MAP4K4 Gene Silencing in Human Skeletal Muscle Prevents Tumor Necrosis Factor-α-induced Insulin Resistance*

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Tumor necrosis factor-α (TNF-α) induces skeletal muscle insulin resistance by impairing insulin signaling events involved in GLUT4 translocation. We tested whether mitogenic-activated protein kinase kinase kinase kinase isoform 4 (MAP4K4) causes the TNF-α-induced negative regulation of extracellular signal-regulated kinase-1/2 (ERK-1/2), c-Jun NH2-terminal kinase (JNK), and the insulin receptor substrate-1 (IRS-1) on the insulin signaling pathway governing glucose metabolism. Using small interfering RNA (siRNA) to suppress the expression of MAP4K4 protein 85% in primary human skeletal muscle cells, we provide evidence that TNF-α-induced insulin resistance on glucose uptake was completely prevented. MAP4K4 silencing inhibited TNF-α-induced negative signaling inputs by preventing excessive JNK and ERK-1/2 phosphorylation, as well as IRS-1 serine phosphorylation. These results highlight the MAPK4K4/JNK/ERK/IRS module in the negative regulation of insulin signaling to glucose transport in response to TNF-α. Depletion of MAP4K4 also prevented TNF-α-induced insulin resistance on Akt and the Akt substrate 160 (AS160), providing evidence that appropriate insulin signaling inputs for glucose metabolism were rescued. Silencing of MAP2K1 and MAP2K4, signaling proteins downstream of MAP4K4, recapitulated the effect of MAP4K4 siRNA in TNF-α-treated cells. Thus, strategies to inhibit MAP4K4 may be efficacious in the prevention of TNF-α-induced inhibitory signals that cause skeletal muscle insulin resistance on glucose metabolism in humans. Moreover, in myotubes from insulin-resistant type II diabetic patients, siRNA against MAP4K4, MAP2K4, or MAP2K1 restored insulin action on glucose uptake to levels observed in healthy subjects. Collectively, our results demonstrate that MAP4K4 silencing prevents insulin resistance in human skeletal muscle and restores appropriate signaling inputs to enhance glucose uptake.

Obesity and type II (non-insulin-dependent) diabetes mellitus are becoming widespread metabolic disorders that are characterized by an overlapping phenotype of impaired insulin action in peripheral tissues (1, 2). Systemic inflammation is also a feature of obesity and type II diabetes, raising the hypothesis that elevated cytokine levels may contribute to peripheral insulin resistance (3–5). Correlative studies provide evidence that type II diabetes is associated with elevated levels of the proinflammatory cytokine tumor necrosis factor-α (TNF-α)2 in adipose tissue (6), skeletal muscle (7), and plasma (8–10). In healthy humans, TNF-α infusion induces skeletal muscle insulin resistance by impairing insulin signaling events involved in GLUT4 translocation without affecting endogenous glucose production (11). The molecular mechanism for TNF-α-induced insulin resistance may involve excessive phosphorylation of extracellular signal-regulated kinase-1/2 (ERK-1/2) and c-Jun NH2-terminal kinase (JNK), concomitant with increased serine and reduced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1).

JNK is a widely recognized target in the TNF-α-induced negative regulation of insulin signaling to metabolic endpoints. Thus, suppressors of JNK signaling may enhance insulin action and prevent defects in glucose metabolism after TNF-α stimulation. JNK is activated via a newly discovered protein named mitogenic-activated protein kinase kinase kinase kinase isoform 4 (MAP4K4) after treatment of cells with TNF-α (12–15) or other chemokine-cytokines such as osteopontin (16). MAP4K4 is a member of the germinal center kinase group related to Saccharomyces cerevisiae MAP4K, Sterile 20 (17, 18). This serine threonine kinase, also named nuclear factor-inducing kinase, phosphorylates ERK-1/2 (16) and JNK through activation of mitogen-activated protein kinase kinase kinase (MEK1) (19). MAP4K4 appears to play a role in TNF-α-induced insulin resistance. For example, deletion of MAP4K4 in 3T3-L1 adipocytes restores GLUT4 trafficking after TNF-α stimulation (13).

We explored the role of MAP4K4 as intermediate kinase for TNF-α action on glucose uptake in human skeletal muscle cells.

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2 The abbreviations used are: TNF-α, tumor necrosis factor-α; ERK-1/2, extracellular signal-regulated kinase-1/2; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK1, MAPK kinase kinase; MAP4K4, MAPK kinase kinase isoform 4; IRS-1, insulin receptor substrate-1; AS160, Akt substrate of 160 kDa; DMEM, Dulbecco’s modified Eagle’s medium; IL-6, interleukin-6; PPARγ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA; MAP2 K1, mitogen-activated protein kinase kinase 1; MAP2 K2, mitogen-activated protein kinase kinase 4.
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and tested the hypothesis that MAP4K4 is involved in JNK and ERK-1/2 activation to modulate insulin sensitivity. Using siRNA to suppress the expression of MAP4K4, we show that TNF-α-induced insulin resistance on signal transduction at the level of Akt and the Akt substrate 160 (AS160), as well as glucose uptake, are prevented. Moreover, we provide evidence that MAP4K4 silencing prevents TNF-α-induced JNK and ERK-1/2 phosphorylation, as well as IRS-1 serine phosphorylation. Silencing MAP2K1 and MAP2K4, intermediary kinases involved in ERK and JNK signaling, respectively, recapitulated the effect of MAP4K4 silencing in TNF-α-treated cells. Thus, strategies to inhibit MAP4K4 are efficacious in the prevention of TNF-α-induced inhibitory signals that cause insulin resistance in skeletal muscle. Finally, we also provide evidence that MAP4K4 silencing in skeletal muscle cells from type II diabetic patients restores insulin-mediated glucose uptake.

EXPERIMENTAL PROCEDURES

Antibodies and Reagent—Antibodies against MAP4K4 was from Abgent (San Diego, CA). Akt (Ser-473), Akt, and ERK-1/2 (p42/44 MAPK) kinase (Thr-202 and Tyr-204) were purchased from New England Biolabs (Beverly, MA). Anti-phospho-(Ser/Thr) Akt substrate, MAP2K1, MAP2K4, anti-phospho-IRS-1 (Ser-312), and IRS-1 (Ser-636, Ser-639) antibodies were obtained from Cell Signaling Technology. Radiochemical 2-[G-3H]deoxy-D-glucose (6.0 Ci mmol⁻¹ x liter⁻¹) was from Amersham Biosciences (Uppsala, Sweden). siRNA primers were purchased from Ambion (Austin, TX). Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-10 medium, fetal bovine serum, penicillin, streptomycin, and fungizone were obtained from Invitrogen (Invitrogen, Stockholm, Sweden). Unless otherwise stated, all other reagents were from Sigma. Radioactive reagents were purchased from Amersham Biosciences.

Subject Characteristics—Skeletal muscle biopsies were obtained with the informed consent of the donors either during scheduled abdominal surgery (rectus abdominus) or during an open biopsy (vastus lateralis) from 10 healthy control subjects (six males, four females; aged 52 ± 4 years; body mass index of 27.8 ± 1.1 kg/m²) were transfected using Lipofectamine 2000 (Invitrogen). Differentiation medium were changed to antibiotic-free growth medium on day 2 of myobute differentiation. On day 3, individual siRNAs (1 μg/ml) were transfected using Lipofectamine 2000 in serum-free DMEM (incubating time >16 h). Myotubes were then washed with phosphate-buffered saline, and DMEM containing 2% fetal bovine serum was added in each well. On day 6, cells were deprived of serum for 16 h before insulin stimulation. Control cultures were similarly prepared but without the addition of siRNA (control) or transfected using a scrambled primer (siRNA Scr). The scrambled construct was similar to the target sequence (same GC content) encoding a nonspecific siRNA without mammalian homology. Different scrambled constructs were tested for each experiment to assure the silencing specificity.

Western Blot Analysis—Aliquots of cell lysate (20 μg of protein) were resuspended in Laemmli sample buffer. Proteins were then separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked with 7.5% nonfat milk, washed with TBST (10 mM Tris-HCL, 100 mM NaCl, 0.02% Tween 20), and incubated with appropriate primary antibodies overnight at 4 °C. Membranes were washed with TBST and incubated with an appropriate secondary antibody for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

2-Deoxyglucose Transport—Glucose uptake was performed as described previously for primary human muscle cells (22). Interleukin-6 (IL-6) Production—IL-6 was measured from cell culture supernatants after different duration of TNF-α stimulation by enzyme-linked immunosorbent assay with the IL-6 human enzyme-linked immunosorbent assay kit (R&D Systems Europe Ltd., Oxon, UK).

Statistics—Data are presented as mean ± S.E. Statistical differences were determined by Students t test or analysis of variance using Fisher’s least significant difference test for post hoc determination.

RESULTS

Effect of TNF-α on IL-6 Release—IL-6 release from human skeletal muscle cells was determined after different time periods of TNF-α stimulation (Fig. 1). During the first 8 h of TNF-α stimulation, IL-6 release was not significantly different from basal. After 12 and 24 h, significant IL-6 release was observed. To avoid any potential effect of IL-6 on signaling and metabolic parameters, experiments were conducted after 2 h of TNF-α stimulation.
MAP4K4 Silencing in Myotubes—The effect of MAP4K4 gene silencing on target protein expression was determined in differentiated myotubes. Two days after induction of the myotube differentiation program, cells were transfected with siRNA against MAP4K4. MAP4K4 protein expression was reduced 85% compared either untransfected cells or cells transfected with scrambled siRNA (Fig. 2). Myotube morphology was unaltered after MAP4K4 silencing (data not shown).

MAP4K4 Gene Silencing Directly Prevents TNF-α-induced Impairment in Glucose Metabolism—The role of MAP4K4 in glucose uptake was determined in differentiated myotubes. Four days after transfection, glucose uptake was assessed under basal and insulin-stimulated conditions before or after 2 h TNF-α treatment. In control or transfected cells with scrambled siRNA, insulin increased glucose uptake 1.7-fold (Fig. 3). Basal glucose transport was unaltered in cells exposed to TNF-α. In contrast, TNF-α treatment completely blocked insulin action on glucose uptake. Silencing of MAP4K4 was without effect on either basal or insulin-stimulated glucose uptake. Importantly, TNF-α-induced insulin-resistant on glucose uptake was rescued by MAP4K4 silencing. We next tested the hypothesis that treatment of cells with siRNA causes release of factor(s), which are in turn responsible for the observed changes in the TNFα effect. Control cells were treated with conditioned medium from transfected cells as described in the legend for Fig. 3. In control cells treated with conditioned media from cells where MAP4K4 was silenced, insulin action on glucose uptake tended to be reduced, although this effect was not statistically significant (Fig. 4). Moreover, TNF-α-induced insulin resistance was not rescued by conditioned media from siMAP4K4 transfected cells (Fig. 4).

MAP4K4 Silencing Prevents TNF-α Action on JNK and ERK-1/2—The role of MAP4K4 in TNF-α-induced JNK and ERK-1/2 signaling was assessed as these targets are implicated in the negative regulation of the insulin signaling cascade to glucose uptake. Insulin increased JNK and ERK-1/2 phosphorylation in control and cells transfected with scrambled siRNA. TNF-α increased basal JNK and ERK-1/2 phosphorylation (Fig. 5). MAP4K4 silencing reduced basal and abolished insulin-stimulated JNK and ERK-1/2 phosphorylation. Similar effects were observed in MAP4K4-depleted cells exposed to TNF-α. MAP4K4 silencing prevented TNF-α-induced JNK and ERK phosphorylation. Protein expression of JNK and ERK-1/2 was unaltered in cells depleted of MAP4K4. Thus, JNK and ERK-1/2 signaling, but not protein expression, are linked to MAP4K4 actions.
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MAP4K4 Silencing Prevents TNF-α-induced IRS-1 Serine Phosphorylation—Elevated levels of TNF-α and excessive JNK and ERK phosphorylation are correlated with increased IRS-1 serine phosphorylation and negative regulation insulin signaling pathways important for glucose metabolism. We next determined whether MAP4K4 gene silencing prevents TNF-α-induced IRS-1 serine phosphorylation. TNF-α increased basal IRS-1 serine phosphorylation on residues 312 and 636/639 (Fig. 6). Depletion of MAP4K4 prevented TNF-α action on basal IRS-1 serine phosphorylation on residues 312 and 636/639. Thus, MAP4K4 is required for TNF-α effects on basal IRS-1 serine phosphorylation. Interestingly, insulin-stimulated serine phosphorylation of IRS-1 at 636/639, but not 312, was prevented in MAP4K4-depleted cells. This MAP4K4 dependence on insulin-stimulated IRS-1 Ser-636/639 phosphorylation was also noted for JNK and ERK1/2. In TNF-α-treated cells, depletion of MAP4K4 restored insulin action on IRS-1 Ser-636/639 phosphorylation when compared with cells transfected with scrambled siRNA. Total protein expression of IRS-1 was unaltered after MAP4K4 silencing, indicating that the observed effects were on changes in phosphorylation rather than protein expression.

TNF-α-induced Insulin Resistance on Akt and AS160 Is Prevented by MAP4K4 Silencing—Insulin increased phosphorylation of Akt Ser-473 and AS160 in control or transfected cells with scrambled siRNA (Fig. 7). Although TNF-α treatment did not alter basal Akt Ser-473 phosphorylation, basal AS160 phosphorylation was increased ~2-fold. Importantly, TNF-α com-
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FIGURE 8. Protein expression of MAP2K1 and MAP2K4 in human skeletal muscle myotubes. Protein expression was measured at day 4 of differentiation after siRNA-mediated depletion of MAP2K1 and MAP2K4. Data are mean ± S.E. *, p < 0.05 versus untransfected control.

FIGURE 9. Effect of MAP2K1 and/or MAP2K4 siRNA on glucose uptake. Glucose uptake was measured on differentiated primary human skeletal muscle myotubes under basal conditions (open box) and after insulin stimulation (closed box). Measurements were performed after silencing of MAP2K1 and MAP2K4. Data are mean ± S.E. *, p < 0.05 versus basal untransfected control, #, p < 0.05 versus insulin-stimulated untransfected control.

FIGURE 10. Protein expression of MAP2K1, MAP2K4, and MAP4K4 in human skeletal muscle myotubes from type II diabetic patients. Protein expression was measured at day 4 of differentiation after siRNA-mediated depletion of MAP2K1, MAP2K4, or MAP4K4. Data are mean ± S.E. *, p < 0.05 versus untransfected control.

FIGURE 11. Effect of MAP2K1, MAP2K4, and MAP4K4 siRNA on glucose uptake in myotubes from type II diabetic patients. Glucose uptake was measured on differentiated primary human skeletal muscle myotubes from type II diabetic patients under basal conditions (open box) and after insulin stimulation (closed box). Measurements were performed after silencing of MAP2K1, MAP2K4, and/or MAP2K4. Data are mean ± S.E. *, p < 0.05 versus basal untransfected control, #, p < 0.05 versus insulin-stimulated untransfected control.

In cells pretreated with TNF-α, insulin action on glucose was partially restored in cells where MAP2K1 or MAP2K4 was suppressed. In cells where both MAP2K1 and MAP2K4 were silenced, TNF-α-induced insulin resistance was prevented. MAP4K4 Silencing Restores Insulin-stimulated Glucose Uptake in Primary Human Skeletal Cells from Type II Diabetic Patients—To test the hypothesis that MAP4K4 silencing improves insulin action in insulin-resistant humans, skeletal muscle cultures were prepared from biopsies obtained from type II diabetic patients and healthy control subjects. siRNA against MAP4K4, MAP2K1, or MAP2K4 significantly reduced target protein expression in human skeletal muscle myotubes from type 2 diabetic patients (Fig. 10). Insulin-mediated glucose uptake was markedly impaired in myotubes from type II diabetic patients versus healthy control subjects (Fig. 11). MAP4K4 siRNA restored insulin action on glucose uptake in myotubes from type II diabetic patients to levels observed in healthy subjects. Silencing of MAP2K1 increased basal glucose uptake and restored insulin action in myotubes from type II diabetic
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patients. Depletion of either MAP2K4 or MAP2K1 and MAP2K4 in myotubes from type II diabetic patients was without effect on basal glucose uptake and completely restored insulin action.

DISCUSSION

We previously provided in vivo evidence that infusion of TNF-α induces skeletal muscle insulin resistance in healthy humans as evident by changes in insulin signaling and whole body glucose uptake during a euglycemic hyperinsulinemic clamp (11). In the present study, we explored the mechanism by which TNF-α induces insulin resistance in skeletal muscle using siRNA. We considered MAP4K4 as a TNF-α target based on earlier work in 3T3-L1 adipocytes where siRNA against MAP4K4 enhances GLUT4 expression and insulin-stimulated glucose transport, concomitant with up-regulation of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein (C/EBPα) expression (13).

MAP4K4 belongs to the Sterile 20 group of protein kinases and is a putative effector of Rap2, a Ras family small GTP-binding proteins that mediates the activation of JNK (19, 23). After osteopontin treatment, MAP4K4 has been shown to phosphorylate JNK through activation of MEKK1 (19), as well as ERK-1/2 (16). The role of MAP4K4 in ERK-1/2 activation has been challenged to resolve because MAP4K4 does not seem to interact with upstream proteins shown to be important for ERK-1/2 activation (19). Furthermore, human tumor cells expressing an inactive mutated form of MAP4K4 and treated with hepatocyte growth factor resulted in a decrease in ERK-1/2 phosphorylation (24). Here we report that MAP4K4 is required for insulin, as well as TNF-α action on JNK and ERK-1/2 phosphorylation in primary human skeletal muscle cells. MAP4K4 silencing (85%) prevented insulin, as well as TNF-α action on ERK-1/2 and JNK, without altering total protein levels. Protein kinases including TAK1, MAP2K4, MAP2K1, and MKK7 acting between MAP4K4, ERK-1/2, and JNK are necessary for activation of this pathway (23). However, these intermediate kinases are not necessarily common in the ERK-1/2 and JNK activating pathways. When either MAP2K4 or MAP2K1 was silenced, TNF-α-mediated insulin resistance was prevented, as observed for MAP4K4. Thus, we show for the first time that MAP4K4 is a common upstream kinase linking ERK-1/2 and JNK signaling pathways in human skeletal muscle cells in response to insulin or TNF-α treatment.

In 3T3-L1 adipocytes, TNF-α stimulation increases MAP4K4 expression, thereby promoting insulin resistance, probably due to cross-talk action with PPARγ signaling (13). Thus, in adipocytes, MAP4K4 appears to contribute to the regulation of glucose metabolism as a suppressor of PPARγ and adipogenesis. We explored the role of MAP4K4 on insulin signaling and acute TNF-α action on metabolic endpoints in human skeletal muscle. Our previous work in healthy human volunteers provides evidence that TNF-α infusion increases skeletal muscle ERK-1/2 and JNK phosphorylation, concomitant with IRS-1 serine phosphorylation and negative regulation of insulin action on phosphatidylinositol 3-kinase, AS160, and glucose uptake (11, 20). We therefore tested the hypothesis that MAP4K4 silencing enhances insulin signaling in TNF-α-treated human skeletal muscle cells. Suppression of MAP4K4 was without effect on basal IRS-1 serine phosphorylation at Ser-636/-639 or -312. However, insulin-stimulated serine phosphorylation of IRS-1 at 636/639, but not 312, was abolished in MAP4K4-depleted cells. This MAP4K4 dependence on insulin-stimulated IRS-1 Ser-636/-639 phosphorylation was also noted for JNK and ERK1/2. These data provide evidence for a previously unrecognized role of MAP4K4 in IRS action to JNK and ERK signaling modules.

The TNF-α-induced increase in phosphorylation of IRS1 at Ser-636/-639 and Ser-312 was completely prevented by MAP4K4 silencing. Thus, TNF-α induces MAP4K4-dependent ERK-1/2, JNK, and IRS-1 serine phosphorylation, eliciting negative signaling input downstream of IRS-1 to impair insulin action on glucose uptake and metabolism in skeletal muscle. Indeed, suppression of MAP4K4 prevented TNF-α-induced insulin resistance on phosphorylation of Akt and AS160. This provides a mechanism for the enhanced insulin-mediated glucose uptake as signal transduction via Akt and AS160 are important for insulin action on glucose uptake and metabolism (25, 26). Our data show that targeted deletion of MAP4K4, an early component of the TNF-α signaling pathway, can enhance insulin signaling and prevent skeletal muscle insulin resistance.
The mechanism responsible for the development of insulin resistance in humans remains unclear. However, defects in insulin signaling and glucose metabolism, as evidenced by excessive serine phosphorylation of IRS-1 and impairments in insulin-mediated glucose uptake, have been observed in cultured skeletal muscle cells from type II diabetic patients, indicating that features of the insulin-resistant state are maintained (20). We determined the role of MAP4K4, as well as other kinases involved in MAPK signaling on insulin action, and tested the hypothesis that silencing of MAP4K4 could reverse insulin resistance in human skeletal muscle cells from type II diabetic patients. MAP4K4 silencing restored insulin action on glucose uptake in myotubes from type II diabetic patients. To confirm whether MAP4K4 is involved in MAPK signaling pathway activation, we also silenced MAP2K1 and MAP2K4, downstream targets of MAP4K4. Silencing of either MAP2K1 or MAP2K4 recapitulated the effect of MAP4K4 silencing to restore insulin-mediated glucose uptake.

In summary, we have identified MAP4K4 as a potential therapeutic target to prevent peripheral insulin resistance. MAP4K4 silencing protects against TNF-α-mediated insulin resistance in myotubes from healthy subjects. Moreover, MAP4K4 silencing restores insulin action in myotubes from insulin-resistant type II diabetic patients. We propose a model where excessive TNF-α levels activate a negative feedback loop on insulin signaling to glucose uptake and metabolism via MAP4K4 (Fig. 12), which couples ERK-1/2 and JNK signaling pathways to IRS-1 serine phosphorylation. Collectively, our results demonstrate that MAP4K4 silencing prevents TNF-α-mediated insulin resistance in human skeletal muscle and restores appropriate signaling inputs to enhance glucose uptake.

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