Oligomeric resistin impairs insulin and AICAR-stimulated glucose uptake in mouse skeletal muscle by inhibiting GLUT4 translocation

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Jørgensen SB, Honeyman J, Oakhill JS, Fazakerley D, Stöckli J, Kemp BE, Steinberg GR. Oligomeric resistin impairs insulin and AICAR-stimulated glucose uptake in mouse skeletal muscle by inhibiting GLUT4 translocation. Am J Physiol Endocrinol Metab 297: E57–E66, 2009. First published May 12, 2009; doi:10.1152/ajpendo.90744.2008.—The hormone resistin is elevated in obesity and impairs glucose homeostasis. Here, we examined the effect of oligomerized human resistin on insulin signaling and glucose metabolism in skeletal muscle and myotubes. This was investigated by incubating mouse extensor digitorum longus (EDL) and soleus muscles and L6 myotubes with physiological concentrations of resistin and assessing insulin-stimulated glucose uptake, cellular signaling, suppressor of cytokine signaling 3 (SOCS-3) mRNA, and GLUT4 translocation. We found that resistin at a concentration of 30 ng/mL decreased insulin-stimulated glucose uptake by 30–40% in soleus muscle and myotubes, whereas in EDL muscle insulin-stimulated glucose uptake was impaired at a resistin concentration of 100 ng/mL. Impaired insulin-stimulated glucose uptake was not associated with reduced Akt phosphorylation or IRS-1 protein or increased SOCS-3 mRNA expression. To further investigate the site(s) at which resistin impairs glucose uptake we treated myotubes and skeletal muscle with the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) and found that, although resistin did not impair AMPK activation, it reduced AICAR-stimulated glucose uptake. These data suggested that resistin impairs glucose uptake at a point common to insulin and AMPK signaling pathways, and we thus measured AS160/TBC1D4 Thr642 phosphorylation and GLUT4 translocation in myotubes. Resistin did not impair TBC1D4 phosphorylation but did reduce both insulin and AICAR-stimulated GLUT4 plasma membrane translocation. We conclude that resistin impairs insulin-stimulated glucose uptake by mechanisms involving reduced plasma membrane GLUT4 translocation but independent of the proximal insulin-signaling cascade, AMPK, and SOCS-3.

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physiological concentrations has significant effects on insulin-regulated glucose uptake in muscle.

A consistent finding in regard to resistin action in skeletal muscle is the suppression of insulin-stimulated glucose uptake and GLUT4 translocation; however, whether or not this effect is dependent on defects in proximal aspects of insulin signal transduction (PI 3-kinase/Akt) is equivocal (8, 23, 25, 26). One of the difficulties in understanding the mechanisms by which resistin induces insulin resistance is that the resistin receptor and the initial signaling steps invoked by resistin have not been identified (20). Some findings suggest that resistin may induce insulin resistance in adipocytes and hepatocytes by increasing the suppressor of cytokine signaling 3 (SOCS-3) (45, 52), a negative regulator of insulin signal transduction (7, 47). Other studies have linked resistin with reduced signaling of the AMP-activated protein kinase (AMPK) (2, 27), a well-established controller of muscle glucose uptake (16, 22).

In the present study, we had three primary aims. The first aim was to determine whether the oligomerized form of resistin impairs insulin-stimulated glucose uptake at physiological concentrations, not only in cultured myocytes but also in differentiated mouse skeletal muscle. The second aim was to identify whether the mechanism of resistin-induced insulin resistance involved impaired insulin signal transduction by examining the effects of oligomerized resistin on proximal insulin signaling (IRS-1, PI 3-kinase, Akt), TBC1D4 phosphorylation, and GLUT4 translocation. The third aim of the study was to investigate whether resistin-induced insulin resistance involved induction of SOCS-3 and/or suppression of AMPK signaling. We hypothesized that a physiological concentration of oligomerized resistin would inhibit both insulin- and AMPK-stimulated glucose uptake and GLUT4 translocation, demonstrating that resistin-induced skeletal muscle insulin resistance is independent of defects in proximal insulin signal transduction.

METHODS

Resistin Production

Wild-type human COOH-terminal Flag-tagged resistin was produced in HEK293 cells as previously described (44), and resistin concentrations were determined using a murine ELISA kit (Cayman Chemical). Flag peptide was used as a vehicle control.

Osmotic Minipump

To test the biological efficacy of our resistin preparation, osmotic minipumps (1007D, ALZET) with a 7-day capacity were loaded with either saline or resistin (3.5 μg·h⁻¹·100 g⁻¹) and implanted subcutaneously (39) in 2.5-mo-old C57/B6 mice. At day 5, an insulin tolerance test was conducted by injecting a bolus of insulin (0.5 IU/kg) and measuring tail blood glucose over the following 2 h (16). At day 7, orbital venous sinus blood was collected for serum resistin measurements (ELISA kit, Cayman Chemical). Animals were subsequently euthanized by cervical dislocation, and the gastrocnemius muscle was collected, freeze-clamped, and stored at −80°C.

Muscle Incubation

Soleus and extensor digitorum longus (EDL) muscles from C57/B6 mice were dissected from anesthetized mice (6 mg pentobarbital sodium/100 g body wt) and transferred to incubation flasks kept at 30°C containing 2 ml of essential buffer for 10 min of preincubation (Krebs-Henseleit buffer, pH 7.5, with 2.0 mM pyruvate, 8 mM mannitol, and 0.1% BSA) and 95% O₂-5% CO₂ (16).

Resistin incubations. After preincubation in essential buffer, muscles were incubated in essential buffer containing vehicle (Flag peptide) or 10, 30, or 100 ng/ml resistin for 130 min with buffer changes after 60 min.

Insulin and AICAR incubations. Following resistin incubations, buffers were replaced by similar buffers with or without insulin (2.8 nM Actrapid; Novo Nordisk, Bagsvaerd, Denmark) or AICAR (2 mM, Toronto Research Chemical, Toronto, ON, Canada), and muscles were incubated for an additional 30 min before the tracer incubations.

Tracer incubation. 2-Deoxyglucose uptake was measured over a period of 20 min by replacing existing incubation buffer with buffer containing tracer and insulin. Muscles were incubated with insulin, AICAR, or both 80 min prior to CH, and insulin (10 nM) or AICAR (2 mM) was added at the indicated time intervals prior to CH.

Vehicle or resistin (100 ng/ml) treatments were for 4 and 2 h in the presence of absence of insulin (10 nM) for the last 5 min before CH.

AMPK signaling. Vehicle or resistin (100 ng/ml) treatments were for 4 h in the presence of absence of AICAR (2 mM) for the last 50 min prior to CH. For measurements of AMPK p-Thr172, acetyl-CoA carboxylase (ACC) p-Ser218, and protein phosphatase 2C (PP2C) expression, resistin (100 ng/ml) was added at the indicated time intervals prior to CH.

TBC1D4 phosphorylation. Vehicle or resistin (100 ng/ml) treatments were for 4 h in the presence of absence of insulin (10 nM) for the last 50 min prior to CH.

GLUT4 translocation assay. To evaluate the effect of resistin on glucose transporter translocation, we utilized L6 myotubes infected with a retrovirus containing an exofacial hemagglutinin epitope-tagged, human GLUT4 construct and performed GLUT4 translocation measurements (ELISA kit, Cayman Chemical). Flag peptide was used as a vehicle control.
Hepatocytes

Isolated hepatocytes from C57/Bl6 mice were prepared by the collagenase perfusion method (37) with minor modifications as recently described (36). Briefly, livers were perfused in situ with HEPES-buffered saline solution for 10 min followed by buffer containing collagenase (1.2 mg/ml) for a further 15 min. Hepatocytes were plated at 10⁶ cells/well in collagen-coated six-well dishes in DMEM supplemented with 10% FBS, glutamine, and antibiotics. Cells were allowed to settle for 24 h and incubated with resistin for indicated periods of time and lysed.

Immunoblotting

Expression and phosphorylation of investigated proteins were determined in lysates by SDS-PAGE and immunoblotting using the following primary antibodies: pan-AMPKα, p-AMPK Thr172, p-ACCβ Ser218 [produced as previously described (6)], STAT3, p-STAT3 Tyr705, Akt, p-Akt Ser473, IRS-1, p85 subunit of PI 3-kinase (Cell Signaling Technology), PP2C (Oxford Biochemical Research), TBC1D4 (Upstate), and p-TBC1D4 T642 (21st Century Biochemistry) containing collagenase (1.2 mg/ml) for a further 15 min. Hepatocytes were plated at 10⁶ cells/well in collagen-coated six-well dishes in DMEM supplemented with 10% FBS, glutamine, and antibiotics. Cells were allowed to settle for 24 h and incubated with resistin for indicated concentrations of insulin and AICAR were as above.

Concentrations and times of exposure of insulin and AICAR were as indicated concentrations of resistin for 4 h prior to translocation assay. Concentrations of insulin and AICAR were as above.

RESULTS

Resistin Production

Physiological circulating resistin is composed of homo-trimers covalently linked through NH₂-terminal disulfide bonds to form the high-molecular-weight hexamers (1, 29). We initially confirmed that our resistin preparation contained the resistin peptide by using a resistin ELISA. To evaluate the multimeric forms of resistin, we first compared electrophoretic mobilities of purified resistin preparations by use of denaturing SDS-PAGE with/without DTT (Fig. 1A). Resistin migrated exclusively as a 13-kDa monomer under reducing conditions and as a 20-kDa dimer under nonreducing conditions (Fig. 1A), suggesting that the preparation under nonreducing conditions consisted entirely of covalently linked resistin dimers, in agreement with previous studies of oligomerized resistin from plasma (29). Using size exclusion chromatography, we estimated that 20–25% of resistin was present as the high-molecular-weight hexameric complexes and the remainder as dimeric resistin complex (Fig. 1B). However, as associations of the resistin head segments are believed to form the trimeric part of the resistin complex spontaneously under physiological conditions as described previously (9, 43). Cells were incubated with indicated concentrations of resistin for 4 h prior to translocation assay. Concentrations and times of exposure of insulin and AICAR were as above.

SOCS-3 and Glucose-6-Phosphatase mRNA

Cells were lysed in TRI Reagent (Sigma-Aldrich) at the time points indicated, and RNA was isolated and reverse transcribed using the thermoscript RT-PCR system (Invitrogen) according to the manufacturers’ recommendations. RNA was analyzed via quantitative real-time PCR (RT-qPCR) on the Rotorgene 3000 (Corbett Research Australia) using Assay-on-Demand gene expression kits (Applied Biosystems). Fluorescent emission data were analyzed for the critical threshold (Ct) values, with the expressions of SOCS-3 and glucose-6-phosphatase normalized to 18S and expressed as 2⁻ΔΔCt.

Statistics

Data are expressed as means ± SE. Statistical evaluations were performed by either Student’s t-test or two-way ANOVA using the Student-Newman-Keuls method as a post hoc test when appropriate. Differences between groups were considered statistically significant if P < 0.05.
condition, the content of trimer-dimer hexameric complexes is probably greater than estimated. We next set out to determine the biological efficacy of the preparation by investigating physiological parameters known to be regulated by resistin (24, 33). We used osmotic minipumps to increase circulating resistin levels fourfold over a 7-day period (Supplementary Fig. S1A) (Supplementary material can be found in the online version of this paper). This resulted in whole body in vivo insulin resistance as assessed by an insulin tolerance test (Supplementary Fig. S1B). In separate experiments, resistin increased the expression of glucose-6-phosphatase mRNA more than 20-fold in cultured hepatocytes (Supplementary Fig. S1C), consistent with previous reports (2, 24). These findings indicate that our resistin preparation mainly contains oligomerized resistin and that it induces metabolic action in accord with previous studies that have utilized mammalian expressed high-molecular-weight resistin (2, 24).

**Physiological Concentrations of Resistin Impair Insulin-Stimulated Glucose Uptake**

We next examined the effects of resistin on insulin-stimulated glucose uptake in mouse skeletal muscles and in L6 myotubes. The concentrations used were chosen to resemble a basal physiological level (10 ng/ml; Supplementary Fig. S1A), the level seen in obese insulin-resistant mice (30 ng/ml) (24), and high physiological concentrations (100 ng/ml). Incubation with a submaximal insulin concentration (2.8 nM) increased glucose uptake in both soleus and EDL muscles approximately twofold compared with basal (Fig. 2, A and B). Incubating the muscles for 3 h with 10 ng/ml resistin did not impair insulin action, as glucose uptake was increased normally with insulin. When the resistin level was increased to 30 ng/ml, insulin-stimulated glucose uptake was decreased by ~50% in soleus but had no effect in the EDL (Fig. 2A&B). At higher resistin concentrations, 100 ng/ml, insulin-stimulated glucose uptake was inhibited in both muscle types by ~50–60% (Fig. 2, A and B). Resistin did not significantly affect basal glucose uptake in either muscle types, even though a tendency ($P = 0.10$) toward an increase was observed in EDL. These data show that the inhibitory effects of resistin on glucose uptake are detected at lower concentrations in soleus (30 ng/ml) than in EDL (100 ng/ml), indicating that muscle types with a higher content of red muscle fibers are more sensitive to resistin than muscle mainly composed of white fiber types.

We also examined the effect of resistin on glucose uptake in L6 myotubes. Basal glucose uptake was not altered with 30 ng/ml resistin but was reduced modestly, by 15%, with 100 ng/ml (Fig. 2C). Consistent with findings in the soleus (Fig. 2A), preincubation with 30 and 100 ng/ml resistin resulted in decreased insulin-stimulated glucose uptake by 40 and 60%, respectively, compared with the isolated effect of insulin (Fig. 2C). Finally, we investigated time-dependent effects of resistin on insulin-stimulated glucose and found that, although 1 h of resistin did not affect insulin-stimulated glucose uptake, 4, 16, and 28 h of resistin exposure decreased insulin-stimulated glucose uptake by 40, 60, and 75%, respectively (Fig. 2D). Thus, more than 50% of the reduction in stimulated glucose had occurred after 4 h of resistin exposure. These findings collectively show that resistin impairs insulin-stimulated glucose uptake in differentiated skeletal muscle and myotubes and that the effect is both time and dose dependent.
Resistin Does Not Inhibit Insulin Signaling

We next investigated whether resistin reduced insulin-stimulated glucose uptake by impairing proximal insulin signaling. We first examined the expression of IRS-1 after 4 h of resistin treatment in myotubes and found that IRS-1 protein expression was not different in myotubes treated with resistin compared with control cells (Fig. 3A). The association of the p85 subunit of PI 3-kinase with IRS-1 is an early step in the insulin-signaling pathway (53), and we assessed whether resistin impaired this association. As expected, insulin increased the association of p85 with IRS-1, but this increase was not impaired by preincubation with 100 ng/ml resistin (Fig. 3B). We also examined the activating phosphorylation (Ser473) of the insulin-signaling molecule Akt and found that resistin did not impair insulin-induced increases in Akt phosphorylation (Fig. 3C). Finally, we examined Akt Ser473 phosphorylation in incubated muscles under the same conditions in which glucose uptake was maximally impaired (100 ng/ml) and found, in agreement with observations in myotubes, that resistin did not impair insulin-stimulated Akt phosphorylation in either muscle type (Fig. 3D). Collectively, these findings suggest that resistin does not impair upstream elements in the insulin-signaling cascade.

Effects of Resistin on AMPK and SOCS-3 Signaling

As SOCS-3 is elevated in skeletal muscle of obese humans and rodents (41, 47, 49) and resistin increases SOCS-3 in adipocytes and hepatocytes (45, 52), we hypothesized that resistin might impair insulin-stimulated glucose uptake by increasing SOCS-3 expression. We treated myotubes with 100 ng/ml resistin for up to 28 h but found no significant increase in SOCS-3 expression under these experimental conditions (Fig. 4A) despite detecting significant increases in SOCS-3 mRNA in mice treated for 7 days with resistin (Supplementary Fig. S1D). These data suggest that resistin-induced impairments in insulin-stimulated glucose uptake ex vivo does not involve an increase in SOCS-3 mRNA expression.

Some findings (27) have suggested that resistin may also impair muscle glucose uptake by reducing AMPK activity, a well-established regulator of muscle glucose uptake (16, 22). We therefore examined AMPK activity in myotubes by measuring αAMPK Thr172 and ACCβ Ser518 phosphorylation exposed to 100 ng/ml resistin for 4 h with and without AICAR. Resistin did not alter AMPK p-Thr172 or ACCβ p-Ser518 basal levels (Fig. 4, B and C) and did not suppress AICAR-induced activation of AMPKα p-Thr172 and ACCβ p-Ser518 (Fig. 4, B and C). Since AMPK activity is negatively regulated by TNFα, induction of PP2C expression (42), we also examined the effect of resistin on PP2C expression and basal AMPKα p-Thr172 for up to 20 h and found, surprisingly, that there was a modest reduction in PP2C expression after 10 h but no change in AMPK phosphorylation (Fig. 4, D and E). Taken together, these data suggest that resistin does not suppress skeletal muscle AMPK and that AMPK does not contribute to impairments in insulin-stimulated glucose uptake in response to resistin.

Resistin Impairs AICAR-Stimulated Glucose Uptake

As AMPK regulates muscle glucose uptake independently of proximal insulin signaling (12), we investigated whether resistin also impairs AMPK-stimulated glucose uptake in EDL muscle and in L6 myotubes. The soleus muscle was not investigated because of the less potent effect of AICAR on glucose uptake in this muscle type (16). As expected, AICAR increased glucose uptake in the EDL muscle by ~2.5-fold; however, this increase was impaired by ~30% with resistin pretreatment (Fig. 5A). In myotubes, AICAR increased glucose uptake by ~50% compared with basal, and resistin impaired this increase by ~50% (Fig. 5B). In agreement with findings shown in Fig. 2, B and C, resistin did not affect basal glucose uptake in the soleus, consistent with the role of resistin in glucose uptake being exerted on proximal insulin signaling.

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Fig. 3. Effect of resistin on insulin signaling in skeletal muscle and L6 myotubes. A: protein expression of IRS-1 in resistin-treated L6 myotubes (n = 2 × 6, 4 h). B: association of IRS-1 with p85 was assessed by immunoprecipitating (IP) IRS-1 and immunoblotting for coprecipitated p85 in lysates from L6 myotubes at basal or treated with 100 ng/ml resistin, 10 nM insulin, or both (I+R) (n = 2, 4 h). C: phosphorylation of Akt Ser473 (S473-P) in lysates from myotubes at basal or stimulated with insulin and resistin to 10 nM insulin or 100 ng/ml resistin (I+R) (n = 2, 4 h). D: Akt Ser473 phosphorylation in lysates from incubated soleus and EDL muscle with either 2.8 nM insulin (INS) or insulin and 100 ng/ml resistin (I+R) (n = 3, 3 h). Values are means ± SE. Ø and ØØ, Significantly different from corresponding basal values, P < 0.05, P < 0.01.

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glucose uptake in incubated skeletal muscle but reduced basal glucose uptake in myotubes by ~20% (Fig. 5B).

Effects of Resistin on TBC1D4 Phosphorylation and GLUT4 Plasma Membrane Translocation

In skeletal muscle, AMPK and insulin signaling to glucose uptake involves phosphorylation and deactivation of GTPase-activating proteins such as AS160/TBC1D4, which facilitates GLUT4 vesicle translocation to the plasma membrane by a relief of inhibition mechanism (4, 9, 21, 46). TBC1D4 is phosphorylated at several sites and Thr642 is considered the most important site in suppressing TBC1D4 inhibition of glucose uptake (34). In myotubes, both AICAR and insulin robustly increased TBC1D4 Thr642 phosphorylation six- and
fivefold, respectively, and these increases were not reduced by resistin at a concentration of 100 ng/ml (Fig. 6, A and B). We next used L6 myotubes stably expressing a hemagglutinin epitope-tagged GLUT4 construct to measure cell surface levels of GLUT4. At concentrations of 100 ng/ml resistin, plasma membrane content of GLUT4 was slightly increased under basal conditions (Fig. 6, C and D). Insulin and AICAR increased cell surface GLUT4 2.5- and 2-fold, respectively, and, consistent with the inhibitory effect of resistin on glucose transport, resistin reduced insulin and AICAR-stimulated GLUT4 translocation by 17 and 30%, respectively (Fig. 6, C and D). The total cellular level of hemagglutinin-tagged GLUT protein was not affected by resistin (data not shown), suggesting that resistin does not lower plasma membrane GLUT4 content by reducing total GLUT4 expression, in agreement with previous reports (8, 26). Taken together, these data suggest that resistin impairs glucose uptake by inhibiting GLUT4 translocation at a step distal to TBC1D4 phosphorylation.

DISCUSSION

Resistin has been shown to be important in regulating metabolic pathways in several tissues and organs, such as the hypothalamus, heart, adipocytes, and liver (10, 24, 38, 44, 45). Studies of cultured L6 myotubes have shown that recombinant bacterial expressed resistin, presumably in the trimeric form but without the interchain Cys-6 disulphide bonds, directly impairs insulin-stimulated glucose uptake (8, 23, 25, 26). In addition, two of these studies (8, 23) used conditioned media from resistin-producing mammalian cells (L6 myotubes and 293T cells) and reported that resistin-containing media impaired insulin-stimulated glucose uptake. However, in these studies the site of resistin action appeared to be fundamentally different [GLUT4 \( V_{\text{max}} \) (23) vs. insulin signaling (8)], which perhaps could be attributed to the use of nonpurified resistin preparations in these experiments. We therefore decided to produce and purify resistin in its oligomerized form to test whether physiological concentrations of biologically relevant resistin impair insulin action not only in cultured cells but also in differentiated mouse skeletal muscle. The present study demonstrates that physiological concentrations of oligomerized resistin acutely impair both insulin and AICAR-stimulated glucose uptake in myotubes and in mouse skeletal muscle. Since insulin stimulates glucose transport via a PI 3-kinase/Akt-dependent pathway (53) whereas AICAR relies entirely on AMPK\(\alpha_2\) (12, 16), our data suggested that the site of action of resistin-induced inhibition of glucose transport lay at, or downstream of, the point where Akt and AMPK signaling converge. Consistent with this notion, we failed to observe any effect of resistin on either the PI 3-kinase/Akt or AMPK signaling pathways. We then measured the point of convergence of these two pathways by measuring TBC1D4 Ser\(^{642}\) phosphorylation in response to insulin and AICAR and found that resistin did not alter the phosphorylation of TBC1D4 but did impair GLUT4 translocation. Hence, we postulate that resistin induces insulin and AICAR resistance at a common step downstream of TBC1D4 involving GLUT4 trafficking. However, it should be noted that our findings do not conclusively rule out additional sites of action of resistin in skeletal muscle glucose uptake.

We showed, using isolated mouse skeletal muscle, that incubation with a resistin concentration similar to levels found in obese mice (24) reduced insulin-stimulated glucose uptake by \(\sim 50\%\) specifically in the red oxidative soleus muscle, whereas glucose uptake in the white, more glycolytic, EDL muscle was decreased only using a high physiological concentration. These findings are the first to show that oligomerized resistin negatively affects insulin actions in differentiated skeletal muscle and that the effect is more pronounced in red muscle types than in white muscle types. The reason for this muscle type difference is currently not known but could be due to...
to higher expression levels of either the resistin receptor or intracellular resistin signaling molecules in red than white muscle types.

In agreement with previous studies (8, 23, 25, 26), we also showed that resistin impaired insulin-stimulated glucose uptake in L6 myotubes. In some of these studies, it was concluded that the major node of insulin resistance occurred at the level of IRS-1 expression/activation and proximal insulin signaling (8, 26), while other studies suggested a reduced transport activity of GLUT4 (23, 25). Interestingly, we found that resistin, in addition to impairing insulin-stimulated glucose uptake, also impaired AICAR-stimulated glucose uptake without affecting AMPK signaling. As AICAR specifically regulates glucose uptake by activating AMPK (16) and does not rely upon the proximal insulin signaling pathway (12), our findings suggest that the major inhibitory site(s) sensitive to resistin lies downstream of where insulin and AICAR signaling converge. Consistent with this notion, we were not able to detect any defect in IRS-1 protein levels, IRS-1-associated P85 subunit of PI 3-kinase or Akt, and TBC1D4 phosphorylation. Although the differences between our studies and previous reports (8, 23, 25, 26) on the effects of resistin on insulin-signaling remain unclear, it is conceivable that this may be related to the nature of the resistin preparation. Interestingly, studies of primary cardiomyocytes reported the efficacy of resistin (produced in 293T cells) to impair insulin-stimulated glucose uptake was greatly reduced if the cysteine necessary for formation of hexameric resistin was mutated (C26A) to alanine (10). These data suggest that oligomerized resistin is the more potent form, at least in heart muscle, and that oligomerization of resistin has significant effects on its biological actions. In agreement with these observations, we detected significant inhibitory effects of resistin in the soleus muscle and myotubes at concentrations as low as 30 ng/ml, which is more than 10 times lower than the dose of bacterial expressed resistin previously reported to induce insulin resistance in myotubes (23). Interestingly, other studies have reported that the monomeric form of resistin is the most potent in inducing hepatic insulin resistance (29), suggesting tissue specific actions of different resistin forms similar to reported for adiponectin (48).

How does resistin impair insulin- and AICAR-stimulated glucose uptake? The plasma membrane translocation and membrane fusion of GLUT4 is a pivotal event in regulated glucose uptake in skeletal muscle (9, 54). Consistent with previous reports (8, 26), we found that resistin reduced GLUT4 translocation in response to insulin. However, importantly, we found that AICAR-stimulated GLUT4 translocation was also reduced with resistin in myotubes. Although it is difficult to equate these reductions in plasma membrane GLUT4 precisely with measurement of glucose transport due to the expression of multiple glucose transporter isoforms in muscle cells (19), these data clearly indicate that the GLUT4 translocation process is a likely convergence point for the inhibitory effects of resistin. These observations are supported by studies of cardiomyocytes reporting that mammalian expressed oligomerized resistin reduces both insulin-stimulated glucose uptake and plasma membrane GLUT4 content and vesicle trafficking without altering insulin signaling (10). It is also noteworthy that resistin increased GLUT4 plasma membrane content under basal conditions, also in accord with observations in cardiomyocytes (10), whereas glucose uptake was slightly reduced in L6 myotubes in the same condition. It has also been suggested that resistin may directly modify the Vmax of GLUT4 (23, 25), and we certainly cannot exclude the possibility that resistin, in addition to the effect on GLUT4 translocation, also reduced the Vmax of GLUT4. It is intriguing that in a recent study of multiple models of insulin resistance defects in insulin-dependent GLUT4 translocation were observed without defects in upstream elements of the insulin signaling cascade (13). Hence, it will be interesting to ascertain whether resistin converges at the same point as these models. Recent studies have shown that insulin, in addition to the classical PI 3-kinase/Akt pathway, regulates glucose uptake by facilitating GLUT4 translocation to the plasma membrane via actin remodeling (32). Thus, one may speculate that resistin could impair insulin- and AICAR-stimulated glucose uptake by interfering with actin structures essential for GLUT4 translocation.

To the best of our knowledge, neither the resistin receptor nor the downstream signaling pathways conveying resistin transduction have yet been identified. We measured phosphorylation of the inhibitory κB kinase, c-jun NH2-terminal kinase, and signal transducer and activator of transcription-3 (STAT3), as markers of inflammatory pathways (data not shown) and did not detect any increases in phosphorylation in accord with previous findings (44). Previous studies in adipocytes and hepatocytes (45, 52) have suggested that SOCS-3 is increased by resistin; however, we did not detect any increases in SOCS-3 mRNA in response to up to 28 h of resistin exposure in myotubes. As SOCS-3 inhibits IRS-1 signaling, our findings seem consistent with resistin not altering insulin signaling in skeletal muscle. The reason why resistin increases SOCS-3 expression in adipocytes and hepatocytes but not skeletal muscle is not known. Interestingly, we did observe an increase in muscle SOCS-3 after 7 days of in vivo hyperresistinemia (Supplementary Fig. S1D), which may suggest that increases in SOCS-3 may be dependent on additional circulating factors not available to the ex vivo cultured myotubes.

Resistin has been shown to reduce AMPK activity in L6 myotubes (27) and adipocytes (18). Chronic in vivo models have also shown that hyperresistinemia decreases AMPK phosphorylation in rat skeletal muscle and in liver and adipose tissue (35) and, conversely, that deletion of resistin is associated with an increase in liver AMPK activity (2). As activation of AMPK in muscle increases muscle glucose uptake (16, 22), we hypothesized that a reduction of AMPK with resistin would lead to a reduction in glucose uptake. However, we found that exposure of myotubes to resistin did not lead to detectable changes in AMPK signaling. Thus, despite the metabolic action of resistin impairing skeletal muscle glucose uptake, the signaling molecules coordinating these responses remain to be identified.

In summary, we have investigated the actions of resistin on insulin- and AICAR-stimulated glucose uptake in isolated differentiated skeletal muscle and in cultured L6 myotubes by using oligomerized resistin at concentrations found in obese animals. Interestingly, resistin impaired not only insulin- but also AICAR-stimulated glucose uptake without impairing either the PI 3-kinase/Akt or the AMPK signaling pathway or TBC1D4 phosphorylation. Importantly, resistin reduced GLUT4 plasma membrane content with insulin and AICAR and thus seems to impair insulin-stimulated glucose uptake, at least in part, by
hindering trafficking of GLUT4. We speculate that resistin impairs glucose transport in muscle at a common step downstream of TBC1D4 that is intimately involved in GLUT4 translocation to the plasma membrane.

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