

Defective Signaling Through Akt-2 and -3 But Not Akt-1 in Insulin-Resistant Human Skeletal Muscle

Potential Role in Insulin Resistance

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Recent evidence has shown that activation of phosphatidylinositol-3-kinase (PI3K) and Akt, necessary for insulin stimulation of glucose transport, is impaired in insulin resistance. It is unknown, however, which Akt isoform shows impaired activation in insulin resistance. Additionally, related growth factors (epidermal or platelet-derived vascular) also stimulate PI3K, but it is unknown whether production of 3,4,5 phosphatidylinositol is sufficient to stimulate glucose transport in insulin-resistant muscle. Moreover, these studies were performed in rodents, and little data exists from humans. Hence, we investigated the stimulation of PI3K and Akt-1, -2, and -3 by insulin and epidermal growth factors (EGFs) in skeletal muscles from lean and obese insulin-resistant humans. Insulin activated all Akt isoforms in lean muscles, whereas only Akt-1 was activated in obese muscles. Insulin receptor substrate (IRS)-1 was associated with PI3K activity, which is necessary for Akt activation by insulin, and was reduced in obese muscles, and this was accompanied by decreased IRS-1 expression. In contrast, insulin- or EGF-stimulated phosphotyrosine-associated PI3K activity was not different between lean and obese muscles. These results show that a defect in the ability of insulin to activate Akt-2 and -3 may explain the impaired insulin-stimulated glucose transport in insulin resistance. Additionally, these data also show that different upstream or downstream signals may regulate the activity of the various Akt isoforms. *Diabetes* 52:935–941, 2003

Type 2 diabetes afflicts over 45 million Americans and is due to an inability of insulin to stimulate glucose transport in peripheral tissues. Intensive research efforts have determined that the defect in insulin-stimulated glucose transport that occurs in type 2 diabetes is distal to the insulin receptor. However, the exact nature of this defect is still unknown. Insulin stim-

ulates skeletal muscle glucose transport by causing the translocation of the insulin responsive glucose transporter (GLUT4) to the plasma membrane (1–3). Insulin has been shown to initiate its signal through tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, and although much evidence exists suggesting that activation of phosphatidylinositol-3-kinase (PI3K) is crucial for insulin action, the exact identity of the downstream players in stimulation of glucose transport by insulin remains largely unknown (4–6). A potential candidate for a downstream regulator of insulin action is Akt, a serine/threonine kinase that is activated by insulin and other growth factors. Recent evidence has implicated this kinase as a possible mediator of insulin-stimulated glucose transport in skeletal muscle (7–12). Studies have indicated that Akt/protein kinase B stimulation by insulin is impaired in insulin-resistant states; however, the majority of these experiments have been performed in diabetic rat skeletal muscle, and there is little data on the regulation of this kinase in insulin-resistant human skeletal muscle (13,14). Further, there are three known isoforms of Akt in insulin sensitive tissues (1, 2, and 3), and it is unknown which of these isoforms displays impaired insulin-stimulated activation in insulin-resistant human muscle (15,16). Therefore, the primary purpose of the present study was to investigate the regulation of Akt activity by insulin in insulin-resistant human skeletal muscle.

Although activation of PI3K appears to be necessary for activation of glucose transport by insulin, there is evidence that other growth factor receptors that also stimulate PI3K lack this ability (17–22). This has led to the hypothesis that the 3,4,5 phosphatidylinositol (PIP₃) that is generated by these other receptors is sequestered in a different compartment from that generated by the insulin receptor or that PI3K activity is not essential for insulin stimulation of glucose transport (23–25). This hypothesis has not been examined in insulin-resistant muscle, where the ability of insulin to stimulate PI3K is severely impaired, while that of other growth factors should be unaffected. Therefore, an additional purpose of the present study is to examine whether activation of PI3K is sufficient for stimulation of glucose transport or whether the PI3K that is activated by other growth factors is incapable of interacting with the required signaling cascade.

The results of the present study provide evidence that the Akt isoforms 2 and 3 may play a role in the defects in insulin signaling that occur in insulin-resistant skeletal muscle. Additionally, these results show that generation of

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2DG, 2-deoxyglucose; EGF, epidermal growth factor; GSK, glycogen synthase kinase; IRS, insulin receptor substrate; KHB, KREBS-Henseleit buffer; PIP₃, 3,4,5 phosphatidylinositol; PI3K, phosphatidylinositol-3-kinase; PVDF, polyvinylidene fluoride; Py, phospho-tyrosine; TTBS, Tween-tris-buffered saline.

PIP₃ by PI3K may not be the crucial event in insulin stimulation of glucose transport, but it may be more important to target PIP₃ to the required signaling machinery.

RESEARCH DESIGN AND METHODS

Muscle incubations. Human rectus abdominus strips were obtained from patients undergoing elective total abdominal hysterectomy or aortic aneurysm vessel bypass (lean patients) or gastric bypass surgery (obese patients). We have done several comparisons of muscle from the lower abdominal wall (hysterectomy) and that from the upper abdominal wall (gastric bypass) and find no differences in their glucose transport response. Patients were informed of risks involved and signed informed consent. The Human Studies Committee of East Carolina University approved the protocol used. Muscle strips were incubated in triplicate in KREBS-Henseleit buffer (KHB) in the presence or absence of 10⁻⁷ mol/l insulin or 10⁻⁷ epidermal growth factor (EGF). The muscles were preincubated for 30 min in KHB with 0.1 BSA and 1 mmol/l pyruvate and then were either frozen for use in insulin signaling assays or used for measurement of 2-deoxyglucose (2DG) transport as described previously (26).

Briefly, for measurement of 2DG transport, the muscles were transferred to medium containing 5 mmol/l 2DG, 20 mmol/l sorbitol, 0.4 μ Ci/mmol [³H]2DG, and 0.4 μ Ci/mmol [¹⁴C]sorbitol for 10 min. All incubations were performed at 37°C, and the muscles were continuously gassed with 95% O₂/5%CO₂. The muscles were then washed twice for 5 min in ice-cold KHB, blotted, weighed, digested, and counted by liquid scintillation for ³H and ¹⁴C.

Akt and GSK3 kinase activity measurements. For measurement of Akt kinase activity and western blotting, frozen muscles were homogenized in lysis buffer (50 mmol/l HEPES, pH 7.5, 2 mmol/l EDTA, 30 mmol/l NaPO₄, 1% Triton X-100, 10% glycerol, 10 mmol/l NaF, 150 mmol/l NaCl, 2 mmol/l NaVO₃, 5 μ g/ml leupeptin, 1.5 mg/ml benzamide, 0.5 mg/ml pepstatin A, 2 μ g/ml aprotinin, 1 mmol/l AEBSF, and 10 μ g/ml antipain). Lysates were spun at 15,000g for 15 min, and aliquots of the supernatant corresponding to 2 mg of protein were immunoprecipitated with an antiphospho-Akt-1(α), anti-Akt-2(β), anti-Akt-3(γ) (UBI, Lake Placid, NY), or anti-GSK3 β (Transduction Labs, Lexington, KY) antibodies.

For kinase assays, immunoprecipitates were washed and then incubated for 30 min in kinase assay buffer (20 mmol/l HEPES, pH 7.2, 5 mmol/l MgCl₂, 1 mmol/l dithiothreitol, 2 μ mol/l ATP, 2 μ g/reaction PKI, 25 μ g/reaction histone 2B, 5 μ Ci/reaction ³²P ATP, 5,000 Ci/mmol). Samples were lysed with Laemmli sample buffer and loaded on a 12.5% SDS-PAGE gel. The resolved proteins were transferred to nitrocellulose and quantified on a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

For glycogen synthase kinase 3 (GSK3) activity, following washing of the immunocomplex, an immunocomplex kinase assay was run using a commercially available kit (UBI, Lake Placid, NY) using glycogen synthase peptide as a substrate, following the manufacturers instructions. Reactions were terminated by spotting on phosphocellulose squares followed by extensive washing in 0.75% phosphoric acid. The squares were then dried and the radioactivity incorporated determined by liquid scintillation counting. Activity was calculated as picomoles of phosphate incorporated per minute per milligram protein.

AKT and IRS-1 Western blotting. For Akt and IRS-1 Western blotting, 200 μ g of protein was run on a 7.5% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membrane. Resolved proteins were transferred to PVDF membranes and blocked in 5% nonfat dry milk in Tween-tris-buffered saline (TTBS). The membranes were then incubated in primary antibody overnight, rinsed in TTBS, and incubated in horseradish peroxidase-coupled goat anti-rabbit antibody. The resolved bands were detected by enhanced chemiluminescence and quantified on a Molecular Dynamics phosphorimager.

PI3K assays. For PI3K assays, 1 mg of lysate protein was immunoprecipitated overnight with 4 μ g of α -IRS-1 or α -phospho-tyrosine (Py) antibody (UBI), and captured with protein A or G agarose beads. The immunocomplexes were washed and incubated with 50 μ l of the final wash buffer containing 20 μ g of phosphatidylinositol, 100 mmol/l MgCl₂, 880 μ mol/l ATP, and 30 μ Ci γ -[³²P]ATP (NEN, Boston, MA). The lipid products were resolved by thin-layer chromatography and quantified on a Molecular Dynamics phosphorimager.

Statistical analysis. The data were analyzed by ANOVA to test the effect of treatment (basal, insulin, and EGF) on muscle glucose uptake, Akt, and GSK3 activity. When a significant *F* ratio was obtained, a Newman-Keuls post hoc test was used to identify statistically significant differences (*P* < 0.05) between the means.

TABLE 1

Physical and metabolic characteristics of subjects involved in study

	Lean	Obese
<i>n</i>	17	15
Age (years)	39.9 \pm 2.0	35.5 \pm 2.0
BMI	24 \pm 0.8	47.6 \pm 2.7*
Weight (lbs.)	150.0 \pm 4.6	289.0 \pm 25.7*
Fasting plasma glucose (mmol/l)	4.9 \pm 0.2	5.6 \pm 0.4*
Fasting plasma insulin (nmol/l)	3.0 \pm 0.6	12.2 \pm 1.6*
Sex (F/M)	16/1	14/1

*Significantly different from corresponding lean value (*P* < 0.05).

RESULTS

Subject characteristics. Characteristics of subjects involved in the study are given in Table 1. The obese patients had significantly greater body weights and BMI than lean patients. Additionally, the obese patients had significantly greater fasting blood glucose than the lean subjects; however, they did not show frank fasting hyperglycemia. The lack of fasting hyperglycemia was due to the fact that these patients showed marked fasting hyperinsulinemia as compared with the lean subjects.

2DG transport. Rectus abdominus muscle strips removed from lean and obese insulin-resistant patients were used in isolated muscle incubations for measurement of glucose transport. Rates of insulin- and EGF-stimulated 2DG transport are shown in Fig. 1. Insulin significantly increased the maximal rate of insulin-stimulated 2DG transport in lean muscles (2.8 \pm 0.3 to 4.8 \pm 0.5 nmol \cdot min⁻¹ \cdot g⁻¹) and was without effect in muscles from obese patients (2.3 \pm 0.3 to 3.2 \pm 0.3 nmol \cdot min⁻¹ \cdot g⁻¹) showing the marked insulin resistance in these patients. EGF stimulation did not significantly increase 2DG transport in muscle from normal or obese patients (2.8 \pm 0.8 and 2.7 \pm 0.4 nmol \cdot min⁻¹ \cdot g⁻¹, respectively).

PI3K activity. Examination of an early event in insulin signaling (PI3K) that may be crucial for activation of Akt by insulin showed that the insulin stimulation of IRS-1-associated PI3K activity was significantly reduced in muscles from obese patients as compared with lean subjects

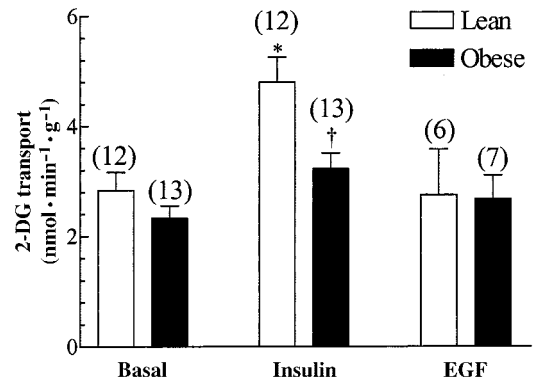


FIG. 1. Effects of insulin and EGF on glucose transport in human skeletal muscle. 2DG transport in isolated human rectus abdominus muscles was measured as described in the text in the absence (basal) or presence of 10⁻⁷ mol/l insulin or 10⁻⁷ mol/l EGF for 30 min. Numbers in parentheses indicate number of observations. Values are the means \pm SE. *Significantly different from basal; †significantly different from lean insulin (*P* < 0.05).

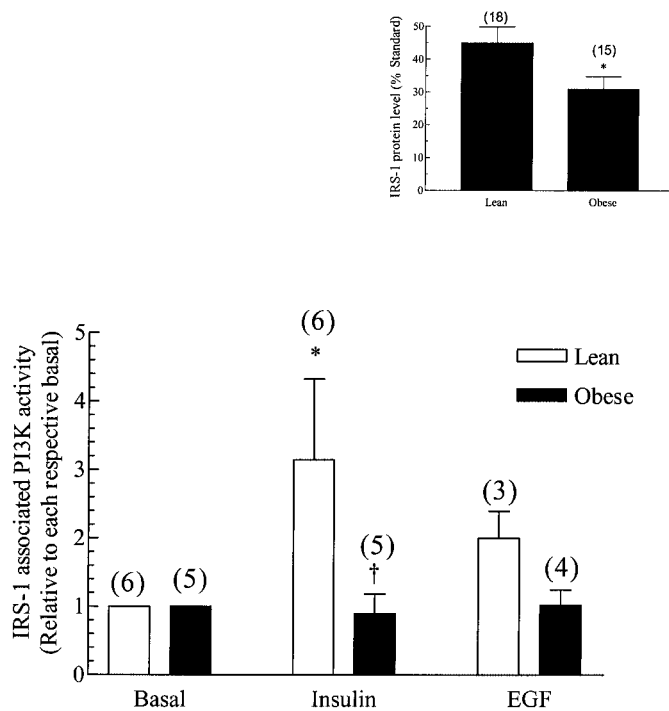


FIG. 2. IRS-1 precipitable PI3K activity in human skeletal muscle stimulated with 10^{-7} mol/l insulin or 10^{-7} mol/l EGF. IRS-1 associated PI3K kinase activity was immunoprecipitated from 2 mg of muscle lysate protein overnight, the immunocomplexes were captured with protein A agarose, and PI3K activity was measured as described in RESEARCH DESIGN AND METHODS. Values are in relative units with the basal set = 1 and are presented as means \pm SE. *Significantly different from basal; †significantly different from lean insulin ($P < 0.05$). Inset shows IRS-1 protein levels in human skeletal muscle. Muscles were processed as described in RESEARCH DESIGN AND METHODS except that 200 μ g of crude lysate protein was loaded on a 7.5% SDS-PAGE gels and transferred to PVDF membrane, probed with anti-IRS-1 antibody, visualized with enhanced chemiluminescence reagents, and quantified on a phosphorimager (Molecular Dynamics). Values are in relative units expressed as a percent of a standard that was run on each gel and are presented as means \pm SE. Numbers in parentheses indicate number of observations. *Significantly different from lean ($P < 0.05$).

(Fig. 2), whereas basal (nonstimulated) PI3K activity was not different between lean and obese subjects (data not shown). This impaired PI3K activity was accompanied by an $\sim 30\%$ decline in IRS-1 protein levels in the muscles of the obese patients (Fig. 2, inset). EGF did not produce a significant increase in IRS-1-associated PI3K activity in either lean or obese patients. Intriguingly, examination of PY-associated PI3K activity (Fig. 3) shows that insulin significantly increased PI3K to a similar level in muscles from both lean and obese patients. Additionally, although not statistically different, EGF produced a twofold increase in PY-associated PI3K in both lean and obese muscles.

Akt kinase activity. Insulin stimulation caused an increase in the kinase activity of all three Akt isoforms in the muscle strips from the lean patients (Fig. 4). However, only Akt-1 activity was significantly increased by insulin in the muscle strips of the obese patients. Although it appears that there is no difference in kinase activity between isoforms, owing to the differences in affinity of the various antibodies for the Akt isoforms, these comparisons are not appropriate. In contrast to the Akt-1 kinase activity measurements, Western blotting of muscle lysates with an antiphospho(s473)Akt-1 antibody showed a significant re-

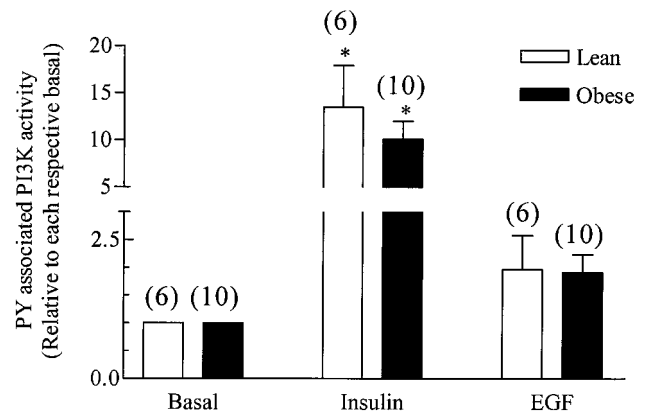


FIG. 3. Phosphotyrosine precipitable PI3K activity in human skeletal muscle stimulated with 10^{-7} mol/l insulin or 10^{-7} mol/l EGF. PY-associated PI3K kinase activity was immunoprecipitated from 2 mg of muscle lysate protein overnight, and the immunocomplexes were captured with protein G agarose. PI3K activity was measured as described in RESEARCH DESIGN AND METHODS. Values are in relative units with the basal set = 1 and are presented as means \pm SE. Numbers in parentheses indicate number of observations. *Significantly different from basal ($P < 0.05$).

duction ($\sim 44\%$) in insulin-stimulated phosphorylation of Akt-1 in the insulin-resistant muscle (Fig. 5). Additionally, EGF also significantly increased Akt-1 activity in both lean and obese muscles, but was without effect on Akt-2 or -3 activities in these muscles. These differences were not due to a decrement in basal activity, as basal activity did not differ between groups. Furthermore, the differences in kinase activity of Akt-1 and Akt-2 could not be attributed to differences in expression since no difference was detected by Western blotting (data not shown). Unfortunately, the antibody used for immunoprecipitation of Akt-3 is not suitable for Western blot analysis, and thus Akt-3 expression levels could not be determined.

GSK3 activity. Insulin stimulation caused a significant reduction in mean GSK3 β activity in muscle strips from lean but not obese patients (Fig. 6). Total GSK3 β protein levels were not significantly different between the lean and obese muscle strips; therefore, the differences in kinase activity could not be attributed to differences in protein expression (data not shown).

DISCUSSION

Type 2 diabetes is a metabolic disorder characterized by the ineffectiveness of insulin to stimulate glucose disposal. Insulin stimulates this process in vivo by activating a cascade of downstream kinases, ultimately resulting in translocation of the GLUT4 glucose transporter to the plasma membrane (1,3,4,13,22,27–29). Although research efforts in the past several years have mapped out many of the downstream kinases involved in insulin signaling, the nature of the defects that are involved in insulin resistance remain elusive. Recent evidence has suggested that the serine-threonine kinase Akt plays a role in insulin-stimulation of GLUT4 translocation (7,8,11,30,31). As such, the primary purpose of the present study was to investigate insulin signaling through Akt to examine the role of this kinase in insulin resistance in human skeletal muscle.

In addition to Akt, PI3K has also been shown to be necessary for a large number of insulin's actions (32–36). Examination of IRS-1-associated PI3K activity in the

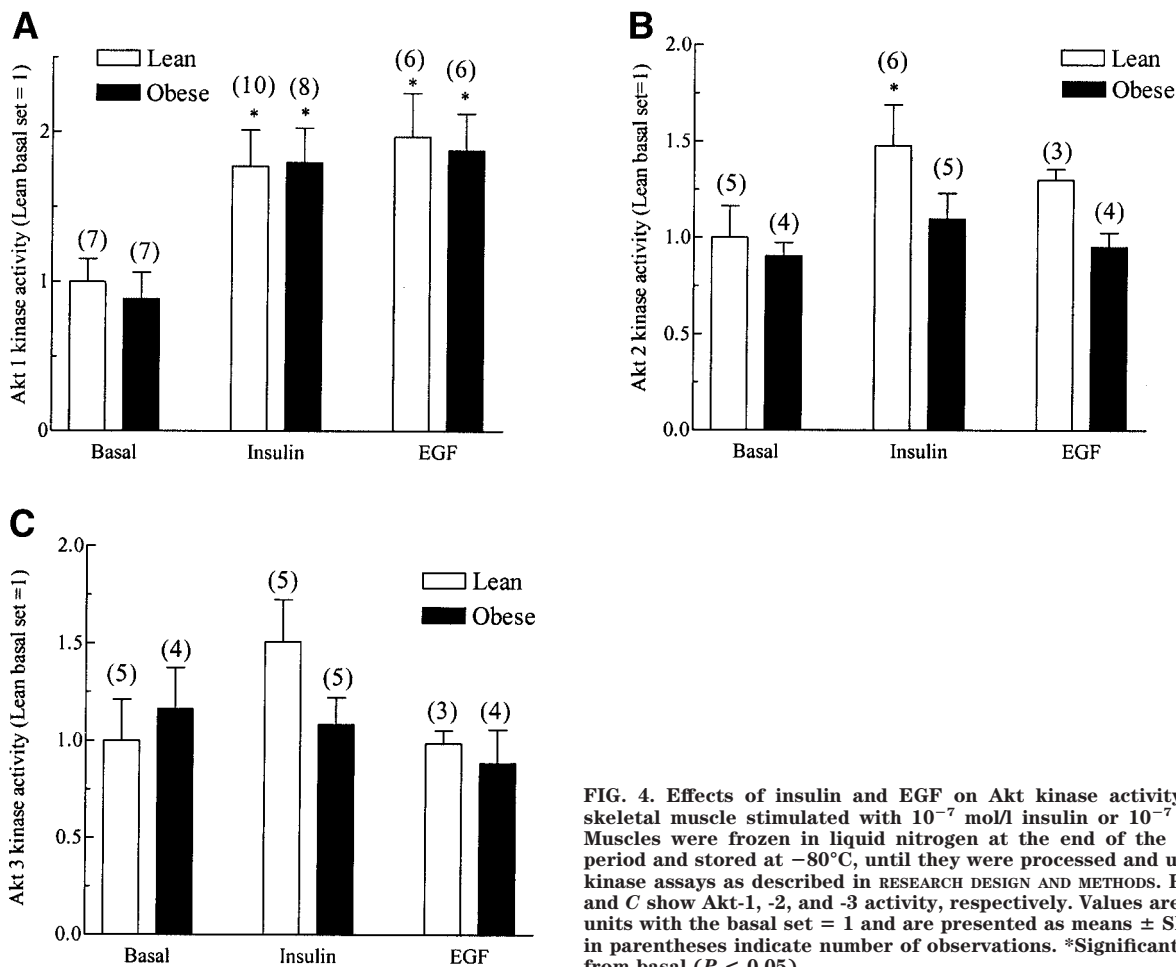


FIG. 4. Effects of insulin and EGF on Akt kinase activity in human skeletal muscle stimulated with 10^{-7} mol/l insulin or 10^{-7} mol/l EGF. Muscles were frozen in liquid nitrogen at the end of the stimulation period and stored at -80°C , until they were processed and used for Akt kinase assays as described in RESEARCH DESIGN AND METHODS. Panels A, B, and C show Akt-1, -2, and -3 activity, respectively. Values are in relative units with the basal set = 1 and are presented as means \pm SE. Numbers in parentheses indicate number of observations. *Significantly different from basal ($P < 0.05$).

present study showed that insulin, but not EGF, significantly increased PI3K activity in muscles of lean individuals. In contrast, the ability of insulin to stimulate IRS-1-associated PI3K activity was severely impaired and was not significantly elevated by insulin in muscles of obese patients. This was accompanied by a decreased expression of IRS-1, but the small decline in expression cannot fully explain the large decline in PI3K activity that is observed in the obese muscles. In light of this idea, recent evidence by Clark et al. (37) has shown that induction of insulin resistance in 3T3-L1 adipocytes is associated with a distribution of IRS-1 to a different cellular compartment than in the normal state. This idea is supported in the present study by the PY-associated PI3K activity, which surprisingly, is elevated by both insulin and EGF in both lean and obese skeletal muscle. These data support the hypothesis that one of the causes of insulin resistance may be the association of PI3K and PIP_3 with an incorrect cellular compartment, where it cannot interact with other downstream elements of insulin signaling.

It is also interesting to note that EGF, a related growth factor receptor that stimulates PI3K independent of IRS-1, produced a twofold increase in PY-associated PI3K activity in both lean and obese muscles. Despite the fact that both insulin and EGF stimulated PI3K activity, albeit to varying degrees, only insulin significantly elevated 2DG transport. In contrast to the present data, it was recently reported that EGF does stimulate glucose transport in

human adipocytes (38). Although the difference between these results and the present work may simply reflect differences in the responses of adipocytes versus skeletal muscle to growth factors, this remains to be determined by further study. Nevertheless, various hypotheses have been proposed to explain the differential abilities of EGF and insulin to stimulate PI3K and glucose transport, but the most attractive is compartmentalization of the PI3K activity and PIP_3 that is produced from insulin stimulation from that generated by EGF (24,35,36,39). The present data provide further support for this hypothesis.

The downstream serine-threonine kinase Akt has three isoforms that are present in mammalian skeletal muscle (15). Examination of the activity of these three isoforms showed that insulin stimulation activated all three isoforms to varying degrees in lean skeletal muscle. In contrast, in obese muscles, insulin stimulation of the Akt isoforms 2 and 3 was impaired; however, insulin retained a normal ability to stimulate Akt-1. This suggests that Akt-1 may not be involved in insulin stimulation of glucose transport in insulin-sensitive tissues, but may be involved in signaling to other insulin-stimulated processes (40,41). It is also interesting to note that despite the impairment in Akt-1 phosphorylation in the obese muscle, there was no difference in Akt-1 kinase activity. This indicates that a small difference in phosphorylation may not be reflected in a similar decline in kinase activity. This idea is supported by the fact that the correlation between Akt-1 kinase

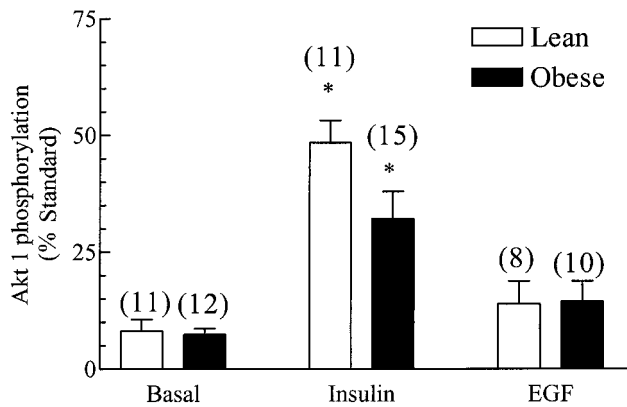


FIG. 5. Effect of insulin on Akt phosphorylation in human skeletal muscle stimulated with 10^{-7} mol/l insulin or 10^{-7} mol/l EGF. Phosphorylated Akt western blotting was performed on muscles that had been treated as described in Fig. 2. Muscles were processed as described in Fig. 2 except that the membranes were blotted with antiphospho-(serine 473)-Akt antibody. Values are in relative units expressed as a percent of a standard that was run on each gel and are presented as means \pm SE. Numbers in parentheses indicate number of observations. *Significantly different from basal; †significantly different from lean insulin ($P < 0.05$).

activity and phosphorylation gives an r^2 of 0.39 (data not shown). Thus, only a portion of Akt-1 may need to be phosphorylated to get full kinase activity. Alternatively, the phosphorylation site at threonine 308, or an alternative activation or deactivation pathway, may be more important in the regulation of Akt-1 activity. In support of this idea, EGF stimulated Akt-1 kinase activity to the same degree as insulin but only produced a twofold increase in Akt phosphorylation at serine 473. Thus, other mechanisms for activating Akt-1 may exist in human skeletal muscle.

The present results differ from those of Kim et al. (42), who found that insulin induced a normal activation of Akt-1, -2, and -3 in muscle biopsies from obese patients undergoing euglycemic clamps. However, these authors used an antibody that did not discriminate between Akt-1 and -2, so it is possible that they missed a defect in insulin-stimulated Akt-2 activation. For Akt-3, in the present study we observed no insulin-stimulated activa-

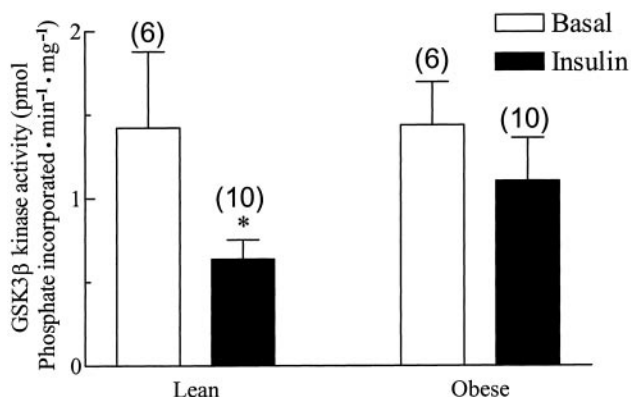


FIG. 6. GSK3 β activity in human skeletal muscle stimulated with 10^{-7} mol/l insulin. Muscles were frozen in liquid nitrogen at the end of the stimulation period and stored at -80°C , then they were processed and used for immunoprecipitation and subsequent GSK3 β kinase assays as described in RESEARCH DESIGN AND METHODS. Values are in pmol phosphate incorporated $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein and are presented as means \pm SE. Numbers in parentheses indicate number of observations. *Significantly different from basal ($P < 0.05$).

tion of this isoform, while Kim et al. (42) saw normal activation by insulin. The reason for this discrepancy is unclear, but could be related to the muscle type used (vastus lateralis biopsy versus rectus abdominus), muscle sampling protocol (punch biopsy versus incubated muscle strip), or the patient population used (moderately obese and normoinsulinemic versus severely obese and hyperinsulinemic). To the last point, it is interesting to note that in the study of Kim et al. (42), insulin-stimulated IRS-1-associated PI3K activity was normal, whereas in the present study this activity was severely impaired. This may point to the fact that there is a heterogeneity in insulin-resistant humans such that different patient populations may exhibit distinct signaling defects. Nonetheless, which of these possibilities is responsible for the differences between the present work and that of Kim et al. remains to be determined by additional study.

The other interesting aspect of the normal activation of Akt-1 in obese muscles is that this occurs despite the severe impairment in insulin-stimulated IRS-1-associated PI3K activity, which is thought to be necessary for activation of the upstream Akt activator, PDK-1. It is possible the activation of IRS-2-associated PI3K activity or non-IRS-associated PI3K activity could explain the seemingly normal activation of Akt-1 in the obese muscles. Although previous data from human muscle by Kim et al. (42) (muscle punch biopsies) and Krook et al. (43) (isolated incubated human muscle) have measured IRS-2-associated PI3K activity in human skeletal muscle, we have been unable to detect this activity in the present study (data not shown). While the discrepancy between the previous work of Kim et al. (42) and the present study may be due to the fact that these authors measured IRS-associated PI3K activity in punch biopsies from patients undergoing euglycemic clamps, it is unclear why our results differ from those of Krook et al. (43), since these authors used a similar muscle preparation to that of the present work. Although previous studies in rats (44) have shown that insulin-stimulated IRS-2-associated PI3K activity is reversed much more rapidly than that of IRS-1, the data of Krook et al. show that even though insulin-stimulated IRS-2-associated PI3K activity begins to decline after 20 min, it is still elevated after 40 min of stimulation. Although the reasons for the difference between our results and those of Krook et al. are not readily apparent, it may be related to differences in signaling protein expression or in the time course for insulin signaling in the muscles used in the two studies (vastus lateralis versus rectus abdominus). In support of this hypothesis, although other authors have reported the ability to Western blot IRS-2 protein in rectus abdominus muscles, we have not been successful with our samples (43,45). Thus, this could indicate that in muscles from patients in the present study, IRS-2 protein levels are extremely low and may have contributed to our inability to measure this activity.

Alternatively, it is also possible that non-IRS-associated PI3K may have stimulated Akt-1 in the obese muscles. Interestingly, EGF also stimulated Akt-1 activity in both lean and obese muscles. Since EGF stimulates PI3K independent of the IRS proteins, this would seem to support this hypothesis. While the degree of stimulation of PI3K was significantly lower for EGF (twofold) than for insulin

(15-fold), it may be that there is a threshold of PIP₃ accumulation that must occur to get stimulation of PDK. Alternatively, the location of the PIP₃ that is produced may be the most important factor in activation of PDK. Thus, it may be that the PI3K activity that is not associated with the IRS proteins is in the correct cellular location in human skeletal muscle to activate PDK and Akt-1.

Much previous work has implicated Akt as playing a role in insulin-stimulated glucose transport (8,14,30,31,46,47). However, as previously mentioned, the present work shows that in vitro, Akt-1 may not be involved in insulin stimulation of glucose transport in human skeletal muscle, while Akt-2 and -3 cannot be ruled out from playing a role in this process. However, these data cannot rule out the possibility that Akt-1 activation is necessary, but not sufficient, for stimulation of glucose transport, or that it is not in the correct compartment to interact with signaling to glucose transport. Much of the previous work that has implicated Akt-1 in regulation of glucose transport has relied on in vitro cell culture over-expression systems, which may not be representative of in vivo signaling. In light of these data, it is interesting to note that it has recently been reported that a muscle-specific knock-out of Akt-2 causes diabetes in mice, while muscle-specific Akt-1 knock-out mice display impaired growth but normal glucose tolerance (40,41). This supports the hypothesis that the Akt-2 and -3 isoforms are more involved in insulin signaling of glucose metabolism, while Akt-1 is involved in regulation of cellular growth.

The immediate downstream kinase from Akt is GSK3, of which the major isoform in skeletal muscle is the β -isoform (48–50). This kinase is constitutively active in the absence of insulin and is inactivated by phosphorylation at serine threonine by Akt (49), and it has been proposed that GSK3 β may play a role in insulin resistance by serine-threonine phosphorylation of IRS-1, which impairs its ability to interact with PI3K (48). In the present work, we have found that insulin has an impaired ability to inhibit the activity of this kinase, which supports this hypothesis. An additional interesting aspect of these data are that despite the increase in activity of Akt-1 by insulin in the obese patients, there was still an impaired ability of insulin to inhibit GSK3 β . This indicates that insulin stimulation of glycogen synthesis (and the other metabolic aspects of insulin signaling) in human skeletal muscle likely occurs through Akt-2, and possibly Akt-3, whereas the insulin-stimulated mitogenic signals are likely transduced through Akt-1, in support of the data of Cho et al. (40,41).

In summary, the results of the present studies have shown that insulin has an impaired ability to stimulate glucose transport in muscle from insulin-resistant obese humans. This impairment was due to defects in insulin-stimulated IRS-1-associated PI3K activity, which was accompanied by a decline in total IRS-1 protein levels. Intriguingly, total PY-associated PI3K activity was not different between lean and obese muscles, suggesting that the cellular location of the PIP₃ that is produced is crucial for stimulation of PDK and downstream signaling. In support of this hypothesis, EGF stimulation produced a twofold increase in PY-associated PI3K activity, without affecting glucose transport or Akt-2 or -3 activity. Finally, it appears that Akt-2 and -3 are likely involved in insulin-

stimulated glucose transport in human skeletal muscle, while activation of Akt-1 may not be necessary for this process. The exact roles of Akt-2 and -3 in glucose transport and their downstream targets remain to be discerned by further study.

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