Effects of Insulin, Contraction, and Phorbol Esters on Mitogen-Activated Protein Kinase Signaling in Skeletal Muscle From Lean and *ob/ob* Mice

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Effects of diverse stimuli, including insulin, muscle contraction, and phorbol 12-myristate-13-acetate (PMA), were determined on phosphorylation of mitogen-activated protein kinase (MAPK) signaling modules (c-Jun NH_2-terminal kinase [JNK], p38 MAPK, and extracellular signal–related kinase [ERK1/2]) in skeletal muscle from lean and *ob/ob* mice. Insulin increased phosphorylation of JNK, p38 MAPK, and ERK1/2 in isolated extensor digitorum longus (EDL) and soleus muscle from lean mice in a time- and dose-dependent manner. Muscle contraction and PMA also elicited robust effects on these parallel MAPK modules. Insulin action on JNK, p38 MAPK, and ERK1/2 phosphorylation was significantly impaired in EDL and soleus muscle from *ob/ob* mice. In contrast, muscle contraction–mediated JNK, p38 MAPK, and ERK1/2 phosphorylation was preserved. PMA effects on phosphorylation of JNK and ERK1/2 were normal in *ob/ob* mice, whereas effects on p38 MAPK were abolished. In conclusion, insulin, contraction, and PMA activate MAPK signaling in skeletal muscle. Insulin-mediated responses on MAPK signaling are impaired in skeletal muscle from *ob/ob* mice, whereas the effect of contraction is generally well preserved. In addition, PMA-induced phosphorylation of JNK and ERK1/2 are preserved, whereas p38 MAPK pathways are impaired in skeletal muscle from *ob/ob* mice. Thus, appropriate MAPK responses can be elicited in insulin-resistant skeletal muscle via an insulin-independent mechanism. *Diabetes* 53:1436–1444, 2004

Multiple mechanisms contribute to the regulation of glucose metabolism and gene expression in skeletal muscle. Insulin signaling along the insulin receptor substrate (IRS)/phosphatidylinositol (PI) 3-kinase pathway is considered to be important for the major metabolic actions of insulin, whereas mitogen-activated protein kinase (MAPK) cascades constitute signaling networks by which insulin regulates gene expression (1). Accumulating evidence suggests that insulin signaling defects along metabolic pathways involving IRS/PI 3-kinase are associated with skeletal muscle insulin resistance in obesity, type 2 diabetes, and gestational diabetes. In contrast, little is known of the regulation of MAPK cascades in insulin-resistant states, such as diabetes.

MAPK cascades are activated by a variety of stimuli, including hormones, growth factors, cytokines, and environmental stress, and transduce extracellular signals to multiple cellular responses, including proliferation, differentiation, apoptosis, and gene expression (2–5). MAPK pathways consist of at least three parallel cascades: extracellular signal–regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK (6). ERK1/2 is activated primarily by growth factors, whereas JNK and p38 MAPK are mainly activated in response to cellular stress (7). Insulin also leads to a rapid increase in JNK activity and p38 MAPK phosphorylation in skeletal muscle (8–12). However, insulin action on these diverse MAPK cascades in skeletal muscle has not been characterized in the context of type 2 diabetes.

Several insulin-independent factors increase MAPK signaling. Emerging evidence suggests that muscle contraction through exercise increases ERK1/2, JNK, and p38 MAPK signaling directly in skeletal muscle (13–20). ERK1/2, JNK, and p38 MAPK phosphorylation is increased in human skeletal muscle after exercise (17,19,20) and in rat skeletal muscle in response to contraction (18,21,22), exercise (13), muscle overload (23), and mechanical stretch (21). This provides evidence that insulin-independent factors lead to a robust stimulation of MAPK cascades. Phorbol esters are functional analogs of diacylglycerol (DAG) that have been used to stimulate MAPK signaling via insulin-independent pathways. Incubation of epitrochlearis muscle with 12-*O*-tetradecanoylphorbol 13-acetate increases ERK1/2 phosphorylation (18). In contrast, effects of phorbol esters on JNK and p38 MAPK phosphorylation in skeletal muscle are unclear. Given the profound effect of exercise and phorbol esters on MAPK signaling, alternative insulin-independent mechanisms may conceivably elicit positive effects on ERK, JNK, and/or p38 MAPK in insulin-resistant skeletal muscle.

MAPK cascades have been implicated in skeletal muscle remodeling and gene regulatory responses (24). To date, the effects of insulin on parallel MAPK signaling pathways in skeletal muscle have not been completely resolved.

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DAG, diacylglycerol; EDL, extensor digitorum longus; ERK, extracellular signal–related kinase; IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate.

Moreover, the extent to which insulin-dependent and insulin-independent stimuli increase MAPK phosphorylation in insulin-resistant skeletal muscle is unknown. Thus, we determined the effects of diverse stimuli (insulin, contraction, and phorbol 12-myristate-13-acetate [PMA]) on JNK, p38 MAPK, and ERK phosphorylation in skeletal muscle from insulin-sensitive lean (C57/BL6) and insulin-resistant obese diabetic *ob/ob* mice.

RESEARCH DESIGN AND METHODS

Animals. Female C57/BL6 and *ob/ob* mice (12–16 weeks old) were purchased from B&K Universal (Sollentuna, Sweden) and housed for at least 1 week before use. All mice were maintained on a 12-h light-dark cycle and had free access to water and standard rodent diet. The Ethics Committee on Animal Research in Northern Stockholm approved all protocols.

Muscle isolation and incubation. Animals were studied after an overnight fast to control for food intake. Mice were anesthetized by an intraperitoneal injection of 2.5% Avertin (0.02 ml/g body wt). Extensor digitorum longus (EDL) and soleus muscle were isolated for in vitro incubation, as previously described for rat epitrochlearis muscle (25). All incubation media were prepared from a pregassed (95% O_2/5% CO_2) stock of Krebs Henseleit buffer supplemented with 5 mmol/l HEPES, 0.1% BSA (radioimmunoassay grade), 5 mmol/l glucose, and 15 mmol/l mannitol. After a 15-min preincubation (30°C), muscles were incubated in the absence or presence of insulin or PMA as described in the figures. The gas phase during the incubation was maintained at 95% O_2/5% CO_2.

In vitro muscle contraction. After preincubation, EDL muscles were placed inside a temperature-controlled (30°C) chamber and immersed in 4 ml of Krebs Henseleit buffer identical to the preincubation condition. Each muscle was positioned between two platinum electrodes, with the distal tendon mounted to the bottom of the chamber (18). The proximal tendon was connected to a jeweler's chain, which was fixed to an isometric force transducer (Harvard Apparatus, South Natick, MA). Resting tension was adjusted to 0.5 g. Isometric tension development during the contraction protocol was recorded using a compact 2-Channel Student Oscillograph (Harvard Apparatus). Muscles were stimulated at a frequency of 100 Hz (0.2 ms pulse duration, 10 volt amplitude), delivered at a rate of one 0.2-s contraction every 2 s (0.2 s/2 s) for 10 min. Pulses were generated by a Tektronix TM 503 Power Module (Beaverton, OR) and amplified on a 4-Channel Power Amplifier (Somedic, Sollentuna, Sweden). Basal muscles were treated as described above, minus electrical stimulation. Muscles were frozen in liquid nitrogen immediately after being contracted.

Muscle homogenization. Muscles were homogenized on ice in 0.3 ml of buffer (50 mmol/l Tris-HCl [pH 7.5], 0.1% Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l NaF, 10 mmol/l β-glycerophosphate, 5 mmol/l sodium pyrophosphate, 0.1% β-mercaptoethanol, 1 μmol/l microcystin, and 10 μg/ml aprotinin and leupeptin). Homogenates were sonicated (two times for 5 s), rotated for 40 min, and centrifuged (12,000*g* for 10 min at 4°C). Supernatants were collected, and protein concentration was determined using the Bradford method (BioRad, Richmond, CA). Aliquots that contained 50 μg of protein were suspended in Laemmli buffer.

Western blot analysis. Proteins were separated by SDS-PAGE (10% resolving gel), transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 7.5% nonfat milk. Membranes were incubated overnight (4°C) with a phospho-specific antibody, followed by a 1-h wash with Tris-buffered saline with Tween (10 mmol/l Tris, 140 mmol/l NaCl, 0.02% Tween 20 [pH 7.6]). The following phospho-specific antibodies (1:1,000; Cell Signaling, Beverly, MA) were used: phospho-JNK antibody, recognizing JNK phosphorylation at Thr^{183} and Tyr^{185}; phospho-p38 MAPK antibody, recognizing p38 phosphorylation at Thr^{180} and Tyr^{182}; and phospho-p44/42 MAPK antibody, recognizing ERK1/2 phosphorylation at Thr^{202} and Tyr^{204}. Membranes were probed with a secondary antibody (1:25,000, goat anti-rabbit IgG horseradish peroxidase conjugate; BioRad) and washed in Tris-buffered saline with Tween for 1 h. Phosphorylated proteins were detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) and quantified by densitometry (BioRad). After determination of protein phosphorylation, membranes were incubated in stripping buffer (1 mol/l Tris [pH 6.7], 10% SDS, and β-mercaptoethanol; 45 min at 60°C), washed extensively, and subjected to immunoblot analysis to determine JNK, p38, or ERK protein expression using antibodies that recognize JNK (1:1,000; Cell Signaling), p38 MAPK (1;1,000; Cell Signaling), or pan-ERK (1:5,000; Cell Signaling), respectively. The phospho-specific JNK antibody used in this study readily detected the p46 isoform and poorly detected the p54 isoform; thus, results are reported for p46 JNK.

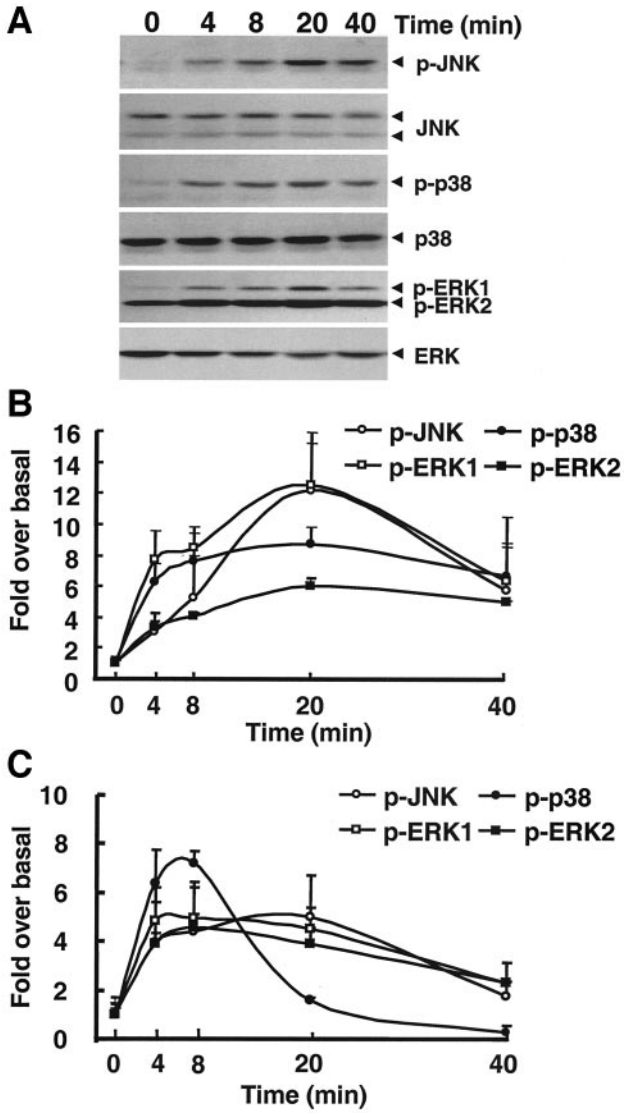

FIG. 1. Time course of MAPK phosphorylation in response to insulin. *A*: Representative immunoblot for MAPK phosphorylation (EDL). Isolated EDL (*B*) or soleus (SOL) (*C*) muscle from lean mice was incubated in the absence or presence of insulin (120 nmol/l). Phosphorylation of JNK, p38 MAPK, and ERK1/2 was determined by Western blot analysis using phospho-specific antibodies as indicated in RESEARCH DESIGN AND METHODS. Membranes were stripped and reblotted to ensure equal loading. Mean ± SE fold response for $n = 3$ muscles.

Statistics. Data are presented as mean ± SE. Differences between groups were determined by one-way ANOVA. Fisher's least significant differences post hoc analysis was used to identify significant differences ($P < 0.05$).

RESULTS

Time course for phosphorylation of JNK, p38 MAPK, and ERK1/2. A time course for the effects of insulin on JNK, p38 MAPK, and ERK1/2 phosphorylation was determined. EDL and soleus muscle were incubated in the absence or presence of insulin (120 nmol/l) for the times indicated (Fig. 1). Phosphorylation of JNK, p38 MAPK, and ERK1/2 was determined. Equal loading of gels was confirmed by immunoblot analysis for JNK, p38 MAPK, and ERK1/2 protein expression (data shown for EDL). Basal phosphorylation of JNK, p38 MAPK, and ERK1/2 was unchanged during the incubation protocol (data not shown). Insulin led to a rapid and transient phosphorylation of MAPK signaling in both EDL and soleus muscle. In EDL

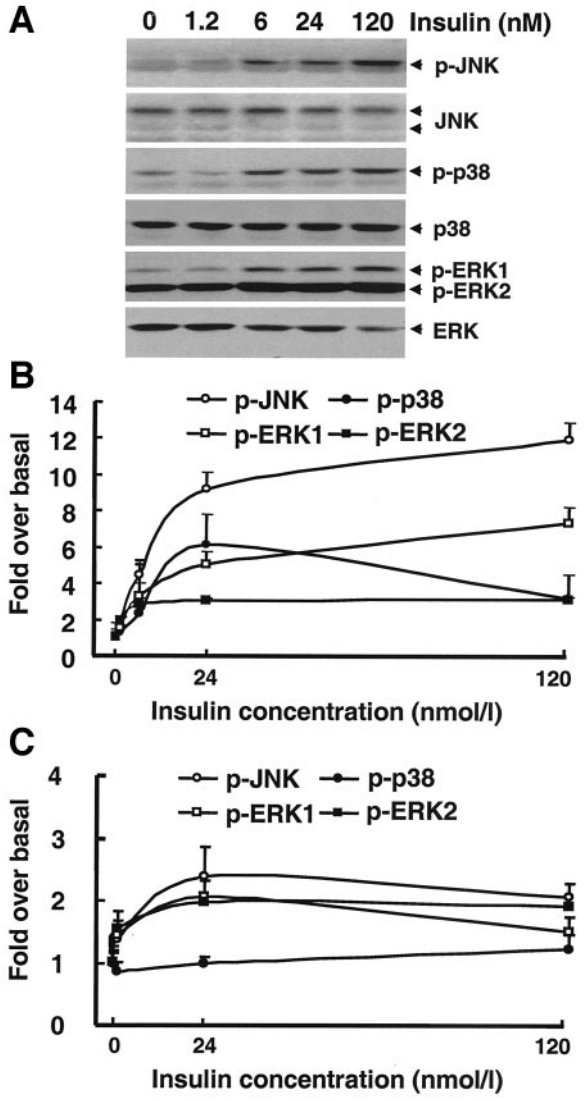

FIG. 2. Insulin dose-response curve for MAPK phosphorylation. *A*: Representative immunoblot for MAPK phosphorylation (EDL). Isolated EDL (*B*) or SOL (*C*) muscle from lean mice was incubated in the absence or presence of insulin (1.2, 6, 24, or 120 nmol/l) for 20 min. Phosphorylation of JNK, p38 MAPK, and ERK1/2 was determined by Western blot analysis using phospho-specific antibodies as indicated in RESEARCH DESIGN AND METHODS. Membranes were stripped and reblotted to ensure equal loading. Mean ± SE fold response for $n = 3$ muscles.

muscle, JNK, p38 MAPK, and ERK1 and ERK2 phosphorylation was increased after 4 min (3.0-, 6.2-, 7.7-, and 3.3-fold, respectively), with maximal effects observed after 20 min. In soleus muscle, JNK, p38 MAPK, and ERK1 and ERK2 phosphorylation was increased after 4 min (4.0-, 6.4-, 4.8-, and 3.2-fold, respectively), with maximal effects of JNK and ERK observed at 20 min and p38 MAPK observed between 4 and 8 min.

Insulin response for phosphorylation of JNK, p38 MAPK, and ERK1/2. EDL and soleus muscle were incubated for 20 min in the absence or presence of insulin (1.2, 6, 24, or 120 nmol/l; Fig. 2). Equal loading of gels was confirmed by immunoblot analysis for JNK, p38 MAPK, and ERK1/2 protein expression (data shown for EDL). Insulin induced a dose-dependent increase in JNK, p38 MAPK, and ERK1/2 phosphorylation in both EDL and soleus.

Comparison of insulin-mediated MAPK signaling between different skeletal muscle fiber types. Insulin action on JNK, p38 MAPK, and ERK1/2 phosphorylation was directly compared between EDL (glycolytic) and soleus (oxidative) skeletal muscle (Fig. 3). Skeletal muscle was incubated for 20 min in the absence or presence of 120 nmol/l insulin. Samples were processed in parallel to allow for direct comparisons of MAPK signaling between different fiber types. Insulin action on JNK and p38 MAPK phosphorylation was greater in EDL versus soleus muscle, whereas ERK1 and ERK2 phosphorylation was similar between muscle fiber types. Because the majority of the skeletal muscle groups in mice characteristically have a greater proportion of glycolytic versus oxidative fibers (26), we focused our analysis of MAPK signaling on EDL muscle.

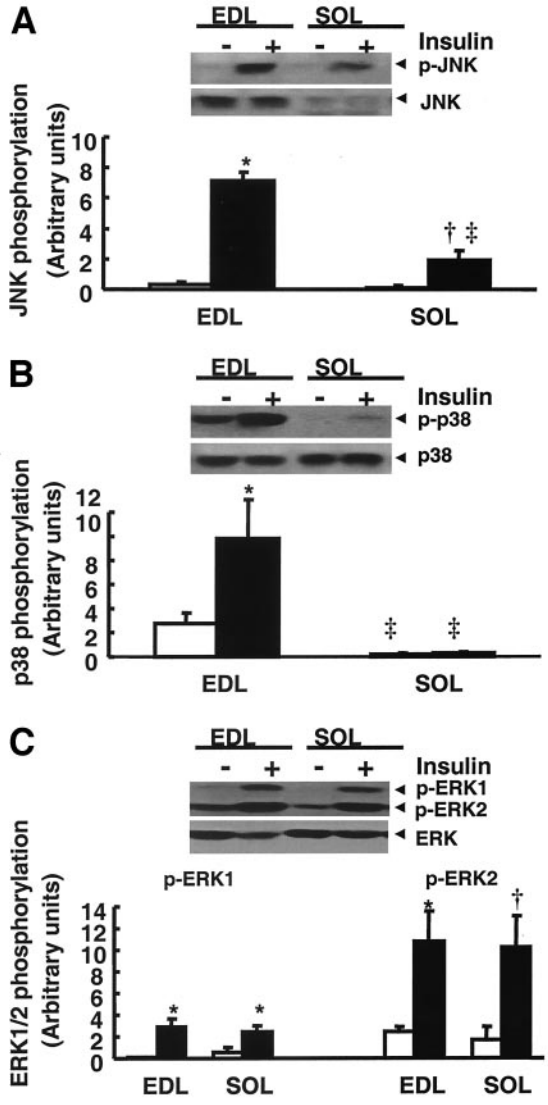

FIG. 3. Effect of insulin on MAPK phosphorylation in EDL and SOL muscle. Isolated EDL or SOL muscle from lean mice was incubated without (□) or with (■) 120 nmol/l insulin for 20 min. Phosphorylation of JNK (*A*), p38 MAPK (*B*), and ERK1/2 (*C*) was determined, as described in RESEARCH DESIGN AND METHODS. Membranes were reprobed after stripping to determine protein expression of the respective MAPK. Representative immunoblot for phosphorylation or protein expression of the specified MAPK (*upper panels*), and mean ± SE fold response for $n = 3$ muscles (*lower panels*). *$P < 0.05$ vs. EDL basal; †$P < 0.05$ vs. SOL basal; ‡$P < 0.05$ vs. EDL muscle.

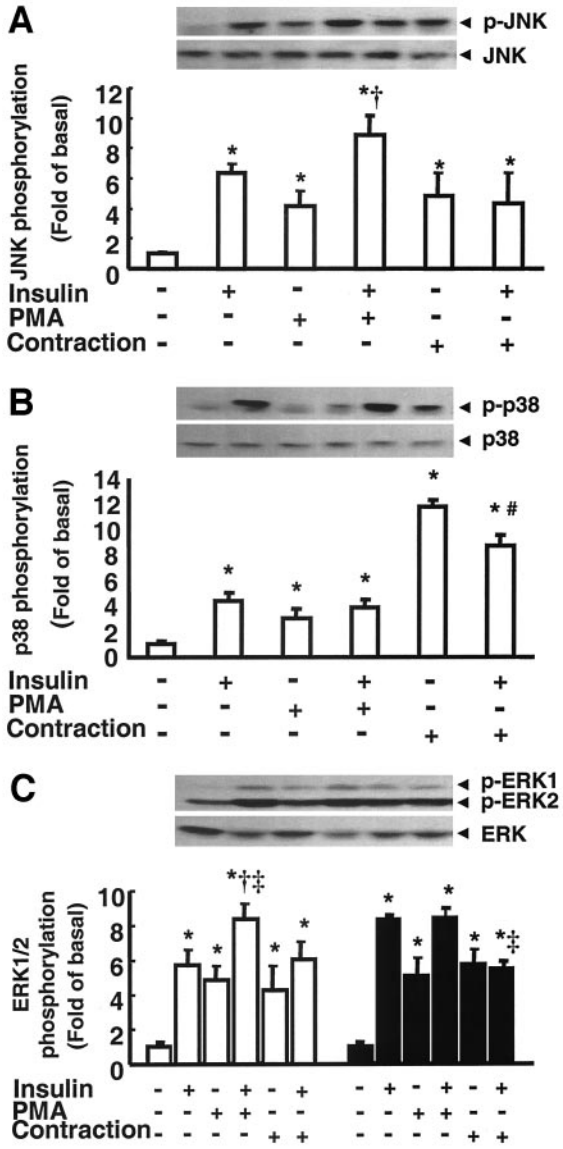

FIG. 4. Effect of insulin, PMA, and contraction on MAPK phosphorylation. Isolated EDL muscle from lean mice was incubated in the absence or presence of 120 nmol/l insulin, with either an addition of 2 μmol/l PMA or subsequent exposure to electrical stimulation, as described in RESEARCH DESIGN AND METHODS. Phosphorylation of JNK (*A*), p38 MAPK (*B*), and ERK1/2 (*C*: □, ERK1; ■, ERK2) was determined by Western blot analysis. Representative immunoblot for MAPK phosphorylation or protein expression (*upper panels*), and mean ± SE fold response for $n = 4–8$ muscles (*lower panels*). *$P < 0.05$ vs. basal; †$P < 0.05$ vs. PMA; ‡$P < 0.05$ vs. insulin; #$P < 0.001$ vs. contraction

Effect of contraction and PMA on phosphorylation of JNK, p38 MAPK, and ERK1/2. Muscle contraction increased phosphorylation of JNK (Fig. 4*A*), p38 MAPK (Fig. 4*B*), and ERK1/2 (Fig. 4*C*) in EDL muscle. Contraction-induced ERK1 phosphorylation was similar to that achieved in response to either insulin or PMA, whereas contraction-induced JNK and ERK2 phosphorylation was similar to that achieved in response to PMA. Contraction-induced p38 MAPK phosphorylation was nearly threefold greater compared with either insulin- or PMA-stimulated effects in EDL muscle. It is interesting that the combined effect of insulin and contraction on p38 MAPK phosphorylation was 27% reduced, as compared with the contraction stimulation alone ($P < 0.001$). Moreover, insulin and contraction did not have an additive effect on JNK or ERK1/2 phosphorylation. PMA treatment increased phosphorylation of JNK (Fig. 4*A*), p38 MAPK (Fig. 4*B*), and ERK1/2 (Fig. 4*C*) in EDL muscle. The effect of PMA on phosphorylation of p38 MAPK and ERK1 was similar to the insulin-mediated response, whereas the effect on JNK and ERK2 was less robust. Insulin and PMA had additive effects on JNK and ERK1 phosphorylation. In contrast, PMA and insulin failed to elicit an additive effect on p38 MAPK and ERK2 phosphorylation. Thus, stimulation with insulin, contraction, or PMA leads to profound increases in JNK, p38 MAPK, and ERK1/2 phosphorylation.

Insulin-mediated MAPK phosphorylation. We determined whether insulin action on MAPK signaling is impaired in skeletal muscle from obese diabetic *ob/ob* mice. EDL (Fig. 5) muscle was incubated for 20 min in the absence or presence of 120 nmol/l insulin, and phosphorylation of JNK, p38 MAPK, and ERK1/2 was determined.

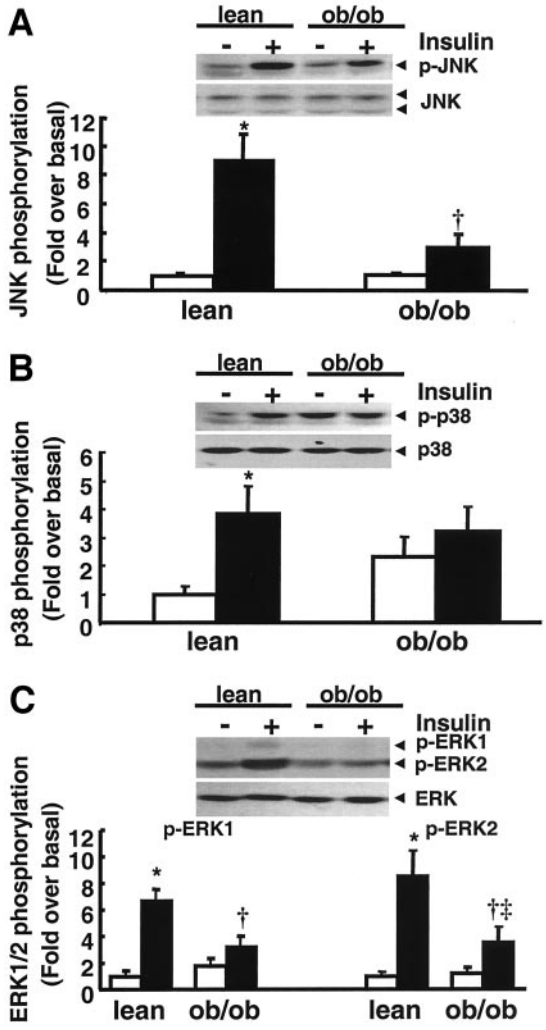

FIG. 5. Effect of insulin on MAPK phosphorylation in EDL muscle from lean and *ob/ob* mice. Isolated EDL muscle from lean and *ob/ob* mice was incubated without (□) or with (■) 120 nmol/l insulin for 20 min. Phosphorylation of JNK (*A*), p38 MAPK (*B*), and ERK1/2 (*C*) was determined as described in RESEARCH DESIGN AND METHODS. Membranes were reprobed after stripping to determine protein expression of the respective MAPK. Representative immunoblot for phosphorylation or protein expression of the specified MAPK (*upper panels*), and mean ± SE fold response for $n = 9–11$ muscles (*lower panels*). *$P < 0.05$ vs. lean basal; †$P < 0.05$ vs. lean insulin; ‡$P < 0.05$ vs. *ob/ob* basal.

JNK protein expression in EDL muscle was similar between lean and *ob/ob* mice (100 ± 6% vs. 98 ± 10% for lean vs. *ob/ob* mice; NS). Basal JNK phosphorylation in EDL muscle was similar between lean and *ob/ob* mice. Insulin increased JNK phosphorylation in EDL muscle 9.0- and 2.8-fold from lean and *ob/ob* mice, respectively. Insulin action on JNK phosphorylation was significantly impaired in EDL muscle from *ob/ob* mice ($P < 0.05$). In contrast to JNK, p38 protein expression was reduced 29% in EDL muscle from *ob/ob* mice (100 ± 11% vs. 71 ± 8% for lean vs. *ob/ob* mice; $P < 0.05$). Insulin increased p38 phosphorylation 3.9-fold ($P < 0.05$) in lean but not in *ob/ob* mice. Despite an apparent failure of insulin to increase p38 phosphorylation in *ob/ob* mice, the absolute insulin response was similar to that achieved in lean mice. Protein expression of ERK1/2 in EDL muscle was similar between lean and *ob/ob* mice (data not shown). Basal ERK1/2 phosphorylation was similar between lean and *ob/ob* mice. Insulin increased phosphorylation of ERK1 and ERK2 in lean mice 6.7- and 8.5-fold, respectively ($P < 0.05$ vs. basal for ERK1 and ERK2, respectively), and in *ob/ob* mice 1.9- (NS) and 3.0-fold ($P < 0.05$ vs. basal), respectively. Insulin action on ERK1 and ERK2 was impaired in *ob/ob* mice ($P < 0.05$ vs. lean mice).

For comparative purposes, additional experiments were performed to assess insulin action on JNK, p38 MAPK, and ERK1/2 in soleus (oxidative) muscle (Fig. 6). Basal JNK phosphorylation in soleus muscle was similar between lean and *ob/ob* mice. Insulin increased JNK phosphorylation fourfold in lean mice ($P < 0.05$ vs. basal), whereas this effect was severely blunted in *ob/ob* mice ($P < 0.05$ vs. lean). Basal p38 MAPK phosphorylation was similar between lean and *ob/ob* mice. In contrast to EDL muscle, insulin was without effect on p38 MAPK phosphorylation in soleus muscle from lean and *ob/ob* mice. This is likely due to the incubation protocol, which was optimized for EDL muscle. Basal ERK1/2 phosphorylation was similar between lean and *ob/ob* mice. Insulin increased phosphorylation of ERK1 and ERK2 in lean mice 2- and 1.8-fold, respectively ($P < 0.05$ vs. basal), and in *ob/ob* mice 1.9- (NS) and 1.3-fold (NS), respectively. Insulin action on ERK1 was normal, whereas insulin action on ERK2 was impaired in soleus muscle from *ob/ob* mice ($P < 0.05$ vs. lean mice).

Contraction-mediated MAPK phosphorylation. The effects of muscle contraction on MAPK phosphorylation were determined in EDL muscle from lean and *ob/ob* mice (Fig. 7). Basal phosphorylation of JNK, p38 MAPK, and ERK was compared between muscle incubated at resting tension and in the quiescent state. We observed similar levels of basal phosphorylation for JNK and p38 MAPK between muscles incubated at resting tension versus the quiescent state (2.1 ± 0.5 vs. 3.4 ± 0.4 arbitrary units for JNK and 7.3 ± 1.7 vs. 11.8 ± 1.8 arbitrary units for p38 MAPK, respectively; NS). ERK1 and ERK2 phosphorylation was increased in muscle incubated at resting tension (0.17 ± 0.04 vs. 0.59 ± 0.11 arbitrary units for ERK1 and 5.4 ± 0.5 vs. 8.2 ± 0.7 units for ERK2, respectively; $P < 0.05$). To clarify data presentation, basal data for contraction experiments were presented. Forced contraction in response to electrical stimulation led to a 2.6-fold increase in JNK phosphorylation in lean mice, with a similar effect

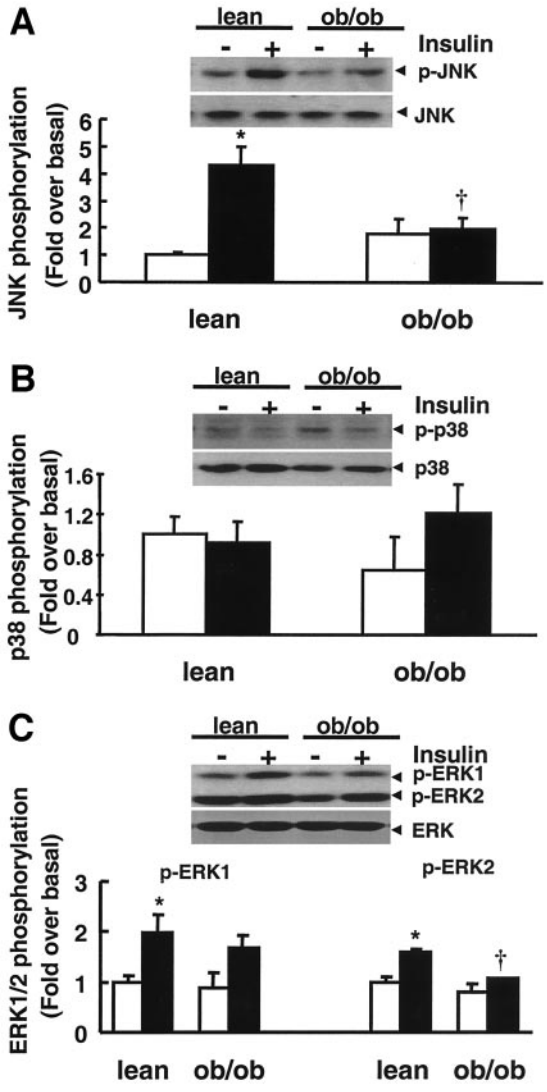

FIG. 6. Effect of insulin on MAPK phosphorylation in SOL muscle from lean and *ob/ob* mice. Isolated SOL muscle from lean and *ob/ob* mice was incubated without (□) or with (■) 120 nmol/l insulin for 20 min. Phosphorylation of JNK (*A*), p38 MAPK (*B*), and ERK1/2 (*C*) was determined as described in RESEARCH DESIGN AND METHODS. Membranes were reprobed after stripping to determine protein expression of the respective MAPK. Representative immunoblot for phosphorylation or protein expression of the specified MAPK (*upper panels*), and mean ± SE fold response for $n = 5$–7 muscles (*lower panels*). *$P < 0.05$ vs. lean basal; †$P < 0.05$ vs. lean insulin.

in *ob/ob* mice (Fig. 7*A*). Thus, although insulin action on JNK is impaired in *ob/ob* mice, the muscle contraction effect is retained. Contraction effects on p38 MAPK phosphorylation were also determined (Fig. 7*B*). Contraction increased p38 MAPK phosphorylation 12.6- and 10.5-fold in lean and *ob/ob* mice, respectively ($P < 0.05$ vs. basal). Although the effect of contraction on p38 MAPK phosphorylation was 17% lower in *ob/ob* mice, this finding, although not significant, may be related to the reduction in p38 MAPK protein expression in EDL muscle from *ob/ob* mice. Thus, in contrast to insulin, contraction elicited a normal response on p38 MAPK in *ob/ob* mice. ERK1 and ERK2 phosphorylation was determined in response to contraction (Fig. 7*C*). Similar to JNK, contraction-mediated ERK1 and ERK2 phosphorylation was preserved in *ob/ob* mice. Contraction increased ERK1 phosphorylation 1.9- and 2.2-fold and ERK2 phosphorylation 1.9- and 2.0-fold in lean

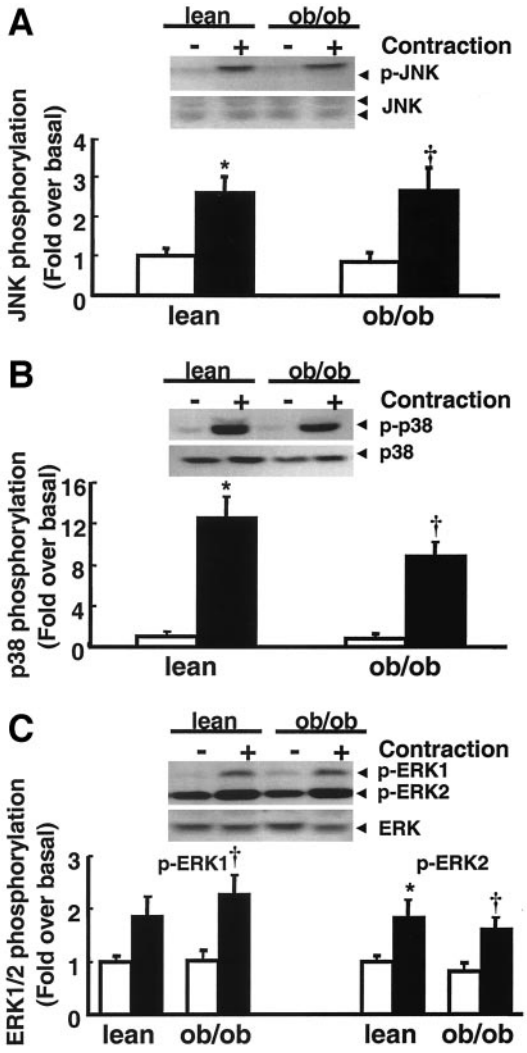

FIG. 7. Effect of muscle contraction on MAPK phosphorylation in lean and *ob/ob* mice. Isolated EDL muscle from lean and *ob/ob* mice was incubated under basal conditions (□) or forced to contract (■), as described in RESEARCH DESIGN AND METHODS. Phosphorylation of JNK (*A*), p38 MAPK (*B*), and ERK1/2 (*C*) was determined as described in RESEARCH DESIGN AND METHODS. Membranes were reprobed after stripping to determine protein expression of the respective MAPK. Representative immunoblot of phosphorylation or protein expression of the specified MAPK (*upper panels*), and mean ± SE fold response for n = 6–8 muscles (*lower panels*). *$P < 0.05$ vs. lean basal; †$P < 0.05$ vs. *ob/ob* basal.

and *ob/ob* mice, respectively ($P < 0.05$ vs. basal). Thus, insulin effects on ERK1/2 are impaired, whereas contraction-mediated effects are retained.

PMA-mediated MAPK phosphorylation. We next determined whether PMA effects on MAPK signaling are impaired in EDL muscle from *ob/ob* mice (Fig. 8). PMA exposure increased JNK phosphorylation (Fig. 8*A*) 3.5-fold in lean and 2.5-fold in *ob/ob* mice ($P < 0.05$ vs. basal). Although the effect of PMA on JNK phosphorylation was 29% lower in *ob/ob* versus lean mice, this reduction was not statistically significant. Thus, insulin action on JNK phosphorylation is impaired in *ob/ob* mice, whereas effects of PMA and contraction are preserved. We next assessed the effect of PMA on p38 MAPK phosphorylation (Fig. 8*B*). PMA increased p38 MAPK phosphorylation 4.3-fold in lean mice ($P < 0.05$), with no apparent increase in *ob/ob* mice. In contrast to JNK, PMA-stimulated p38 MAPK phosphorylation was impaired in *ob/ob* mice. ERK1 and ERK2 phosphorylation was determined in response to PMA (Fig. 8*C*). PMA increased ERK1 phosphorylation 4.8- and 3.1-fold in lean and *ob/ob* mice, respectively ($P < 0.05$ vs. basal). PMA increased ERK2 phosphorylation 3.6- and 2.4-fold in lean and *ob/ob* mice, respectively ($P < 0.05$ vs. basal). The absolute magnitude of the PMA effect on ERK1 and ERK2 was similar between lean and *ob/ob* mice, indicating that PMA elicits a normal response on ERK phosphorylation in insulin-resistant muscle.

DISCUSSION

Components of the MAPK signaling cascades are expressed in all muscle cell types, including skeletal, cardiac, and smooth muscle (14,27,28). However, relatively little is known of the regulation of these signaling cascades in skeletal muscle. Moreover, even less is known of the

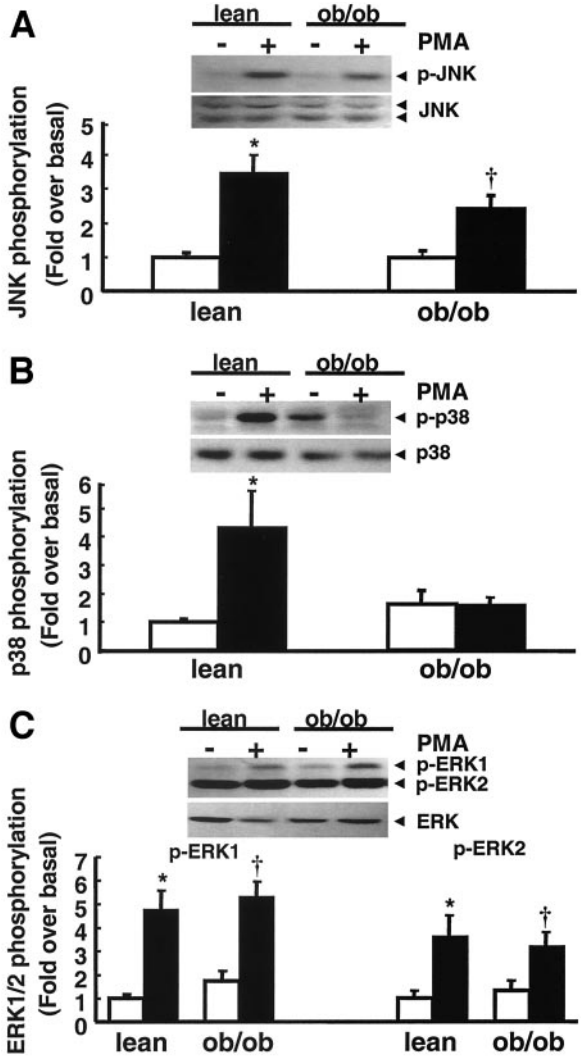

FIG. 8. Effect of PMA on MAPK phosphorylation in lean and *ob/ob* mice. Isolated EDL muscle from lean and *ob/ob* mice was incubated without (□) or with (■) 2 μmol/l PMA for 30 min. Phosphorylation of JNK (*A*), p38 MAPK (*B*), and ERK1/2 (*C*) was determined as described in RESEARCH DESIGN AND METHODS. Membranes were reprobed after stripping to determine protein expression of the respective MAPK. Representative immunoblot for phosphorylation or protein expression of the specified MAPK (*upper panels*), and mean ± SE fold response for n = 8–11 muscles (*lower panels*). *$P < 0.05$ vs. lean basal; †$P < 0.05$ vs. *ob/ob* basal.

regulation of MAPK cascades in insulin-resistant skeletal muscle. Here we characterized the effects of insulin on JNK, p38 MAPK, and ERK1/2 phosphorylation in isolated EDL and soleus muscle from lean mice. In addition, we determined the differential effects of insulin-dependent and insulin-independent stimuli (contraction and PMA) on phosphorylation of parallel MAPK modules in EDL muscle from lean and *ob/ob* mice.

We performed a detailed time-course study of insulin action on MAPK cascades in skeletal muscle. Insulin increased phosphorylation of JNK, p38 MAPK, and ERK1/2 in a similar time- and dose-dependent manner in isolated EDL and soleus muscle. Phosphorylation of all three MAPKs in response to insulin was increased in EDL and soleus muscle after 4 min, with maximal effects at 20 min. A previous in vivo study provides evidence that the time course for insulin-induced activation of the three MAPKs is distinctly different, with activation of JNK occurring within seconds, p38 MAPK within 2 min, and ERK occurring within 4 min (8). Thus, there seems to be a different time course for MAPK activation between in vivo and in vitro models. Because insulin induces a variety of hemodynamic responses in vivo, including increased vasodilation, this may account for a greater exposure of the muscle fibers to insulin, allowing for a more rapid activation of these kinases in vivo. Our in vitro model provides evidence that insulin increases phosphorylation of JNK, p38 MAPK, and ERK1/2 directly in glycolytic and oxidative skeletal muscle. This is particularly interesting in light of the general belief that JNK and p38 MAPK are primarily activated in response to cellular stress, rather than growth factor stimulation. Moreover, our results highlight additional regulatory mechanisms for phosphorylation of these MAPK modules in skeletal muscle. Insulin action on p38 MAPK was more transient and less robust in oxidative versus glycolytic muscle, suggesting fiber-type–specific regulation, a finding consistent with a previous report that showed that contraction-induced ERK signaling was greater in glycolytic versus oxidative skeletal muscle (29). Fiber-type–specific responses have been identified along insulin-signaling cascades; oxidative (soleus) fibers have greater insulin action on insulin receptor activity, IRS1 and IRS2 tyrosine phosphorylation, PI 3-kinase activity, and Akt serine phosphorylation compared with glycolytic (EDL) fibers (30).

Insulin, contraction, and PMA presumably increase the phosphorylation of JNK, p38 MAPK, and ERK1/2 by different mechanisms. We determined whether insulin-dependent and insulin-independent factors elicit additive responses on MAPK phosphorylation. The only evidence for an additive response on MAPK phosphorylation was observed between insulin and PMA on JNK and ERK1. Insulin and contraction did not elicit additive effects on any of the MAPKs studied. It is interesting that the combined effect of insulin and contraction on p38 MAPK phosphorylation was blunted, consistent with previous reports of reduced insulin action on PI 3-kinase in exercised skeletal muscle (31,32). This was an unexpected observation, because insulin and contraction have additive effects on glucose transport (1). Our data suggest that for mitogenic signaling, insulin and contraction signal via common pathways, whereas contraction and PMA seem to increase MAPK phosphorylation by insulin-independent mechanisms. Indeed, contraction-stimulated ERK phosphorylation is unaltered in the presence of calphostin C, a cell-permeable, irreversible protein kinase C (PKC) inhibitor that blocks the action of DAG-sensitive PKC isoforms (33). Although the precise mechanism by which insulin, PMA, and contraction induce MAPK phosphorylation is unknown, all three stimuli elicit a striking effect on MAPK.

PI 3-kinase and MAPK couple insulin signaling to metabolic and mitogenic events, respectively. Insulin-stimulated PI 3-kinase activity and glucose transport are impaired in skeletal muscle from type 2 diabetic patients (34–36) and from animal models of the disease, including *ob/ob* mice (37,38). In type 2 diabetic patients, insulin action on ERK1/2 phosphorylation is preserved in skeletal muscle (35,36). In contrast, in obese diabetic Zucker rats, insulin-stimulated ERK phosphorylation is reduced (39). Little is known of the effects of insulin on MAPK signaling at the level of JNK and p38 MAPK in insulin-resistant skeletal muscle. Here we provide evidence that insulin action on JNK, p38 MAPK, and ERK phosphorylation is impaired in isolated EDL and soleus muscle from *ob/ob* mice. In *ob/ob* mice, basal p38 MAPK phosphorylation tended to be increased and protein expression was significantly reduced. Thus, the stoechiometric phosphorylation of p38 MAPK would be expected to be higher than illustrated, suggesting that basal p38 MAPK phosphorylation may be even greater in *ob/ob* versus lean mice. This is consistent with previous reports of elevated basal p38 MAPK phosphorylation in skeletal muscle from moderately obese type 2 diabetic patients (12). Moreover, basal p38 MAPK phosphorylation was shown to be elevated in adipocytes from type 2 diabetic patients, and inhibition of this pathway prevented the insulin-stimulated decrease in GLUT4 protein level (40). The observation of increased basal p38 MAPK phosphorylation in important insulin target tissues (skeletal muscle and adipose tissue) supports the hypothesis that p38 MAPK may play a role in the pathogenesis of insulin resistance in type 2 diabetes.

Defects in insulin signaling in obese diabetic rodents involve impairments at the level of all three MAPKs studied. The insulin-signaling defect was observed in both glycolytic and oxidative skeletal muscle and is likely related to a receptor defect, because reduced number and activity of insulin receptors have been observed in skeletal muscle from *ob/ob* mice (41,42). Moreover, IRS1 tyrosine phosphorylation and protein expression are also reduced in skeletal muscle from *ob/ob* mice (43). Thus, a defect in the proximal signaling machinery may contribute to the impaired insulin action on MAPK pathways in skeletal muscle from *ob/ob* mice, because a global defect was observed at the level of JNK, p38 MAPK, and ERK.

In insulin-resistant rodents, muscle contraction/exercise elicits a normal response on glucose transport (44–47). Thus, we hypothesized that the contraction-mediated response on MAPK kinase signaling would be preserved in skeletal muscle from insulin-resistant *ob/ob* mice. We used an in vitro system, devoid of neural influence and systemic factors, to examine the direct role of muscle contraction on MAPK signaling in lean and *ob/ob* mice. Electrical stimulation increased JNK, p38 MAPK, and ERK1/2 phosphorylation in skeletal muscle from lean mice to a level

similar to (JNK and ERK1) or greater than (p38 MAPK) insulin-stimulated values. It is interesting that contraction-mediated effects on MAPK phosphorylation were preserved in insulin-resistant *ob/ob* mice. The detailed mechanism by which muscle contraction elicits signal transduction to downstream responses along MAPK cascades is currently unknown. Whereas ERK signaling is dispensable for glucose uptake (29,33), p38 MAPK has been linked to GLUT4 activation (48). Thus, p38 MAPK may constitute a common step along insulin and contraction pathways that regulate glucose uptake. Although we are limited in the translation of our finding of normal MAPK kinase signaling in insulin-resistant *ob/ob* mice to specific metabolic and gene-regulatory responses, the data provide evidence to suggest that regular exercise may circumvent impairments in insulin action along MAPK cascades in skeletal muscle.

Insulin and contraction independently increase PKC activity in skeletal muscle (49,50). Experimentally, phorbol esters have been widely used to activate DAG-sensitive PKC isoforms. PKC has been implicated as a signal transducer mediating gene-regulatory responses through activation of MAPK (51,52). Here we provide evidence that PMA increased phosphorylation of JNK, p38 MAPK, and ERK1/2 in lean mice. Furthermore, PMA-induced JNK and ERK1/2 phosphorylation was preserved in insulin-resistant *ob/ob* mice, whereas effects on p38 MAPK were completely abolished. The lack of effect of PMA on p38 MAPK cannot be completely explained by the reduction in p38 MAPK protein expression, because the contraction-mediated effects were normal. Because insulin and PMA activate different PKC isoforms (49), our findings suggest that DAG-independent PKC signaling or, alternatively, a PKC-independent mechanism may be impaired in *ob/ob* mice.

In summary, insulin increased JNK, p38 MAPK, and ERK1/2 phosphorylation in isolated EDL and soleus muscle from lean mice in a time- and dose-dependent manner. MAPK phosphorylation was also increased by PMA and contractions, indicating that insulin-dependent and -independent pathways mediate MAPK signaling in skeletal muscle. Important is that insulin action on MAPK was impaired in skeletal muscle from *ob/ob* mice, whereas contraction-mediated effects were preserved. It is interesting that PMA elicited divergent effects on MAPK signaling in *ob/ob* mice; JNK and ERK1/2 phosphorylation were preserved, whereas p38 MAPK phosphorylation was refractory. Thus, PMA does not mimic all of the effects of contraction on MAPK signaling. In conclusion, appropriate MAPK response can be elicited in insulin-resistant skeletal muscle via insulin-independent mechanisms. Muscle contraction through regular exercise may circumvent aberrant MAPK signaling in insulin-resistant skeletal muscle.

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