Enhancement of muscle glucose uptake by the vasopeptidase inhibitor, omapatrilat, is independent of insulin signaling and the AMP kinase pathway

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Abstract

Omapatrilat (OMA), a vasopeptidase inhibitor (VPI), presently being tested in clinical trials for its anti-hypertensive properties, inhibits both angiotensin-converting enzyme and neutral endopeptidase, and raises tissue bradykinin levels. Recent studies from our laboratory and those of others have demonstrated that VPIs enhance muscle glucose uptake in animal models, and this effect is mediated by the bradykinin–nitric oxide pathway. The mechanism of the effect of OMA on muscle glucose uptake, however, is presently unknown. To investigate the effect of OMA on insulin signaling, soleus muscle was isolated 2 or 5 min after an i.v. bolus of insulin or saline from male Zucker fatty rats (8–10 weeks of age), following a 5-day treatment period of oral OMA (15 mg/kg per day) or drug vehicle (placebo). OMA resulted in significantly lower systolic blood pressure compared with the placebo-treated group (84±7.52 mmHg in OMA vs 112±2.18 mmHg in controls, P<0.01). Immunoprecipitation and Western blot analysis of insulin receptor substrate 1 (IRS-1) revealed no changes in protein mass with OMA treatment. OMA did not enhance basal or insulin-stimulated IRS-1 tyrosine phosphorylation or its subsequent association with the p85 regulatory subunit of phosphatidylinositol 3-kinase. Under basal and insulin-stimulated conditions, OMA treatment did not alter the protein mass or the phosphorylation of Akt/protein kinase B, p42/44 extracellular signal-regulated kinase or adenosine monophosphate-activated protein kinase, or GLUT4 protein expression. We conclude that the ability of OMA to enhance whole body and specifically muscle glucose uptake in Zucker fatty rats is not mediated by enhancing insulin or AMPK signaling. Future studies should examine whether hemodynamic effects of the drug, independent of insulin signaling, enhance glucose uptake in insulin-resistant skeletal muscle.

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Introduction

Insulin resistance is a major pathogenic factor for type-2 diabetes mellitus and is associated with a cluster of atherosclerotic cardiovascular disease risk factors (Reaven 1988). These risk factors include, but are not limited to, hypertension, dyslipidemia, endothelial dysfunction, procoagulant and pro-inflammatory states, abdominal obesity, and hepatic steatosis, a complex that has collectively been termed the metabolic syndrome. The close link between insulin resistance and the development of cardiovascular complications suggests either a direct or an indirect causal mechanism and emphasizes the need to develop therapeutic strategies that improve insulin sensitivity and its co-morbidities.

Vasopeptidase inhibitors (VPIs) are new therapeutic agents that are being developed for the treatment of hypertension and heart failure (Floras 2002). They act primarily by blocking both angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP; Floras 2002). The consequence of dual inhibition of NEP and ACE is a synergistic reduction in vasoconstriction and enhancement of vasodilation, thereby serving to reduce blood pressure (BP) more effectively than ACE inhibitors (ACEI). Of the VPIs, the most advanced in its development as a therapeutic agent is omapatrilat (OMA), although its clinical development has been curtailed severely by a high incidence of serious side effects, including angioedema (Rouleau et al. 2000, Guthrie & Reeves 2002, Lapointe & Rouleau 2002). We recently showed that OMA enhances muscle glucose uptake in Zucker fatty rats and these results have been confirmed by others using another VPI, mixanpril (Arbin et al. 2003, Wang et al. 2003). By using two independent methods, euglycemic hyperinsulinemic clamp and insulin-bolus-mediated 2-deoxyglucose tissue uptake, our laboratory has previously demonstrated enhancement of whole body and muscle glucose uptake by OMA (Wang et al. 2003).
The insulin-sensitizing effects of OMA are blocked by bradykinin B$_2$ receptor antagonist (ectalibant-140) and nitric oxide synthase inhibitor (Danser et al. 2000, Wang et al. 2003).

Following on from our previous observations (Wang et al. 2003), the present study was designed to investigate whether the previously demonstrated OMA-induced improvement in muscle glucose uptake is mediated by enhanced insulin signaling via known pathways of insulin signaling in skeletal muscle. Our results demonstrate that OMA does not affect insulin–signaling molecules under basal or insulin-stimulated conditions. Furthermore, the enhancement of muscle glucose uptake by OMA treatment cannot be attributed to adenosine monophosphate-activated protein kinase $z$ (AMPK$z$) activation or to an increase in total GLUT4 protein expression.

**Materials and Methods**

**Materials**

Polyethylene (PE-50) catheters were obtained from Cay Adams (Aronson et al. 1997a), and each was extended with a segment of silastic tubing (length 3 cm, internal diameter 0.05 cm) from Dow Corning Co. (Midland, MI, USA). OMA was obtained from Bristol-Myers Squibb (#186716-01, Lot9B10310 CA, Princeton, NJ, USA). Human insulin was obtained from Eli Lilly and heparin from Organon (Toronto, Ont., Canada). Protease inhibitors used in this study such as aprotinin, leupeptin, okadaic acid, phenylmethylsulfonyl fluoride (PMSF), and pepstatin were obtained from Sigma. All electrophoresis and transfer processes were carried out in XCell SureLock Novex Mini-Cell and XCell II Blot module obtained from Invitrogen. Nitrocellulose membranes were purchased from Schleicher and Schuell Bioscience (Keene, NH, USA). Anti-phospho-tyrosine antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Agarose-conjugated anti-insulin receptor substrate-1 (anti-IRS-1) and anti-phospho-tyrosine antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-AKT, anti-phospho-AKT (Ser473), anti-phospho-ACC(Ser79), anti-acetyl CoA carboxylase (ACC), and anti-phospho-ACC (Thr172), anti-extracellular signal-regulated kinase 1 and 2 (anti-ERK1/2) mitogen-activated protein kinase (MAPK; p44/p48) and anti-phospho-ERK1/2 MAPK (Thr202/Tyr204), anti-acetyl CoA carboxylase (ACC), and anti-phospho-ACC (Ser79) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal anti-GLUT4 antibody was a generous gift from Dr Amira Klip (Hospital for Sick Children, Toronto, Ont., Canada). Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) was purchased from Cell Signaling Technology, Inc. Enhanced chemiluminescence (ECL) reagents were obtained from Amersham.

**Animals**

Obese (fa/fa) Zucker male rats (Charles River Laboratories, Saint Constant, Quebec, Canada) were studied at the age of 8–10 weeks, as this is the time when Zucker fa/fas progress from insulin-resistance to mild glucose intolerance. Limited studies were also performed in two leanstrains (fa−/− and −/−) for comparison. The rats were housed in the Animal Care Facility of the Toronto General Hospital. They were exposed to a 12 h light:12 h darkness cycle and fed rat chow (Purina 5001, 4.5% fat, Ralston Purina Co., St Louis, MO, USA) and water ad libitum. All procedures were approved by the Animal Care Committee of the University Health Network, University of Toronto.

**Surgery and animal handling**

After 3 days of adaptation to the facility, the rats were anesthetized by i.p. injection of ketamine: xylazine:acepromazine (20:2:1 mg/ml, 1 µl/g body weight) and an indwelling catheter was inserted in the right internal jugular vein for i.v. administration of the insulin or saline bolus. The catheter was tunneled subcutaneously and exteriorized to the back of the neck. All surgeries (vessel cannulation) were performed under sterile conditions. The catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1000 USP/ml) to maintain patency for 5 days and closed at the end with a metal pin. The catheters were flushed with saline once during the 5-day treatment of either OMA or placebo, on day 3.

**Treatment of rats and muscle preparations**

The obese Zucker rats were randomized to receive oral gavage of either OMA (15 mg/kg per day) diluted in saline ($n=12$) or drug vehicle only (control, $n=12$), once daily, for a period of 5 days. This is the same dose of the drug used in our previous studies (Wang et al. 2003), in which we demonstrated an insulin-sensitizing effect of OMA. All animals were given free access to regular chow and water throughout the 5-day treatment period. On the third day of the study, systolic BP was measured by tail cuff in lightly anesthetized rats (approximately two-thirds of the anesthetic dose for surgery as mentioned earlier), 6 h before their oral treatment. Immediately afterwards, the catheter was flushed with saline and filled with polyvinylpyrrolidone and heparin admixture to maintain patency for the remaining days of the treatment. Blood samples were obtained from the tail vein after an overnight fast (food was removed at 1800 h after the final dose of treatments on day 5). Insulin (50 U/kg) was administered as an i.v. bolus through the venous catheter to $n=12$ OMA-treated and $n=12$ placebo-treated rats. Six animals in each of these two treatment groups had muscle biopsies performed 2 min post-i.v. insulin bolus for assessment of IRS-1 activation, whereas six animals in each group had muscle biopsies performed at 5 min for assessment.
of Akt phosphorylation and all other measures of insulin signaling, as described below. We (unpublished observations) and others (Kim et al. 2000, Shauchi et al. 2002) have found that peak stimulation of IRS-1 phosphorylation and p85 association with IRS-1 occurs 2 min after the insulin bolus, whereas downstream Akt peak phosphorylation occurs later, at approximately 5 min. A separate group of OMA- (n = 6) or placebo-treated (n = 6) Zucker fatty rats received an i.v. bolus of saline in order to assess basal (i.e. non-insulin stimulated) tissue levels of the parameters of interest. To determine whether the 12-h time lag following the last dose of OMA could have influenced our results, an additional three Zucker fatty rats (n = 3) pre-treated with OMA received an additional dose of OMA 2 h prior to the insulin bolus.

Immediately after insulin administration, the animal was placed under anesthesia with 5% isoflurane in 100% O2 and maintained during surgery with 1.9-2.1% isoflurane in 100% O2 through a nose cone to minimize stress and excitation. The depth of anesthesia was verified by noting the absence of physical responses to firm paw pinch, and the right and left soleus skeletal muscles were removed from each rat 2 or 5 min after insulin administration, as indicated earlier. We have chosen the soleus muscle to assess insulin signaling, since our previous studies showed a marked increase in glucose uptake in this muscle group (Wang et al. 2003). Tissue samples were removed and snap frozen in liquid nitrogen and stored at −70°C for later analysis. Approximately 50 mg of soleus skeletal muscles were ground in a glass-on-glass homogenizer containing ice-cold lysis buffer (50 mM Tris (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM ethylene glycol tetra acetic acid, 1 mM Na3VO4, 100 mM NaF, 10 mM Na2HPO4, 1 mM okadaic acid, 1 mM phenyl methyl sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Insoluble materials were removed by centrifugation at 2000 r.p.m. for 10 min at 4°C. Protein concentration was determined by the Lowry method using BSA as standard.

In order to determine whether the insulin bolus could have lowered blood glucose within the 5-min time-frame prior to performing the muscle biopsy, thereby eliciting a counter-regulatory response, blood glucose was measured by Sure Step One Touch glucometer (Lifescan, Inc., Johnson & Johnson, Milpitas, CA, USA) in a subgroup of three animals prior to insulin administration and again after 5 min.

**Immunoprecipitation and Western blot analysis**

For detection of tyrosine phosphorylation of IRS-1 and p85 regulatory subunit of PI3K association with IRS-1, muscle lysate containing equal amounts of protein (1 mg) were subjected to immunoprecipitation overnight at 4°C with agarose-conjugated rat polyclonal anti-IRS-1 antibodies. Immune complexes were collected by brief centrifugation and washed three times with lysis buffer as mentioned above. The equivalent amount of protein samples was resuspended in 1×Laemmli sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris (pH 6.8), 0.1% bromophenol blue, 5% β-mercaptoethanol), boiled for 5 min, and separated by SDS-7.5% PAGE under reducing conditions. Proteins on the gels were transferred onto nitrocellulose membranes. Following blocking with Tris-buffered saline/0.1% Tween 20 (TBST) blocking buffer containing 0.1% Tween-20 and 5% non-fat milk or BSA at room temperature for 1 h, the membranes were immunoblotted with anti-IRS-1, anti-phosphotyrosine, or anti-PI3K p85 (1:1000) overnight at 4°C. After washing three times with TBST, the membranes were incubated at room temperature for 1 h with HRP-conjugated secondary antibodies. Bands of interest were visualized by ECL, scanned, and quantified by densitometry. When necessary, some nitrocellulose membranes were stripped subsequently at 50°C for 30 min in stripping buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol) for a second round of immunoblotting.

To determine the protein levels of Akt, phospho-Akt (Ser473 and Thr308), p44/p42 MAPK, phospho-p44/p42 MAPK, AMPK, phospho-AMPK, ACC, and phospho-ACC, equal amounts of protein (40 μg) were prepared from muscle homogenates and subjected to SDS-PAGE and immunoblotting procedures as described above.

To determine the protein levels of GLUT4, 1 mg soleus muscle was weighed and homogenized in ice-cold lysis buffer (20 mM NaHCO3, 250 mM sucrose, 5 mM NaN3, 0.2 mM PMSE, 10 μM E64, 1 μM pepstatin, 1 μM leupeptin). The homogenates were centrifuged at 1300 g for 10 min. The pellets were resuspended in lysis buffer and re-centrifuged at 1300 g for another 10 min. The supernatants from the first and second centrifugations were pooled and spun at 229 000 g for 90 min. The resultant pellets containing the total or crude membranes were re-suspended in lysis buffer and protein concentrations of the extracts were estimated by the Lowry method as described previously. Samples were stored at −70°C until used.

**Other laboratory methods**

Blood glucose was analyzed by Sure Step, One Touch glucometer (Lifescan, Inc., Johnson & Johnson, Milpitas, CA, USA) in a subgroup of three animals prior to insulin administration and again after 5 min.

**Statistical analysis**

All data are presented as means ± S.E.M. Statistical significance was determined for each parameter of interest by unpaired Student’s t-test for OMA- vs placebo-treated animals. A value of P<0.05 was considered significant (as indicated by *) and P<0.01 was considered highly significant (as indicated by **).
Results

Basal parameters in Zucker fatty rats

Characteristics of the animals are shown in Table 1. Body weight was similar between control and OMA-treated groups after 5 days of treatment and there was no difference in fasting blood glucose, insulin, triglycerides (TGs), or free fatty acids (FFAs) between OMA and control groups. BP was reduced significantly in the OMA-treated group compared with the placebo group, verifying that the OMA-treated animals had a biologically significant response to the drug. There was no difference in the blood glucose level before and 5 min after the insulin bolus in an additional three control and three OMA-treated rats (glucose 4.9±0.5 mmol/l pre- vs 6.7±0.5 mmol/l 5-min post-insulin administration in the control group, P=not significant (n.s.); 5.9±0.8 mmol/l pre- vs 5.5±0.8 mmol/l 5-min post-insulin bolus in the OMA-treated group, P=not significant (n.s.)). Such an observation allows us to safely eliminate the possibility that counter-regulatory hormonal changes may have occurred and impacted on insulin signaling.

Table 1 Body weight, fasting plasma glucose, insulin, free fatty acid (FFA), triglyceride (TG) concentrations, and systolic blood pressure (BP) in control and OMA-treated Zucker fatty rats

<table>
<thead>
<tr>
<th>Placebo-treated Zucker fatty rats (n=12)</th>
<th>15 mg/kg per day OMA-treated Zucker fatty rats (n=12)</th>
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<tbody>
<tr>
<td>Weight (g)</td>
<td>382±13.2</td>
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<tr>
<td>Glucose (mmol/l)</td>
<td>5.3±0.12</td>
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<tr>
<td>Insulin (pmol/l)</td>
<td>1163±103</td>
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<tr>
<td>FFA (mmol/l)</td>
<td>0.27±0.02</td>
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<tr>
<td>TG (mmol/l)</td>
<td>4.08±0.27</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>107±3</td>
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<td>86±5*</td>
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*P<0.001 for differences between OMA- and placebo-treated rats.

IRS-1 tyrosine phosphorylation and association with the p85 regulatory subunit of PI3-kinase

Tyrosine phosphorylation of IRS-1 is shown in Fig. 1A. There were no changes in total IRS-1 protein expression in the soleus muscles of control and OMA-treated rats after 5 days. As expected, the insulin bolus significantly increased IRS-1 tyrosine phosphorylation compared with saline in both OMA- and placebo-treated rats, but the OMA treatment neither induced any changes in basal tyrosine phosphorylation of IRS-1 proteins nor did it augment insulin-stimulated IRS-1 tyrosine phosphorylation.

The association of IRS-1 with the p85 regulatory subunit of PI3K was also examined and is shown in Fig. 1B. In parallel with an increase seen in IRS-1 tyrosine phosphorylation induced by insulin, there was an increase in IRS-1 association with the p85 regulatory subunit of PI3K in insulin-treated animals compared with saline treatment. Consistent with the tyrosine phosphorylation results, 5 days of OMA treatment alone did not stimulate basal or insulin-stimulated IRS-1/PI3K association.

The relative impairment of insulin signaling in obese animals compared with Zucker lean rats was demonstrated by measuring insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K association in soleus muscles, 2 min after administration of the insulin bolus (data not illustrated). Zucker fatty rats (n=3) had a clear impairment in IRS-1 tyrosine phosphorylation and p85 association with IRS-1 compared with their lean counterparts (n=2). Fatty rats demonstrated 155.7±29.5 and 168.6±27.6% enhancement of IRS-1 phosphorylation and p85 association respectively after an insulin bolus, when compared with rats administered a saline bolus. In contrast, lean rats demonstrated 430.0±50.7 and 581.1±30.2% enhancement of IRS-1 phosphorylation and p85 association respectively after an insulin bolus, when compared with rats administered a saline bolus. These data are consistent with previous studies (Hotamisligil et al. 1994, Anai et al. 1998).

Akt/protein kinase B (PKB) and p42/44 MAP kinase phosphorylation in Zucker fatty rat skeletal muscle

We next examined insulin-signaling proteins downstream of IRS-1 and the results are illustrated in Fig. 2. Akt protein levels did not differ among the groups in this study. Insulin-stimulated Akt serine phosphorylation was increased significantly compared with the saline-treated group. However, Akt phosphorylation insoleus muscle after OMA treatment was comparable to that seen in placebo-treated rats. OMA treatment did not augment insulin-stimulated Akt serine phosphorylation. Akt phosphorylation at threonine was also examined and yielded similar observations (data not shown). In addition, we also examined Akt phosphorylation in other muscle groups, such as tibialis anterior, gastrocnemius, and quadriceps. Consistently, OMA treatment did not affect serine and threonine phosphorylation of Akt under basal or insulin-stimulated conditions in these other muscle groups (n=5–6, data not shown).

ERK1/2 (p42/44) MAPK protein expression was comparable between the OMA and placebo groups (Fig. 2B). Insulin induced significant phosphorylation of MAPK compared with saline treatment. Five days of OMA treatment alone neither altered the phosphorylation status of ERK1/2 nor did it augment insulin-stimulated ERK1/2 activation.

AMPK protein expression and phosphorylation

We examined whether chronic treatment of OMA could have enhanced glucose uptake through AMPK phosphorylation in skeletal muscle of insulin-resistant rats. The results are shown in Fig. 3. AMPK protein expression was not different between all groups examined. OMA treatment did not enhance AMPK phosphorylation under basal or insulin-stimulated conditions.
To confirm the absence of AMPK signaling in the skeletal muscle, we examined ACC, which is a downstream-signaling molecule of AMPK. Activated AMPK has been shown to induce Ser97 phosphorylation of ACC, thereby inhibiting ACC enzymatic activity (Ha et al. 1994, Park et al. 2002). In accordance with the above AMPK inactivity, differences in total ACC protein and serine phosphorylation of ACC were not observed (Fig. 4).

**Total GLUT4 protein expression**

OMA treatment did not change total cell GLUT4 protein under basal or insulin-stimulated conditions (Fig. 5).

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**Figure 1** Effects of omapatrilat (OMA) on basal and insulin-stimulated (A) IRS-1 tyrosine phosphorylation and (B) IRS-1 association with p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) in soleus muscle of Zucker fatty rats. Skeletal muscles were obtained 2 min after insulin (50 U/kg) or saline bolus. After immunoprecipitation (I.P.) with an anti-IRS-1 antibody, proteins were separated by SDS-PAGE and identified by Western blotting (I.B.) with (A) anti-phosphotyrosine (pY) antibody or (B) anti-p85 antibody as described in Materials and Methods. Control blots indicate that IRS-1 protein was no different between treatment groups. IRS-1 phosphorylation was stimulated by insulin administration, but OMA treatment did not alter IRS-1 phosphorylation or its association with PI3K under basal or insulin-stimulated conditions. Each group was expressed as a percent of control. Representative bands are shown. Values are means ± S.E.M. for six rats per treatment group.

**Figure 2** Effect of OMA treatment on (A) Akt/protein kinase B (PKB) and (B) ERK1/2 MAPK phosphorylation. Zucker fatty rats were treated with either placebo or OMA for 5 days and soleus muscles were extracted 5 min after an i.v. bolus of insulin (50 U/kg) or saline. Treatment of OMA for 5 days did not affect basal or insulin-stimulated Akt/PKB or ERK1/2 MAPK phosphorylation. Each group was expressed as a percent of control. Representative bands are shown. Values are means ± S.E.M. for six soleus muscle samples per group.
Discussion

In our previous study (Wang et al. 2003), we demonstrated that OMA treatment, at the same dose as that used in the present study (15 mg/kg per day for 5 days), induced profound whole-body insulin sensitization as measured by the euglycemic hyperinsulinemic clamp method, and enhanced insulin-stimulated muscle 2-deoxyglucose uptake in insulin-resistant obese Zucker rats. In the present follow-up study, we examined potential mechanisms underlying the improvement in insulin sensitivity by OMA. We were unable to demonstrate an effect of OMA on insulin signaling in the skeletal muscle of Zucker fatty rats. Furthermore, OMA did not alter the activity of AMPK or GLUT4 protein expression in muscle tissue derived from treated animals. Our studies provide clear evidence that the enhanced muscle glucose uptake by OMA cannot be attributed to the improvement in known insulin-signaling pathways or stimulation of AMPK activity.

Numerous studies in animals (Uehara et al. 1994, Henriksen & Jacob 1995, Jacob et al. 1996, Wang et al. 2003) and humans (Pollare et al. 1989, Lithell et al. 1990, Torlone et al. 1993, Falkner et al. 1995, Vuorinen-Markkola & Yki-Jarvinen 1995, Valensi et al. 1996, Tillmann et al. 1997, Fogari et al. 1998, Lender et al. 1999) have reported small improvements in insulin sensitivity by treatment with ACEI and their beneficial effect can be abolished completely by pre-treatment with HOE-140 (B2 kinin receptor blocker) in animal studies (Tomiya et al. 1994). Studies using captopril, an ACEI, increased insulin sensitivity in normal rats but not in kininogen-deficient rats in which kinin formation is severely reduced (Damas et al. 1999). This observation strongly implicates the role of kinins in tissue glucose uptake. The contribution of bradykinin to glucose metabolism has been appreciated for some time, with a number of studies showing that bradykinin can increase glucose uptake in skeletal muscle (Leighton et al. 1996, Henriksen et al. 1998). In skeletal muscles, 64% of the kininase activity is attributed to ACE and the remaining 36% to NEP (Floras 2002), providing an explanation for the greater insulin-sensitizing effect of VPIs than ACEI (Wang et al. 2003).

Figure 3 Effect of 5 days OMA treatment on AMPK protein content and phosphorylation. Soleus muscles were taken 5 min after administration of saline or insulin bolus in placebo or OMA-treated insulin-resistant animals. There were no changes in total AMPK protein levels between any of the treatment groups, as illustrated in the bar graph. Representative bands for AMPK phosphorylation are shown. Values are means ± S.E.M. for six rats per treatment group.

Figure 4 Effect of 5 days OMA treatment on total soleus muscle acetyl CoA carboxylase (ACC) and Ser97 phosphorylation of ACC. There were no changes in total ACC or Ser97 phosphorylation of ACC with OMA treatment, as illustrated in the bar graph. Representative bands for total ACC and serine phosphorylation are shown. Values are means ± S.E.M. for six rats per treatment group.

Figure 5 Effect of OMA treatment on total cellular GLUT4 protein expression. Zucker fatty animals were treated with placebo or OMA for 5 days and soleus muscles were taken 5 min after saline or insulin bolus on the last day. There were no differences in total GLUT4 protein levels between any of the treatment groups shown. Representative bands for GLUT4 expression are shown. Values are means ± S.E.M. for six animals per treatment group.
The mechanism of enhanced muscle glucose uptake by bradykinin remains controversial, with some studies showing that bradykinin mediates its insulin-sensitizing effects via the insulin receptor-signaling pathway (Henriksen et al. 1996, Carvalho et al. 1997, Nawano et al. 1999), and other studies demonstrating that the effects are independent of the insulin receptor (Shiuchi et al. 2002). We found that under basal or insulin-stimulated conditions, OMA treatment has no effect on Akt serine and threonine phosphorylation (data not shown). Our finding is in agreement with the results of a study that demonstrated that treatment of type-2 diabetic KK-Ay mice with temocapril, an ACEI, significantly enhanced 2-deoxyglucose uptake in skeletal muscle in a bradykinin–nitric oxide–dependent fashion without alterations in IRS-1 phosphorylation (Shiuchi et al. 2002). In contrast, Carvalho et al. (1997) reported that injection of captopril or bradykinin enhanced insulin-induced increase in insulin receptor phosphorylation, IRS-1 phosphorylation, and PI3K association with IRS-1 in aged rats. Similarly, Nawano et al. (1999) also reported that the administration of imidapril, another ACEI, also improved insulin sensitivity which was associated with increased insulin–induced IRS-1 tyrosine phosphorylation and PI3K activity in female obese Zucker rats (Nawano et al. 1999). It is possible that differences in therapeutic agents and the animal models may account for the contrasting results.

We found no effect of OMA on total muscle tissue GLUT4 expression. Technically, it is extremely difficult and requires large amounts of muscle tissue to examine GLUT4 translocation from intracellular vesicles to the plasma membrane ex vivo in muscle. In the present study, therefore, we were only able to measure total cellular GLUT4 and found no increase after 5-days OMA treatment. Our findings do not preclude an increase in GLUT4 translocation from intracellular organelles to the plasma membrane with OMA treatment. Indeed, Kishi et al. (1998) examined bradykinin effects on GLUT4 translocation in vitro in L6 myoblasts stably expressing c-mer epitope-tagged GLUT4 (GLUT4c-myc) and found that bradykinin directly triggers GLUT4 translocation, thereby increasing the rate of glucose uptake through an insulin-independent pathway. It remains an intriguing possibility that OMA can increase glucose uptake through direct stimulation of GLUT4 translocation. Future studies using very different experimental model systems to the present study will be required to address this possibility.

In addition to insulin, skeletal muscle glucose transport is also stimulated by exercise. AMPK is a known regulator of glucose metabolism in skeletal muscle in response to exercise (Winder & Hardie 1996, Hutter et al. 1997, Vavvas et al. 1997). Activation of AMPK in muscle leads to an increase in glucose transport, accompanied by increased translocation of GLUT4 to the plasma membrane (Merrill et al. 1997, Hayashi et al. 1998, Kurth-Krakczek et al. 1999). Since AMPK appears to increase glucose metabolism through an insulin-independent mechanism and is possibly nitric oxide-dependent (Lund et al. 1995, Hayashi et al. 1997, 1998), we postulated that activation of this pathway may provide an alternative mechanism to explain VPI-induced insulin-sensitizing effects in insulin-resistant skeletal muscle. OMA, however, had no effect on AMPK α protein content or its phosphorylation using antibodies that recognize both α isoforms. Therefore, it is unlikely that the increase in glucose uptake induced by OMA treatment in Zucker fatty rats is dependent on changes in AMPK activity.

We also examined one component of the MAPK (Au et al. 2003) signaling. Recent evidence has suggested that MAPK-signaling cascades contribute to the regulation of insulin action and protein expression in skeletal muscle (Goodyear et al. 1996, Aronson et al. 1997a, b). Furthermore, muscle contractions have been demonstrated to activate ERK1/2, isoforms of MAPK, and exercise training by obese Zucker rats leads to the upregulation of ERK2 protein expression (Osman et al. 2001). This raises an interesting possibility that enhanced signal transduction via the MAPK pathway may play an important role in the increased expression of glucose catabolic enzymes, GLUT4, and other insulin signaling proteins. We were unable to demonstrate an effect of OMA treatment on basal or insulin-stimulated ERK1/2 phosphorylation.

There was no difference in fasting blood glucose, insulin, TGs, or FFAs between OMA-treated and control groups. These parameters are insensitive indicators of differences in insulin sensitivity in the highly insulin-resistant Zucker fatty rat. In our previous study (Wang et al. 2003), there were also no significant differences in these parameters, whereas OMA treatment was associated with marked improvement in insulin sensitivity as measured by the gold standard method, the euglycemic hyperinsulinemic clamp technique.

How do we reconcile our previous findings, confirmed by others, of a significant increase in insulin-stimulated glucose uptake induced by VPIs, and the present ‘negative’ findings regarding some of the molecular mechanisms known to mediate glucose uptake in myocytes? We used the same animal model in the present and our previous study, as well as the same dose and duration of OMA treatment. The significantly lower BP in the OMA vs placebo-treated rats in the present study, similar to the BP changes noted in our previous study, indicates that the drug was indeed ingested and was biologically active. Although we cannot definitively exclude an effect of BP on insulin signaling, indirect evidence suggests that it does not account for the insulin-sensitizing properties of OMA. In our previous publication (Wang et al. 2003), BP was precisely matched between different pharmacological treatment experimental groups and yet insulin sensitization was markedly increased only by OMA. Changes in BP, therefore, cannot fully explain the insulin-sensitizing effect of OMA. In addition, in the present study, we detected no differences in the cellular insulin–signaling cascade in muscle tissue, despite a significant difference in BP between OMA- and placebo-treated animals.

The dose of insulin used in the present study was in the midrange of doses that have been used by others and indeed insulin administration effectively stimulated phosphorylation of IRS–1, Akt/ PKB and association of the p85 subunit of PI3K with
IRS-1 in the present study. In order to determine whether the 12-h time-period following administration of the last dose of OMA accounted for the absence of effect of OMA on insulin signaling in the present study, additional experiments were performed in three OMA-treated and three placebo-treated rats in which an additional dose of OMA or saline respectively were administered 2 h prior to the insulin bolus. There was no significant difference in the activation of IRS-1 or Akt/PKB in these animals, suggesting that the time-period following the last dose of OMA was not the reason for the negative results of this study. In the present study, we used only the soleus muscle to examine insulin signaling, since we found that soleus had the greatest enhancement of glucose uptake. In addition, we found no effect of OMA on insulin-stimulated Akt/PKB in three other skeletal muscle groups: tibialis anterior, gastrocnemius, and quadriceps (data not shown). We (unpublished observations) and others (Kim et al., 2000, Shiuchi et al. 2002) have found that peak stimulation of IRS-1 phosphorylation and p85 association with IRS-1 occurs 2 min after the insulin bolus, whereas downstream Akt peak phosphorylation occurs later, at approximately 5 min. Therefore, the negative results of the present study cannot be explained on the basis of down-regulation of insulin signaling due to delayed taking of muscle biopsies. The only caveat to consider that may account for a lack of difference is that OMA and ACE inhibitors may alter the timing or extent of insulin induced feedback inhibition of signaling, which occurs following the peak of IRS phosphorylation and Akt activation. Further work delineating a larger time course will answer this question.

It remains for future studies to investigate alternative pathways such as atypical protein kinase Cζ (aPKCζ) (Farese, 2002). Studies suggest that the aPKCζ is a downstream mediator of PI3K and that its activation is required for insulin stimulation of GLUT4 translocation and ultimately glucose uptake (Bandyopadhyay et al., 1997, Standaert et al. 1997, Kotani et al., 1998). aPKCζ is an attractive candidate as several in vitro studies using overexpression of dominant-negative and wild-type kinase can abrogate and enhance insulin-stimulated glucose uptake respectively (Bandyopadhyay et al., 1997, 2000, Etgen et al. 1999). Interestingly, aPKCζ has been implicated in insulin resistance and this is supported by studies in vivo demonstrating impaired insulin-stimulated aPKCζ activity in skeletal muscle tissue of animal models of insulin resistance and diabetes, such as Goto–Kakizaki, high-fat fed rats, and obese diabetic monkeys (Kanoh et al., 2001, 2003, Standaert et al. 2002). Similarly, defective aPKCζ activity in skeletal muscle of obese humans with impaired glucose tolerance and in type-2 diabetes humans has also been reported (Vollenweider et al., 2002, Kim et al. 2003). Since our data do not exclude the possibility that the activity or membrane localization of aPKCζ could also be altered by OMA treatment, it will be interesting to investigate this aspect of insulin signaling.

Another intriguing possibility, muscle capillary bed recruitment, could explain an increase in muscle glucose uptake by OMA in vivo that is not mediated by enhanced insulin signaling. Insulin resistance is closely linked to alterations in vascular function (Laakso et al., 1992, Steinberg et al., 1996, Baron 2002) and insulin itself is vasodilatory, thereby enhancing its own and/or glucose access to muscle tissues (Steinberg et al., 1996, Baron et al. 2000). Clark and colleagues have championed the concept that increased nutrient capillary recruitment in muscle beds plays an important role in mediating glucose delivery to myocytes, and thus it is a critical determinant of whole-body insulin sensitivity (Vincent et al., 2002, 2004, Zhang et al. 2004). Recent studies have demonstrated that insulin’s ability to enhance capillary recruitment is nitric oxide dependent (Shankar et al. 2000, Vincent et al. 2003). Furthermore, Wallis et al. (2002) have shown that the insulin-resistant obese Zucker rat has markedly impaired insulin-stimulated capillary recruitment. Our previous observation that the nitric oxide synthase inhibitor significantly blocked the insulin-sensitizing effects of OMA is consistent with this concept (Wang et al. 2003). It remains an interesting possibility that capillary recruitment can play a vital role in the enhancement of insulin-stimulated glucose transport, brought about by administration of VPIs.

In conclusion, enhancement of muscle and whole-body glucose uptake with the VPI, OMA, cannot be attributed to enhanced insulin signaling, activation of AMPK, or increase in total GLUT4 protein content. We speculate that OMA mediates its effect on glucose uptake by muscle capillary bed recruitment, which increases glucose delivery to myocytes. Future investigations should focus on the link between the hemodynamic changes induced by vasoactive agents, such as VPIs, and their ability to enhance glucose uptake. Although these agents are presently being developed primarily for the treatment of hypertension and heart failure, given the association between insulin-resistant states and cardiovascular disease, this pleotropic effect of VPIs could make them extremely attractive therapeutic tools for the treatment of those with the metabolic syndrome.

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