein A for 1 h at room temperature. All blots were washed with TBS-T for 1 h at room temperature after each incubation, and [125I]protein A-bound proteins were detected and quantified by a phosphoimager using BAS2000 Image File Manager software (version 3.0). To detect tyrosine phosphorylation of p44/42 MAPK (ERK-1/2) and to detect protein levels, total lysates (30 µg protein) from thoracic aorta and soleus muscle samples were separated with 12.5% SDS-PAGE and electrotransferred to PVDF membranes, which were incubated with anti-phospho (Thr202/Tyr204)-ERK-1/2 antibodies (1:1,000 dilution) and anti-ERK-1/2 antibodies (1:1,000 dilution), respectively, overnight at 4°C. Each PVDF membrane was incubated with horseradish peroxidase-linked anti-rabbit immunoglobulin antibodies (1:2,000 dilution) for 1 h at room temperature, and then phosphorylation and protein levels were detected by ECL. All blots were washed with TBS-T for 1 h at room temperature after each incubation.

Statistical Analyses

Analysis of variance was performed for statistical analysis, and p values less than 0.05 were considered to be statistically significant. Data are expressed as the means ± SEM.
muscle were excised and treated as described above to derive protein extracts for use in immunoprecipitation and Western immunoblotting. The extent of tyrosine/threonine phosphorylation of ERK-1/2 in the thoracic aorta was maximal at 10 min after the bolus injection of insulin, and the extent of tyrosine phosphorylation of IRS-1 in the soleus muscle was maximal at 90 s after the bolus injection of insulin (Fig. 1). The extent of tyrosine phosphorylation of IRβ in the thoracic aorta was maximal at 5 min. The extent of tyrosine phosphorylation of IRS-1 in the thoracic aorta was maximal at 15 min, and the extent of tyrosine/threonine phosphorylation of ERK-1/2 in the soleus muscle was maximal at 10 min post-injection (data not shown). These time points were therefore adopted for the examination of phosphorylation.

Protein Levels and Tyrosine Phosphorylation of IRβ in the Thoracic Aorta and Soleus Muscle

There were no significant differences in IRβ protein levels in the thoracic aorta or soleus muscle between FFR and control rats. Tyrosine phosphorylation of IRβ was increased by stimulation with insulin in both the thoracic aorta (by 5.3 fold in control rats and by 6.0 fold in FFR compared with the respective basal levels) and the soleus muscle (by 7.3 fold in control rats and by 7.9 fold in FFR compared with the respective basal levels), but there were no significant differences in the degree of phosphorylation of IRβ in either the thoracic aorta or soleus muscle between FFR and control rats (Fig. 2).

Fig. 1. Time courses of tyrosine/threonine phosphorylation of ERK-1/2 in the thoracic aorta (a) and tyrosine phosphorylation of IRS-1 in the soleus muscle (b) following a bolus injection of insulin. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with ERK-1/2 antibody or tyrosine/threonine phosphorylated ERK-1/2 antibody. Equal amounts of protein were subjected to immunoprecipitation with αIRS-1 antibody and immunoblotted with αIRS-1 antibody or αPY antibody.

Fig. 2. Protein levels and tyrosine phosphorylation of IRβ in the thoracic aorta (a) and soleus muscle (b). Equal amounts of protein were subjected to immunoprecipitation with αIRβ antibody and immunoblotted with αIRβ antibody (top panel) or αPY antibody (middle panel). Data (mean ± SEM; n = 5) are expressed as a percentage of the level (100%) in control rats administered insulin.
Protein Levels and Tyrosine Phosphorylation of IRS-1 in the Thoracic Aorta and Soleus Muscle

There were no significant differences in IRS-1 protein levels in the thoracic aorta or soleus muscle between FFR and control rats. Tyrosine phosphorylation of IRS-1 was increased by stimulation with insulin in the thoracic aorta (by 1.4 fold in control rats and by 1.5 fold in FFR compared with the respective basal levels), and the degree of phosphorylation of IRS-1 in the thoracic aorta was not significantly different between FFR and control rats. Although stimulation with insulin did not alter the extent of phosphorylation of IRβ in the soleus muscle of FFR compared with that in control rats, tyrosine phosphorylation of IRS-1 in the soleus muscle of FFR was reduced to 80 ± 4% (p < 0.001) of that in control rats (Fig. 3).

Protein Levels and Tyrosine/Threonine Phosphorylation of ERK-1/2 in the Thoracic Aorta and Soleus Muscle

There were no significant differences in ERK-1/2 protein levels in the thoracic aorta or soleus muscle between FFR and control rats. After stimulation with insulin, tyrosine/threonine phosphorylation of ERK-1/2 increased in the thoracic aorta (by 1.2 fold in control rats and by 1.3 fold in FFR compared with the respective basal levels) and in the soleus muscle (by 1.2 fold in control rats and by 1.2 fold in FFR compared with the respective basal levels), but there were no significant differences in the degree of tyrosine/threonine phosphorylation of ERK-1/2 in the thoracic aorta or soleus muscle between FFR and control rats (Fig. 4).

Discussion

Insulin resistance has been reported in several animal models, including spontaneously hypertensive rats, Zucker fatty rats, IRS-1-knockout mice, and FFR (11–13). FFR show an acquired form of insulin resistance and hypertension that is diet-induced, but they do not show obesity (14, 15). In the present study, there were no significant differences in body weights, heart rates or fasting blood glucose levels between control rats and FFR. SBP and fasting insulin level were significantly higher in FFR than in control rats. It has been reported that FFR become insulin-resistant due to a change in muscle fiber composition, peripheral vasoconstriction, and impaired intracellular signal transduction (15–17). However, the mechanism of insulin resistance has not been completely elucidated. Also, hyperinsulinemia can stimulate activation of the sympathetic nervous and renin-angiotensin systems, an increase in renal sodium retention, and proliferation of vascular smooth muscle tissue, and it may lead to the development of a hypertensive state (18). The observations of insulin resistance and hyperinsulinemia in the present study suggest that fructose feeding leads to insulin resistance and that compensatory hyperinsulinemia may result in hypertension.
In this study, we investigated intracellular signal transduction in the vasculature and skeletal muscle. The results showed that stimulation with insulin did not alter the extent of phosphorylation of IRβ in the soleus muscle of FFR compared with that in control rats, but that tyrosine phosphorylation of IRS-1 in the soleus muscle of FFR was reduced to 80 ± 4% (p < 0.001) of that in control rats. These findings are similar to those reported by Bezerra et al. (17), who investigated the insulin-stimulated PI3-K pathway in the liver and skeletal muscle of FFR and found that stimulation with insulin did not alter the extent of phosphorylation of the insulin receptor (IR) in skeletal muscle of FFR compared with that in control rats, but that insulin-induced IRS-1 tyrosine phosphorylation in the skeletal muscle of FFR was reduced to 76 ± 5% of that in the control group. They reported that the association between insulin-stimulated IRS-1 and PI3-K in the skeletal muscle of FFR was reduced to 84 ± 4% of that in control rats.

Recent studies have suggested that tumor necrosis factor α (TNF-α), FFA, and angiotensin II inhibit insulin-induced stimulation of the PI3-K pathway (19–23). For example, Togashi et al. showed that skeletal muscle TNF-α was associated with insulin resistance in FFR (24). And other studies have shown that TNF-α inhibits tyrosine phosphorylation of IRβ, IRS-1 and PI3-K activity (21, 25). Similarly, Griffin et al. showed that an increase in FFA concentration causes serine phosphorylation of IRS-1 (26), and serine phosphorylation would reduce the ability of IRS-1 to activate PI3-K. Folli et al. reported that angiotensin II inhibits insulin signaling in cultured rat smooth muscle cells at multiple levels (22, 27). In their studies, angiotensin II inhibited the insulin-stimulated association between IRS-1 and the p85 subunit of PI3-K. Finally, Togashi et al. and Higashiura et al. reported that an angiotensin-converting enzyme inhibitor and an angiotensin II receptor antagonist improved insulin resistance in FFR (24, 28). TNF-α, FFA and angiotensin II may thus play important roles in inhibition of the PI3-K pathway in skeletal muscle of FFR. In the present study, no significant differences were found in the degree of phosphorylation of IRS-1 in thoracic aorta samples between FFR and control rats. Our results demonstrated that there was tissue specificity in the insulin resistance, with the PI3-K pathway, which is related to glucose metabolism, being selectively impaired in skeletal muscle.

On the other hand, insulin stimulates MAPK pathways (29). However, there has been no in vivo study of the insulin-stimulated MAPK pathway in the vasculature of FFR. We here evaluated ERK-1/2 protein levels in the vasculature and soleus muscle of FFR. There were no significant differences in ERK-1/2 protein levels in the thoracic aorta or soleus muscle between FFR and control rats. After stimulation with insulin, tyrosine/threonine phosphorylations of ERK-1/2 in the thoracic aorta and soleus muscle were increased, but no significant differences were found in the degree of tyrosine/threonine phosphorylation of ERK-1/2 in the thoracic aorta or soleus muscle between FFR and control rats. In
an ex vivo study, Jiang et al. found that insulin stimulated tyrosine phosphorylation of ERK-1/2 in the microvessels of epididymal fat in obese Zucker rats to the same degree as in control rats, but that the basal levels of tyrosine phosphorylation of ERK-1/2 were significantly higher in the microvessels of obese Zucker rats than in those of lean control rats (9). In the present study, the basal levels of tyrosine phosphorylation of ERK-1/2 were not significantly different between FFR and control rats. Jiang et al. examined microvessels of obese Zucker rats ex vivo, while we investigated thoracic aortae of non-obese FFR in vivo. The fasting insulin level was remarkably higher in obese Zucker rats than in lean control rats (8.2 and 26.2 $\mu$U/ml in the lean control rats and obese Zucker rats, respectively). The discrepancy between our results and those of Jiang et al. may be due to the difference in experimental conditions. Both our results and those of Jiang et al. suggest that the insulin-stimulated MAPK pathway is not impaired in the vasculature. Pleiffl and Ditschuneit found that the proliferation of cultured human arterial smooth muscle cells was stimulated even by small concentrations of insulin similar to those found in plasma under physiological conditions (30). There is evidence that insulin resistance may be directly atherogenic. The results of an insulin resistance atherosclerosis study (IRAS) suggested that there is a direct relationship between insulin resistance and carotid artery intima/media thickness, even after adjusting for several associated risk factors (31). Tsuchihashi et al. who evaluated the role of insulin resistance in coronary atherosclerosis, reported that hyperinsulinemia is a risk factor for coronary arterial disease and emphasized the severity of coronary atherosclerosis in normal glucose-tolerant subjects (32).

In conclusion, the insulin-stimulated PI3-K pathway was impaired in the skeletal muscle of FFR, while the insulin-stimulated MAPK pathway was not impaired in the aorta of FFR. The PI3-K pathway, which is related to such components of glucose metabolism as glucose transport and glycogen synthesis, is selectively impaired in skeletal muscle, and this impairment may induce hyperinsulinemia. The fasting insulin level in FFR in the present study was about 2.8-fold higher than that in control rats. Hyperinsulinemia stimulates the MAPK pathway related to gene expression and cell growth in the vasculature, and this pathway may be related to progression of atherosclerosis. This may be one of the factors responsible for the high incidence of cardiovascular disease among patients with insulin resistance. Further investigation of these pathways and their cross-talk with other factors or systems such as the renin-angiotensin system, will be needed to understand the etiology of insulin resistance and its complications.

To the best of our knowledge, this is the first report describing the tissue specificity and intracellular signal transduction selectivity of insulin resistance in the vasculature and skeletal muscle in vivo.

References

17. Bezerra RMN, Ueno M, Silva MS, Tavares DQ, Carvalho CRO, Saad MJA: A high fructose diet affects the early steps of insulin action in muscle and liver of rats. J Nutr