Differential Effects of Tumor Necrosis Factor-α on Protein Kinase C Isoforms α and δ Mediate Inhibition of Insulin Receptor Signaling

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Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine that interferes with insulin signaling, but the molecular mechanisms of this effect are unclear. Because certain protein kinase C (PKC) isoforms are activated by insulin, we examined the role of PKC in TNF-α inhibition of insulin signaling in primary cultures of mouse skeletal muscle. TNF-α, given 5 min before insulin, inhibited insulin-induced tyrosine phosphorylation of insulin receptor (IR), IR substrate (IRS)-1, insulin-induced association of IRS-1 with the p85 subunit of phosphatidylinositol 3-kinase (PI3-K), and insulin-induced glucose uptake. Insulin and TNF-α each caused tyrosine phosphorylation and activation of PKCs δ and α, but when TNF-α preceded insulin, the effects were less than that produced by each substance alone. Insulin induced PKCδ specifically to coprecipitate with IR, an effect blocked by TNF-α. Both PKCα and -δ are constitutively associated with IRS-1. Whereas insulin decreased coprecipitation of IRS-1 with PKCα, it increased coprecipitation of IRS-1 with PKCδ. TNF-α blocked the effects of insulin on association of both PKCs with IRS-1. To further investigate the involvement of PKCs in inhibitory actions of TNF-α on insulin signaling, we overexpressed specific PKC isoforms in mature myotubes. PKCα overexpression inhibited basal and insulin-induced IR autophosphorylation, whereas PKCδ overexpression increased IR autophosphorylation and abrogated the inhibitory effect of TNF-α on IR autophosphorylation and signaling to PI3-K. Blockade of PKCα antagonized the inhibitory effects of TNF-α on both insulin-induced IR tyrosine phosphorylation and IR signaling to PI3-K. We suggest that the effects of TNF-α on IR tyrosine phosphorylation are mediated via alteration of insulin-induced activation and association of PKCδ and -α with upstream signaling molecules.
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Tumor necrosis factor-α (TNF-α) (1) is a multifunctional cytokine, produced mainly by activated macrophages and involved in immune and proinflammatory responses. It has been suggested that TNF-α may also play a role in obesity-linked insulin resistance. Thus, adipose tissues of obese animals and human patients (1) produce high levels of TNF-α mRNA accompanied by overexpression of the cytokine. TNF-α is also expressed in human muscle and is found at higher levels in muscle from insulin-resistant and diabetic patients than in normal control subjects (2,3). Moreover, depletion of TNF-α activity, using a chimeric antibody or as occurs in transgenic animals lacking either TNF-α or TNF-α receptor, improves sensitivity to insulin in obese animals (4,5). Although the mechanism of action of TNF-α in these insulin-related effects is not fully understood, it is known that a major component involves TNF-α inhibition of upstream insulin signaling. In this regard, a number of studies have shown that TNF-α both reduces insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) and IR substrate (IRS)-1 and inhibits tyrosine phosphorylation and activity of phosphatidylinositol 3-kinase (PI3-K) (6,7). Inhibition of IR signaling by TNF-α in skeletal muscle is controversial, and although some studies have confirmed this effect (8), others could not demonstrate this pathway (9,10).

Several studies have shown that the protein kinase C (PKC) family of serine threonine kinases may be implicated in various aspects of TNF-α signaling. The PKC family is composed of at least 11 isoforms, which are categorized into three groups (conventional PKCs α, βI, βII, and γ; novel PKCs δ, ε, η, and θ; and atypical PKCs ζ and λ/ι) according to their structure and mechanisms of activation (11,12). Phosphorylation on tyrosine residues is associated with activation of PKCδ, PKCβII, and PKCζ (13–15). It is currently believed that tyrosine phosphorylation may be important in the determination of substrate specificity of the enzyme (16).

Activation and inhibition of PKC by TNF-α in a variety of cell types have been reported (17,18). With regard to the involvement of PKC isoforms in TNF-α inhibition of insulin signaling, it was shown in adipocytes that the cytokine inhibits insulin-induced translocation of PKCβ and -ζ to the membrane (19). In addition, overexpression of PKCε increased the inhibitory effect of TNF-α on insulin signaling in HEK293 cells (20).

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2DG, 2-deoxy-D-glucose; IR, insulin receptor; IRS, IR substrate; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TNF-α, tumor necrosis factor-α.

Recent studies implicate certain PKC isoenzymes in the insulin-signaling cascade. Insulin activates PKCs α, βII, and ζ in several cell types, including cell lines of skeletal muscle (21–24). We recently reported that insulin stimulates glucose uptake and induces tyrosine phosphorylation, translocation, and activation of PKCs βII, δ, and ζ in primary cultures of rat skeletal myotubes (15). Activation of PKCs βII and ζ apparently occurs via a PI3-K–dependent pathway, whereas PKCδ appears to be activated upstream (15,25), perhaps by the IR itself or by the IRS proteins. The various PKC isoforms are both activated by insulin and can also modulate insulin signaling by regulating the kinase activity of IR. Indeed, PKCδ was found to upregulate IR tyrosine phosphorylation in rat skeletal muscle cells (26), whereas PKCs α, δ, and θ were found to inhibit this phosphorylation in HEK293 cells (27). Because various PKC isoforms have been found to interact with both IR and IRS, it is still not clear whether specific PKC isoforms regulate IR directly or through IRS-1.

TNF-α induces serine phosphorylation of IRS-1 and reduces its ability to interact with the juxtamembrane region of IR (28). Whereas IRS-1 from cells treated with TNF-α inhibits insulin-induced IR tyrosine phosphorylation, it is not certain whether this effect occurs directly by interaction of IRS-1 with IR or indirectly through another element in the pathway (29,30). PKC isoforms, which are activated by insulin, are possible candidates for phosphorylating the receptor on serine residues and affecting subsequent signaling.

The preparation of primary skeletal muscle cultures obtained from neonatal mice pups is a useful model for the study of regulation of insulin signaling by TNF-α. These cells, plated initially as individual myoblasts, align and fuse into multinucleated muscle fibers by day 3–4 in vitro. The mature fibers display resting membrane and action potentials that are nearly identical to those seen in vivo. In addition, the physiological expression of a number of membrane proteins in this preparation, in contrast to muscle cell lines such as L6, closely resemble that obtained in vivo (31–33). In this study, we investigated the possibility that TNF-α may differentially affect the interaction of specific PKC isoforms with upstream elements in the IR signaling cascade to modulate IR signaling. The results show that insulin induces PKCδ to associate with IR and causes PKCα, which is constitutively associated with IRS-1, to disassociate from this docking protein. TNF-α interferes with these insulin-induced effects, resulting in a decrease in insulin-induced tyrosine phosphorylation of IR. Overexpression of PKCδ on one hand and inhibition of PKCα on the other reverse the influence of TNF-α on IR autophosphorylation. These results strongly suggest that TNF-α alters IR signaling in skeletal muscle through its effects on PKCα and -δ.

RESEARCH DESIGN AND METHODS

Materials. Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). TNF-α was purchased from Sigma (St. Louis, MO). Antibodies to various proteins were obtained from the following sources: monoclonal antibodies to IRβ were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antiphosphotyrosine was obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies to IRS-1, IRS-2, and specific PKC isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; polyclonal) and from Transduction Laboratories (monoclonal). Horseradish peroxidase and anti-rabbit and anti-mouse IgG were obtained from BioRad (Israel). Leupeptin, aprotinin, PMSF (phenylmethylsulfonyl fluoride), orthovanadate, and pepstatin were purchased from Sigmas. GÖ6976 was purchased from Calbiochem (San Diego, CA).

Methods

Preparation of mouse muscle cell cultures. Skeletal muscle cultures were freshly prepared for each experiment from the limb muscles obtained from 1- to 2-day neonatal mice as described (15,25,34). Fusion of plated myoblasts into mature myotubes occurs 3–4 days after plating. On day 5 in culture, myotubes were transferred to low-glucose (4.5 mmol/l) serum-free DMEM containing 1% BSA for 24 h before study.

Immunoprecipitation. Culture dishes (90 mm; Nunc) containing the muscle cultures were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached in RIPA (radioactive immunoprecipitation assay) buffer (50 mmol/l Tris HCl, 150 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l NaF, 1% Triton X-100, 0.1% SDS, and 1% Na deoxycholate; pH 7.4) containing a cocktail of antiproteases (20 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mmol/l PMSF, and 1 mmol/l dithiothreitol) and antiphosphatases (200 mmol/l orthovanadate and 2 μg/ml pepstatin). After scraping, the preparation was centrifuged at 20,000*g* for 20 min at 4°C. The supernatant was used for immunoprecipitation as described (15,25).

Western blot analysis. Crude and fractionated lysates of control and treated cultures were subjected to SDS-PAGE and electrophoretic transfer to Immobilon-P (Millipore) membranes. The membranes were subjected to standard blocking procedures and incubated with monoclonal and polyclonal antibodies (25).

Glucose uptake. Glucose transport was evaluated by measuring 2-deoxy-D-glucose (2DG) uptake as described (15,25). After appropriate treatment, cells were washed three times with 0.5 ml PBS, and the final wash was replaced immediately with 0.5 ml PBS containing 2 mmol/l glucose and tracer amounts (1 μCi/ml) of [^{3}H]-2DG. Cells were then incubated for 15 min at 37°C, washed four times with 0.5 ml cold PBS, and then detached from the wells by addition of 300 μl Triton X-100 (1%) and incubation for 30 min. The contents of each well were transferred to counting vials, and 3.5 ml scintillation fluid was added to each vial. Samples were counted in the [^{3}H] window of a Tricarb scintillation counter. Nonspecific uptake was determined in the presence of excess (100 mmol/l) D-glucose. Experiments were carried in triplicate. Net specific uptake was then calculated as the difference between the total and nonspecific values.

PKC recombinant adenoviruses and viral infection of cultures. The recombinant adenovirus vectors were constructed as described (35). The dominant-negative mutant of mouse PKCδ was generated by substitution of the lysine residue at the ATP binding site with alanine (36). The mutant δ cDNA was cut from the SRD expression vector with *Eco*RI and ligated into the pAxCA1w cosmid cassette to construct the Ax vector. Dominant-negative activity was demonstrated by abrogation of autophosphorylation (36).

After differentiation of cultured rat myoblasts into myotubes, the culture medium was aspirated and cultures were infected with medium containing PKC recombinant adenoviruses as described (25).

PKC activity. Specific PKC activity was determined in freshly prepared immunoprecipitates from mature muscle cultures after appropriate treatments as described (15,25). These lysates were prepared in RIPA buffer without NaF. Activity was measured using the SignaTECT PKC Assay System (Promega, Madison, WI). The kit contains phosphatidylserine and diacylglycerol and uses Neurogranin as substrate.

RESULTS

Characterization of TNFα effects on upstream IR signaling in cultured skeletal muscle. TNF-α has been shown to inhibit insulin-induced phosphorylation of the receptor tyrosine kinase in several cell types (6,30,37). Figure 1 shows the effects of TNF-α on insulin-induced tyrosine phosphorylation in cultured mouse skeletal muscle. Under control conditions, immunoprecipitated IR displayed an increase in tyrosine phosphorylation within 1 min of insulin stimulation. This remained elevated for at least 5 min. IR phosphorylation then decreased toward prestimulation levels by 15 min. As the maximum level of IR tyrosine phosphorylation occurred within 1–5 min of insulin stimulation, this time period of insulin stimulation was used in all subsequent experiments. Effects of TNF-α on insulin-induced tyrosine phosphorylation of IR are shown in Fig. 1B. Pretreatment of cells with TNF-α (1

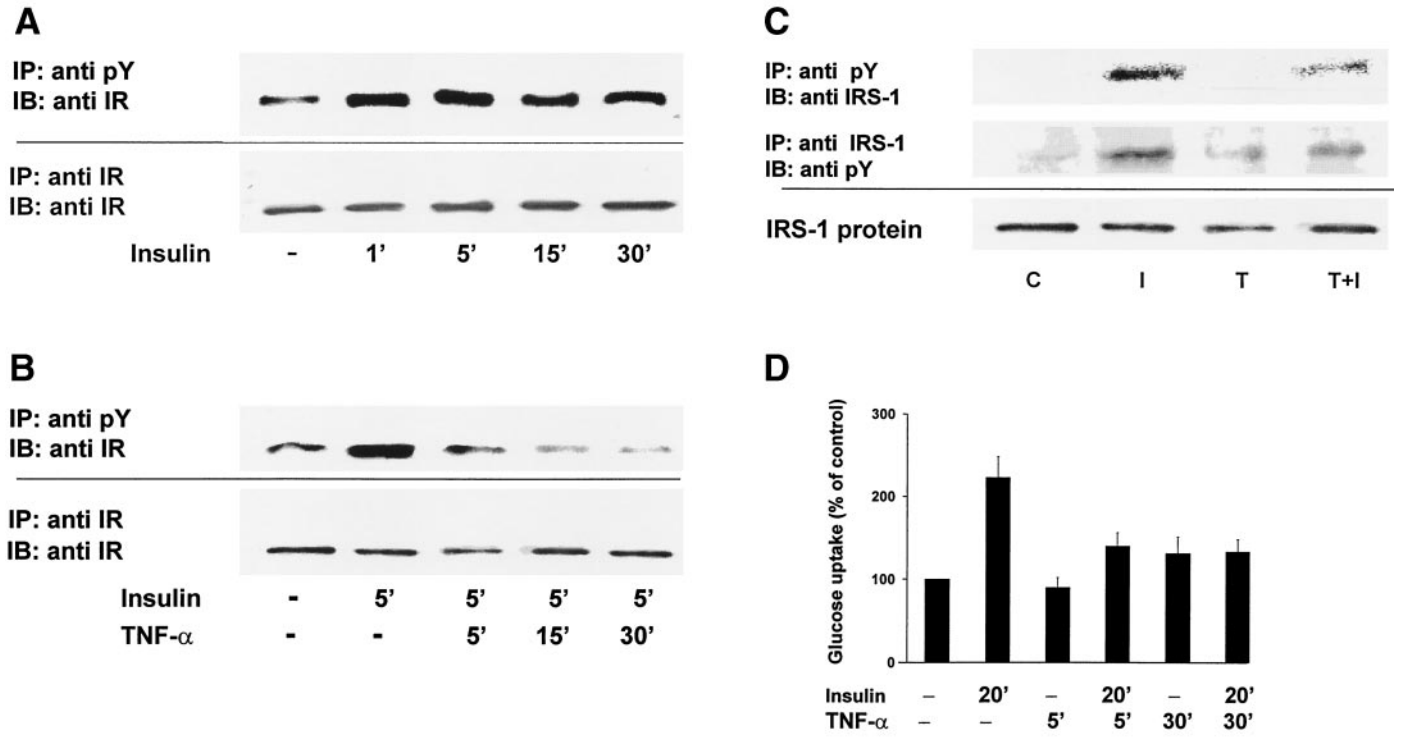

FIG. 1. TNF-α inhibits insulin-induced tyrosine phosphorylation of IR in cultured mouse skeletal myotubes. Studies were performed on 6-day-old cultured myotubes, which were transferred to serum-free low-glucose Eagle's medium, 24 h before experiments were conducted. *A*: Time course of insulin-induced tyrosine phosphorylation of IR in control cells. Protein extracts from untreated cultures or insulin-stimulated cultures treated for various time periods (1, 5, 15, or 30 min) were immunoprecipitated with specific antiphosphotyrosine antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti-IR antibodies. *B*: Effects of pretreatment with TNF-α on insulin-induced tyrosine phosphorylation of IR. Protein extracts were prepared from untreated cultures or cultures stimulated with insulin for 5 min with and without prior treatment with TNF-α (1 nmol/l) for 5, 15, or 30 min. Extracts were immunoprecipitated with specific antiphosphotyrosine antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti-IR antibodies. The Western blots shown are representative of three blots from three separate cultures. *C*: TNF-α inhibits insulin-induced tyrosine phosphorylation of IRS-1 in cultured mouse skeletal myotubes. Cells were either untreated (C) or treated with insulin for 5 min (I), with 1 nmol/l TNF-α for 5 min (T), or with TNF-α for 5 min before addition of insulin for 5 min (T+I). Extracts were immunoprecipitated with specific antiphosphotyrosine antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti–IRS-1 antibody. The Western blots shown are representative of three blots from three separate cultures. *D*: TNF-α inhibits insulin-induced glucose uptake. Myotubes were transferred to serum-free low-glucose medium for 24 h. Cells were either untreated or treated with insulin for 20 min, with 1 nmol/l TNF-α for 5 or 30 min, or with TNF-α before addition of insulin. The results are described as percent of control. Glucose transport was evaluated by measuring [^{3}H]-2DG for 15 min as described in RESEARCH DESIGN AND METHODS. Data are presented as percent of basal (18 ± 1.3 pmol · mg protein^{-1} · min^{-1}), which was set at 100%. Each bar represents the means ± SE of triplicate values in each of three experiments performed on separate cultures.

nmol/l) for 5 min completely inhibited insulin-induced phosphorylation of IR. Longer pretreatment (15–30 min) with TNFα not only blocked the insulin-induced phosphorylation of IR but also reduced the level of phosphorylation to below pretreatment levels.

The first element downstream of IR is the docking family of IRS proteins. In cultured mouse skeletal myotubes, the primary IRS protein involved in insulin signaling is IRS-1, which is also rapidly phosphorylated on tyrosine. As shown in Fig. 1*C*, immunoprecipitated IRS-1 is tyrosine phosphorylated within 5 min after insulin stimulation. Pretreatment of cells with TNF-α (1 nmol/l for 5 min) markedly reduced phosphorylation of IRS-1 by insulin. In addition, TNF-α inhibited insulin-induced association of IRS-1 with the p85 subunit of PI3-K (Fig. 9, left panel). Effects of TNF-α on the uptake of 2-deoxy-D-glucose (2-DG) were also studied (Fig. 1*D*). Treatment of myotubes with TNF-α for 5 min did not alter basal 2-DG uptake but significantly reduced the stimulatory effect of insulin. Treatment with TNF-α for 30 min caused a slight but significant increase in basal 2-DG uptake, which was not further increased by insulin. Thus, these findings in cultured mouse skeletal myotubes confirm that TNF-α inhibits early upstream insulin signaling as well as glucose transport.

TNF-α affects insulin-induced association between specific PKC isoforms and upstream elements. Several studies indicate that TNF-α inhibition of insulin signaling is mediated by other, as yet unknown, elements. Among the possible candidates is the PKC family of serine-threonine kinases, because TNF-α is known to affect certain members of this family, and some PKC isoforms can modulate IR autophosphorylation. In preliminary studies, we found that cultured mouse skeletal muscle cells express PKC isoforms α, β2, δ, ε, θ, and ζ, in agreement with other studies on mammalian skeletal muscle in vivo and in culture (15,38–40). In a recent study on rat skeletal muscle in primary culture, we showed that insulin induces a rapid and specific physical association between IR and PKCδ and that this physical linkage is essential for the continuation of the IR signaling cascade (26). We therefore reasoned that interference of PKC interactions with upstream elements might be involved in TNF-α effects on IR signaling. Accordingly, in this series of experiments, we

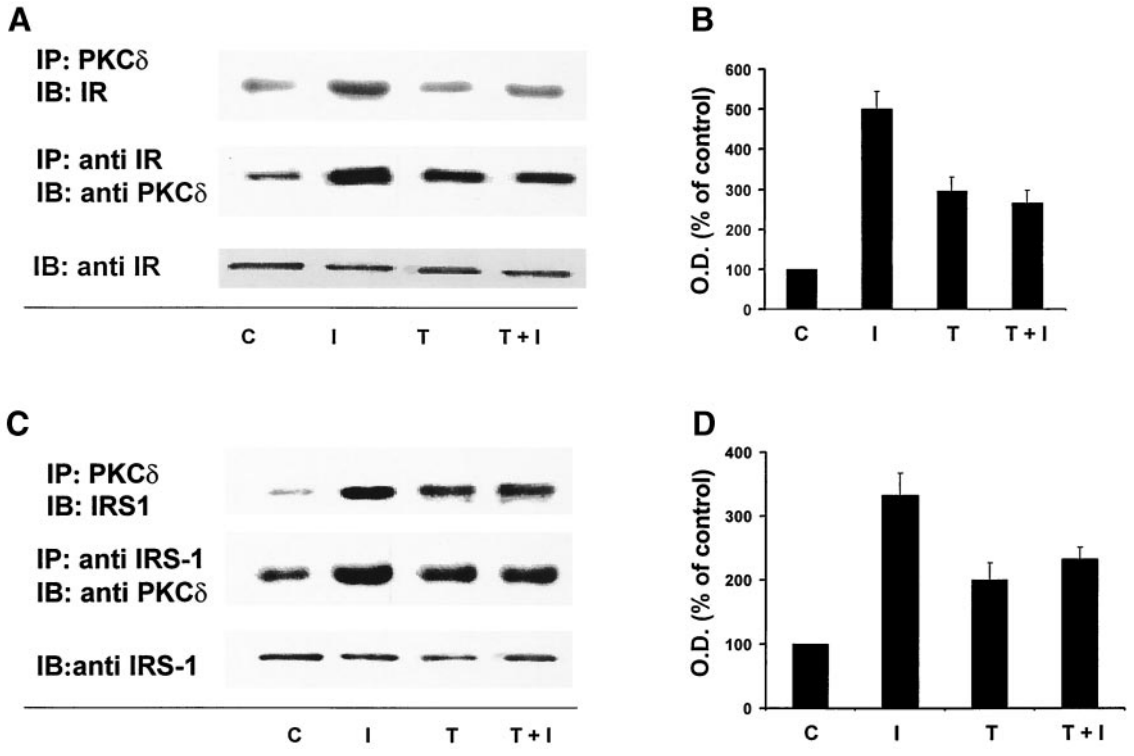

FIG. 2. TNF-α inhibits insulin-induced coimmunoprecipitation of PKCδ with IR (*A* and *B*) and IRS-1 (*C* and *D*). Cells were either untreated (C) or treated with insulin for 5 min (I), with 1 nmol/l TNF-α for 5 min (T), or with TNF-α for 5 min before addition of insulin for 5 min (T+I). Cell lysates were immunoprecipitated with anti-IR or anti–IRS-1 antibodies and then subjected to SDS-PAGE and immunoblotted with specific anti-PKCδ antibodies. *A* and *C*: Western blots of PKCδ induced to coimmunoprecipitate with IR and IRS-1, respectively. *B* and *D*: Optical density measurements of the Western blots. The values represent the means ± SE of three measurements in three separate cultures.

sought to determine whether TNF-α affects any physical interactions induced by insulin between various PKC isoforms and IR, IRS-1, and IRS-2. In these studies, we immunoprecipitated IR, IRS-1, and IRS-2 from control cells, insulin-stimulated cells, and insulin-stimulated cells after pretreatment with TNF-α. After SDS-PAGE and transfer, the immunoprecipitated proteins were probed with specific anti-PKC antibodies. Of the PKC isoforms examined (PKCs α, βII, δ, ε, and ζ), only PKCs α and δ were found to coimmunoprecipitate with upstream elements. PKCδ was constitutively associated with both IR and IRS-1, and this association was increased within 5 min by insulin stimulation. PKCα was found to be constitutively associated with IRS-1 only, and this association was decreased within 5 min by insulin stimulation (Figs. 3 and 4). None of the other PKC isoforms examined coassociated with IR, IRS-1, or IRS-2. Moreover, IRS-2 did not coimmunoprecipitate with either PKCδ or -α under basal or insulin-stimulated conditions (not shown). Therefore, all subsequent studies were done on IR and IRS-1 interactions with PKCs δ and α.

Treatment of muscle cells with TNF-α for 5 min before insulin stimulation had opposite effects on PKC association with IR and IRS-1. Thus, on one hand, as shown in Fig. 2, TNF-α increased the association between IR and IRS-1 with PKCδ and reduced the effect of insulin to increase coassociation of these elements with PKCδ. On the other hand, as illustrated in Fig. 3, this cytokine, while also inducing an increase in PKCα-IRS-1 association, prevented the ability of insulin to cause IRS-1 to dissociate from PKCα.

TNF-α activates specific PKC isoforms. Previous results from our laboratory have shown that insulin stimulation of cultured rat skeletal muscle cells results in activation of specific PKC isoforms (15). We therefore considered the possibility that the inhibitory effect of TNF-α on IR tyrosine phosphorylation might involve modulation of the activity of PKCs δ and α. Accordingly, we examined the effects of insulin and TNF-α on the activity of these PKC isoforms. Figure 4 shows the effects of insulin and TNF-α, separately and in combination, on the activity of specific PKC isoforms. In these studies, PKC isoforms were immunoprecipitated with specific antibodies from control cells and from cells treated with insulin, or with insulin after TNF-α, and the PKC activity of the immunoprecipitated protein was measured by an activity assay, as described in RESEARCH DESIGN AND METHODS. As can be seen, both insulin and TNF-α increased the activity of each of the PKC isoforms, the effect of the latter being at least as effective as that of the former. However, the activity of each of the enzymes from cells treated with TNF-α before insulin was less than that in cells treated with each substance alone.

Activation of PKC isoforms in skeletal muscle by insulin is associated with an increase in the tyrosine phosphorylation state (15). Accordingly, we examined effects of insulin and TNF-α on tyrosine phosphorylation of PKCα and -δ. In one series of experiments, we immunoprecipitated specific PKC isoforms and performed Western blotting with antiphosphotyrosine antibodies. The results are exemplified in Fig. 5, which shows that within 5 min after insulin stimulation, there was a strong increase in tyrosine

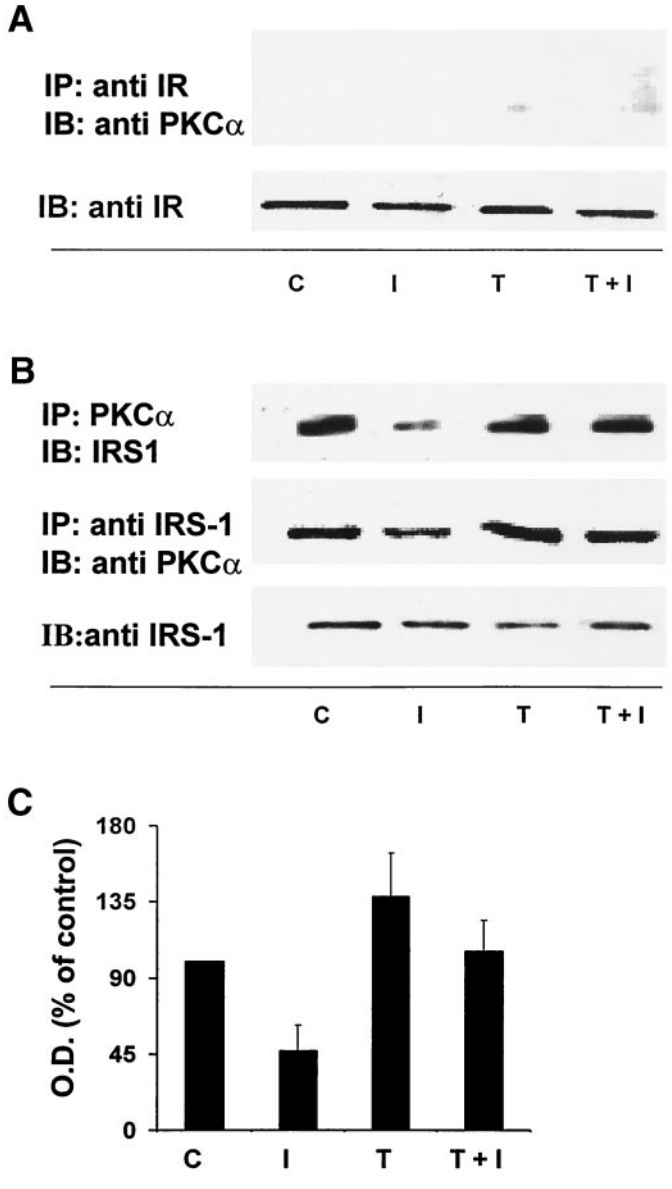

FIG. 3. TNF-α prevents insulin-induced decrease in PKCα/IRS-1 association. Cells were either untreated (C) or treated with insulin for 5 min (I), with 1 nmol/l TNF-α for 5 min (T), or with TNF-α for 5 min before addition of insulin for 5 min (T+I). Cell lysates were immunoprecipitated with anti-IR or anti–IRS-1 antibody, subjected to SDS-PAGE, and then immunoblotted with anti-PKCα antibodies. A: lack of either constitutive or insulin-induced association between PKCα and IR. B: Effects of TNF-α on insulin-induced dissociation between PKCα and IRS-1. The Western blots shown are representative of results obtained in three separate experiments on different cultures. C: Graphs of optical density measurements of the Western blots. The values represent the means ± SE of three measurements in three separate cultures.

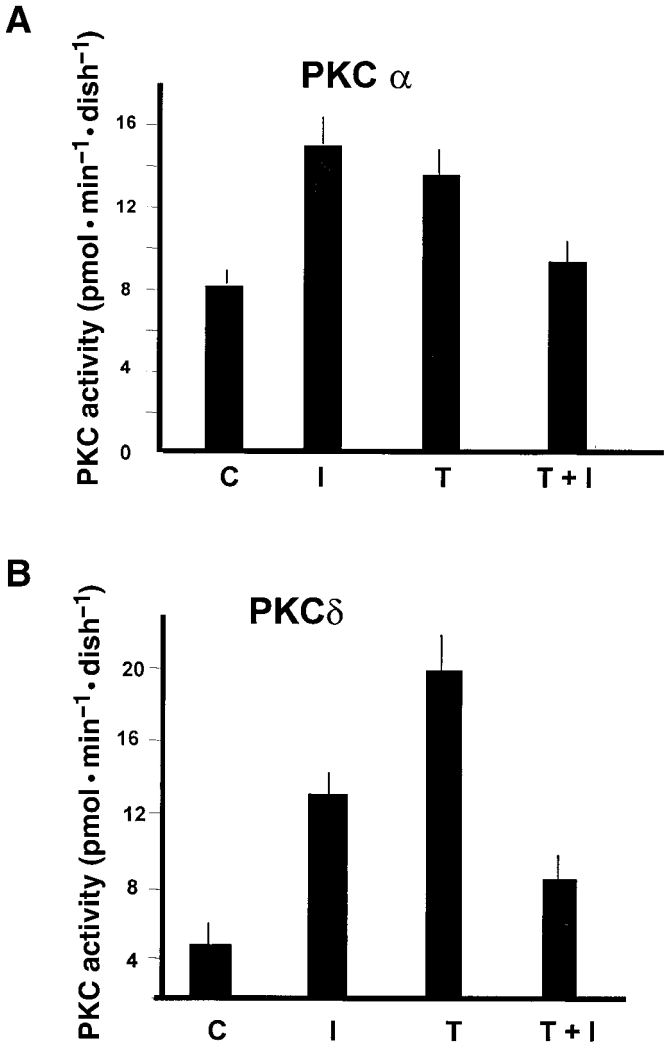

FIG. 4. TNF-α reduces insulin-induced activity of specific PKC isoforms in cultured mouse skeletal myotubes. Cells were either untreated (C) or treated with insulin for 5 min (I), with 1 nmol/l TNF-α for 5 min (T), or with TNF-α for 5 min before addition of insulin for 5 min (T+I). Cell lysates were immunoprecipitated with specific antibodies against PKCα (*A*) or PKCδ (*B*). Activity was determined with a SignaTECT PKC Assay System. The values represent the means ± SE of three measurements in three separate cultures.

phosphorylation of PKCs α and δ. Similar to insulin, TNF-α also induced phosphorylation of the α and δ isoforms within 5 min. As both insulin and TNF-α induced tyrosine phosphorylation of PKC isoforms α and δ, we expected that addition of TNF-α followed by insulin might produce an additive effect on these isoforms. However, when myotubes were treated with TNF-α before insulin, the level of tyrosine phosphorylation of each of the PKC isoforms induced by insulin stimulation was lower than that induced by insulin alone. In another series of studies, we immunoprecipitated with antiphosphotyrosine antibodies and immunoblotted with specific anti-PKC antibodies to each of the isoforms. The results were essentially the same as those obtained with immunoprecipitation of the PKC isoforms.

Overexpression of specific PKC isoforms modifies effects of TNF-α on IR signaling. The upstream locations of PKC isoforms α and δ in the insulin signaling cascade suggest that these isoforms may play a direct role in the regulation of IR function. Indeed, our results so far have shown that TNF-α not only affects the interaction of the α and δ isoforms with IR and IRS-1 but also modifies the effects of insulin on these interactions. Thus, it is possible that TNF-α modulation of the activity of these PKC isoforms may affect IR autophosphorylation. We therefore examined more directly whether the ability of TNF-α to reduce insulin-induced activity of PKCs α and δ might play a role in the effects of this cytokine on IR autophosphorylation. To this end, we overexpressed these isoforms using an adenovirus expression system. When

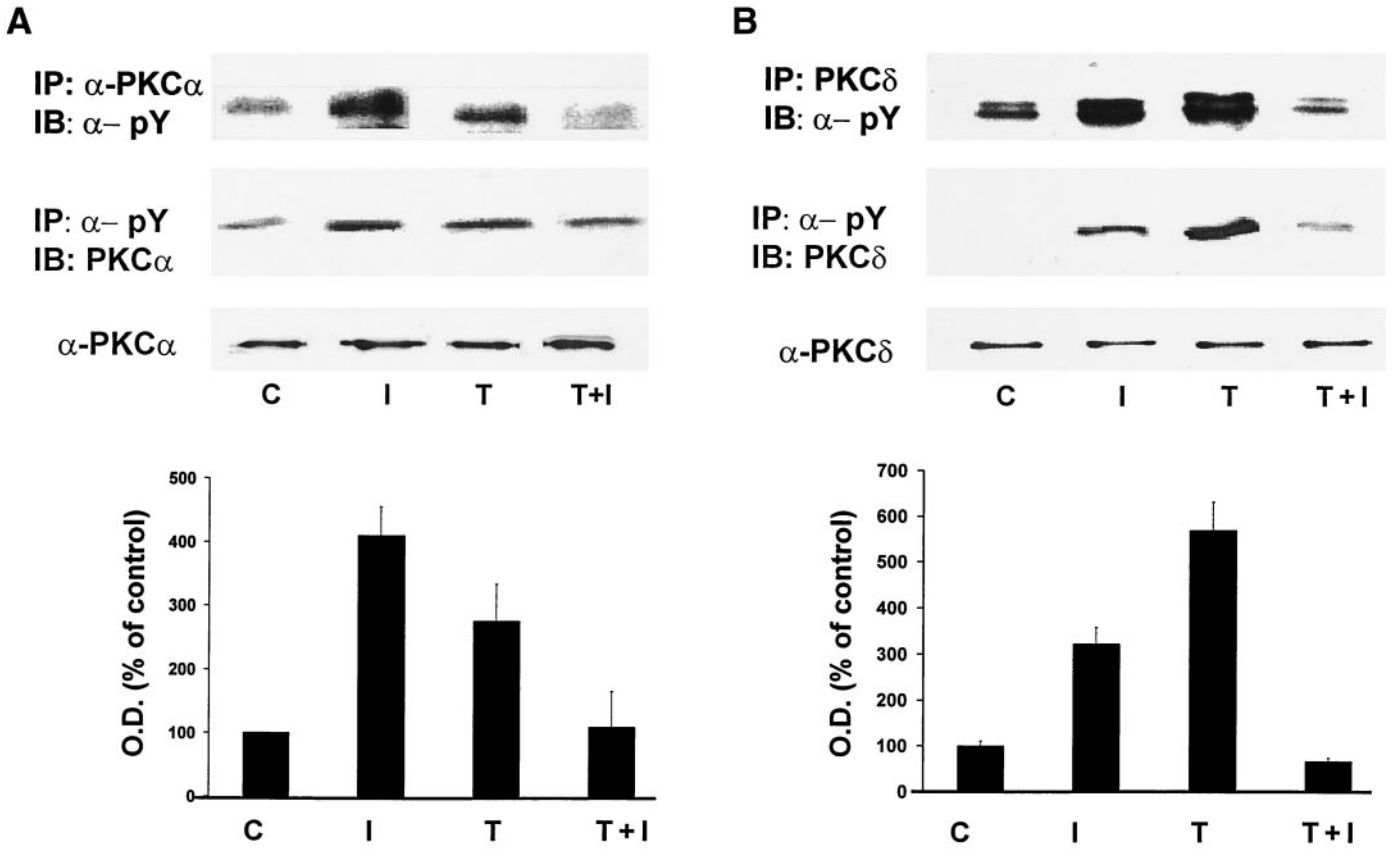

FIG. 5. TNF-α reduces insulin-induced tyrosine phosphorylation of specific PKC isoforms in cultured mouse skeletal myotubes. Cells were either untreated (C) or treated with insulin for 5 min (I), with 1 nmol/l TNF-α for 5 min (T), or with TNF-α for 5 min before addition of insulin for 5 min (T+I). Extracts were immunoprecipitated with either specific antiphosphotyrosine or anti-PKC antibodies. Immunoprecipitates were run on SDS-PAG and transferred to Immobilon-P membranes. Anti-PKC immunoprecipitates were immunoblotted with antiphosphotyrosine antibodies, and antiphosphotyrosine immunoprecipitates were immunoblotted with specific antibodies against PKC-α (*A*) or PKCδ (*B*). The Western blots shown are representative of three blots from three separate cultures. The lower row in each blot shows the protein level of each individual PKC isoform.

infected with recombinant adenovirus constructs containing cDNA for wild-type PKCδ, kinase-inactive dominant-negative PKCδ, or wild-type PKCα, myotubes displayed elevated protein expression of the transfected isoform compared with the expression of the endogenous isoforms (Fig. 6). Overexpression of each isoform, with the exception of dominant-negative PKCδ, also resulted in elevated kinase activity of the specific PKC isoform transfected (data not shown).

Next, we studied the effect of this specific overexpression of PKCα or -δ on insulin-induced IR tyrosine phosphorylation. As shown in Fig. 7*A*, overexpression of PKCδ resulted in an increase in tyrosine phosphorylation of IR in control and TNF-α treated cells (*lanes 1*, *2*, *7*, and *8*). This effect was not additive to that of insulin, since insulin stimulation in cells overexpressing PKCδ failed to further increase the phosphorylation state (*lanes 2–4*). Furthermore, overexpression of PKCδ prevented the inhibitory effect of TNF-α on insulin-induced IR tyrosine phosphorylation (*lanes 5* and *6*). To confirm that PKCδ overexpression increases IR tyrosine phosphorylation, we infected cells with kinase-inactive dominant-negative PKCδ. The infected cells displayed high protein levels of the inactive PKCδ (Fig. 6). Overexpression of dominant-negative PKCδ abrogated both basal and insulin-induced IR tyrosine phosphorylation (Fig. 7*B*, *lanes 1–4*). In addition, the

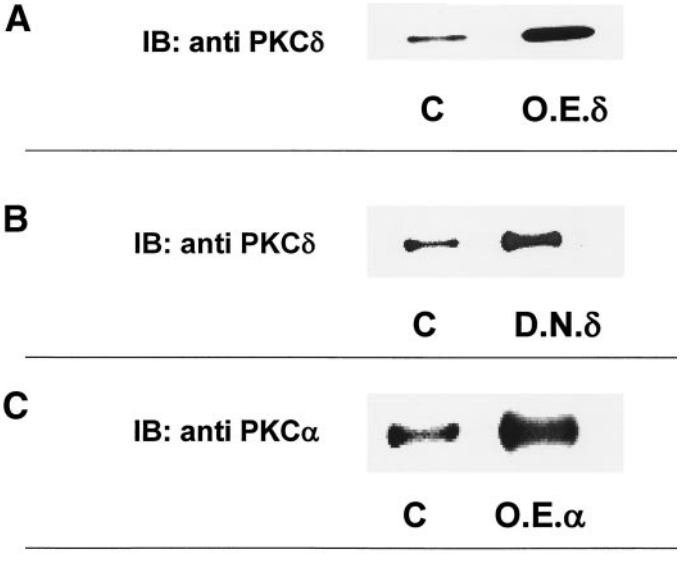

FIG. 6. Overexpression of specific PKC isoforms. Five-day-old myotube cultures were infected with adenovirus constructs of specific PKC isoenzymes for 12 h. Whole-cell lysates of nonoverexpressing and specific PKC isoform-overexpressing cells were subjected to SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti-PKC antibodies. Western blots of wild-type PKCδ (*A*; O.E.δ), dominant-negative PKCδ (*B*; D.N.δ), and wild-type PKCα (*C*; O.E.α) in noninfected (C) and adenovirus infected cells (right-side blots). The blots show strong overexpression of the PKC isoforms in adenovirus-infected compared with wild-type noninfected cells.

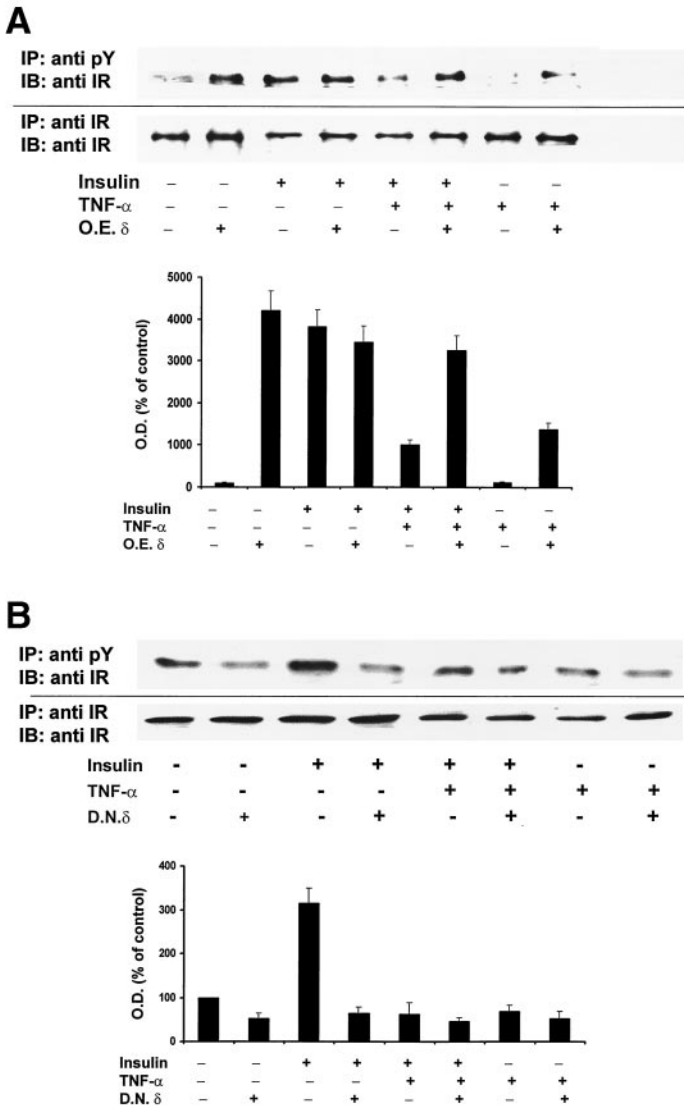

FIG. 7. Effects of overexpression of WTPKCδ and dominant-negative PKCδ on insulin-induced tyrosine phosphorylation of IR. Control noninfected and infected cells overexpressing wild-type PKCδ (*A*) and dominant-negative PKCδ (*B*) were treated for 5 min separately with insulin or TNF-α or 5 min with TNF-α followed by 5 min with insulin. Cell lysates were immunoprecipitated with antiphosphotyrosine antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti-IR antibodies. The Western blot shown is representative of results obtained in four separate experiments on four separate cultures.

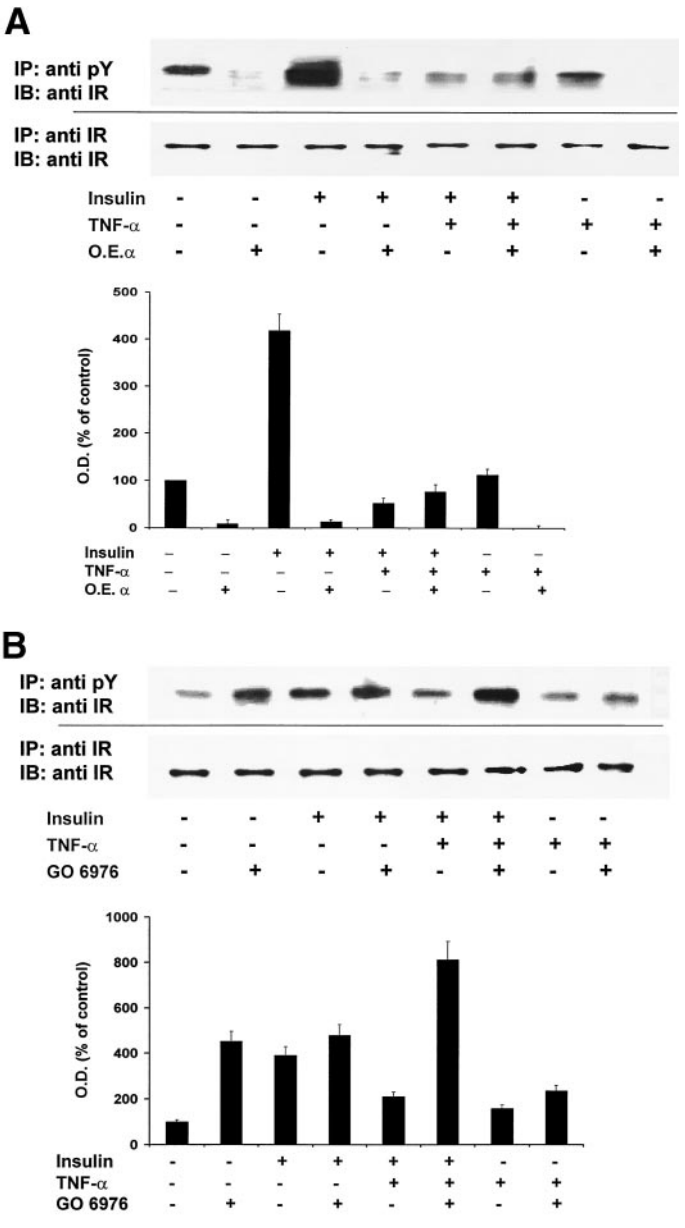

FIG. 8. Effects of overexpression and blockade of PKCα on TNF-α inhibition of insulin-induced tyrosine phosphorylation of IR. Cell lysates were prepared from cells treated for 5 min with insulin or TNF-α or 5 min with TNF-α followed by 5 min with insulin. The lysates were immunoprecipitated with antiphosphotyrosine antibodies, and immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti-IR antibodies. *A*: Overexpression of PKCα inhibits insulin-induced autophosphorylation of IR. Lysates were prepared from control noninfected and PKCα-overexpressing cells. *B*: Blockade of PKCα prevents TNF-α inhibition of IR autophosphorylation. Lysates were prepared from control cells and cells pretreated for 20 min with GÖ6976 (10 nmol/l). The Western blots are representative of results obtained in three separate experiments on three separate cultures.

effect of TNF-α on both basal and insulin-induced tyrosine phosphorylation in cells overexpressing dominant-negative PKCδ was not significantly greater than either TNF-α or dominant-negative PKCδ alone (Fig. 7*B*, *lanes 5–8*).

In contrast to the effects of PKCδ overexpression, overexpression of PKCα inhibited both basal and insulin-induced IR tyrosine phosphorylation, as shown in Fig. 8*A* (*lanes 1–4*). Interestingly, insulin did appear to increase tyrosine phosphorylation of IR in PKCα-overexpressing cells that had been treated initially with TNF-α (*lanes 6* and *8*), but the level was considerably less that that in control noninfected myotubes (*lane 1*). To determine whether PKCα is necessary for the inhibitory function of TNF-α on IR autophosphorylation, we used a selective inhibitor of the α and βI PKC isoforms, GÖ6976 (Fig. 8*B*). (Because PKCβI is not detected in primary cultures of mouse skeletal muscle, we used this inhibitor as a selective inhibitor against PKCα in this system.) In contrast to effects of PKCα overexpression, inhibition of this isoform increased basal tyrosine phosphorylation of IR (*lanes 1* and *2*). In addition, inhibition of PKCα abrogated the ability of TNF-α to inhibit IR autophosphorylation (*lanes 5* and *6*). The results indicate that one component of the effects of TNF-α on IR signa ling involves the activity of

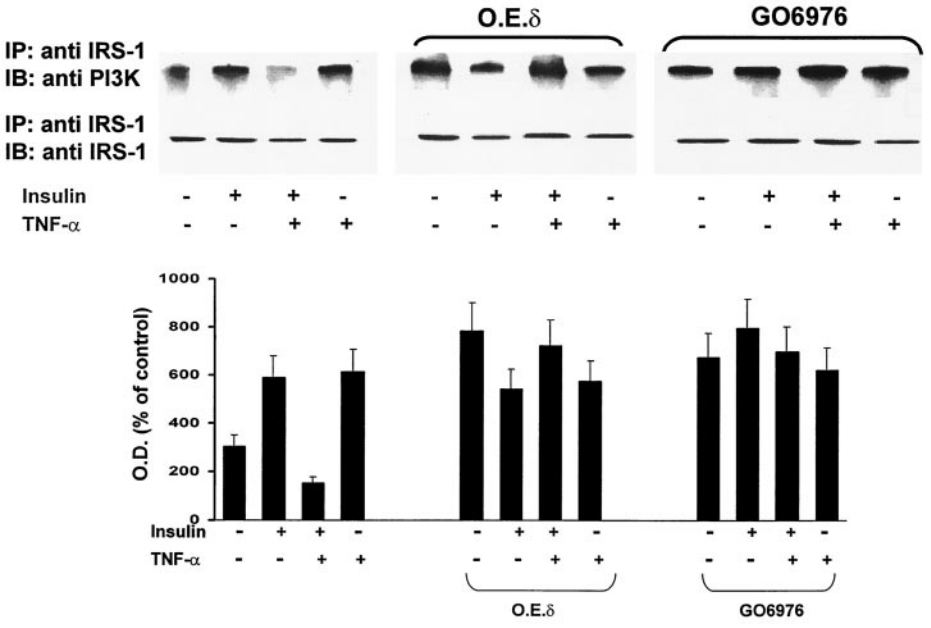

FIG. 9. Effects of overexpression of PKCδ and blockade of PKCα on insulin-induced association between IRS-1 and PI3-K. Control noninfected cells (left panel), infected cells overexpressing WTPKCδ (middle panel), and cells treated with GÖ6976 (right panel) were treated for 5 min separately with insulin or TNF-α or 5 min with TNF-α followed by 5 min with insulin. Cell lysates were immunoprecipitated with anti–IRS-1 antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with specific anti-p85 PI3-K antibodies. The Western blot shown is representative of results obtained in three separate experiments on three separate cultures.

PKCα and its association with IRS-1. In parallel, TNF-α reduced responses to insulin by inhibiting insulin-induced activity of PKCδ and its association with IR and IRS-1.

The results therefore show that overexpression of PKCδ and inhibition of PKCα were able to reverse the deleterious effects of TNF-α on IR phosphorylation. To determine whether these treatments of the PKCs δ and α might also influence progression of the IR signal, we examined the association between IRS-1 and PI3-K which is one of the downstream steps in the IR signaling pathway. As shown in Fig. 9 (left panel), insulin induced PI3-K to associate with IRS-1, and TNF-α reduced this effect. Overexpression of PKCδ increased basal association between these two proteins, even in the absence of insulin, and insulin did not further increase this effect. Moreover, the inhibitory effect of TNF-α on IRS-1/PI3-K association was completely abrogated (Fig. 9, middle panel). Effects of inhibition of PKCα by GÖ6976 were essentially the same as those obtained with overexpression of PKCδ (Fig. 9, right panel). Thus, either PKCδ overexpression or PKCα blockade effectively antagonizes the inhibitory effects of TNF-α on signaling from IR to PI3-K.

DISCUSSION

In this study, we have shown that TNF-α has two distinct mechanisms to oppose insulin action on upstream elements in the IR signaling pathway. In each case, the effect is related to interaction with specific PKC isoforms. These interactions are summarized in Fig. 10. Thus, on one hand, insulin induced a physical interaction of PKCδ with IR and IRS-1 (Fig. 10*A*), and TNF-α prevented this interaction (Fig. 10*B*). On the other hand, insulin disrupted the constitutive association that occurs in this preparation between IRS-1 and PKCα (Fig. 10*A*), and TNF-α caused this association to increase (Fig. 10*B*). The effects of insulin and TNF-α on both PKCα and -δ were accompanied by tyrosine phosphorylation and activation. Although it has been shown that stimulation of PKCs α and δ can inhibit IR autophosphorylation, and although various effects of TNF-α on PKC have been reported (17), a link between these effects and TNF-α inhibition of IR tyrosine phosphorylation has not been established.

This is the first report to demonstrate that PKCα may actually be constitutively associated with IRS-1 and that insulin causes these elements to physically dissociate. Others have shown, however, that activation of PKCα not only inhibits IR signaling (41,42) but also that PKCα may require IRS-1 for inhibition of IR tyrosine kinase activity (27). Activation of PKCα and its increased physical association with IRS-1 in response to TNF-α, as reported here, are thus consistent with the involvement of IRS-1 in PKCα inhibition of IR signaling. Moreover, the ability of insulin

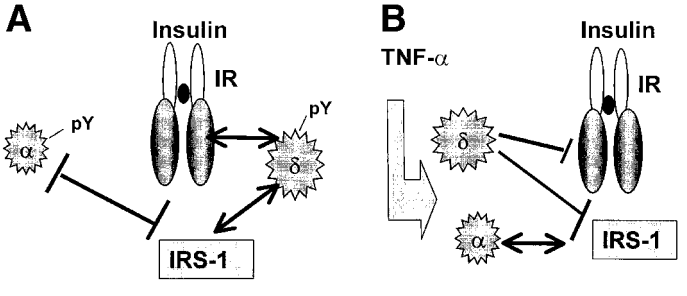

FIG. 10. Schematic diagram of insulin (*A*) and TNF-α (*B*) effects on PKC interactions in insulin signaling. *A*. Insulin induces tyrosine phosphorylation of PKCs α and δ, as well as association of PKCδ with IR and IRS-1, and causes PKCα to dissociate from IRS-1. *B*. Pretreatment with TNF-α reduces insulin-induced tyrosine phosphorylation of PKCs α and δ. In addition, TNF-α interferes with insulin-induced interactions between the PKC isoforms and IR and IRS-1; PKCδ association with IR and IRS-1 is inhibited, whereas association between PKCα and IRS-1 is strengthened.

to cause dissociation between IRS-1 and PKCα may, by reducing serine phosphorylation, allow the insulin signal to continue. The results demonstrating that TNF-α activation of PKCα is involved in inhibition of IR signaling are also consistent with an earlier study conducted on a line of CHO cells overexpressing PKCα. Activation of PKCα in these cells by the phorbol ester, TPA (tetradecanoylphorbol acetate), also inhibited tyrosine phosphorylation of IRS-1 in response to insulin stimulation (42). The results obtained in studies on overexpression and blockade of PKCα indicate that this isoform is essential for the inhibitory effect of TNFα on insulin signaling. Thus, overexpression of PKCα alone was sufficient to block both basal and insulin-induced IR tyrosine phosphorylation, and treatment with a PKCα inhibitor elevated basal IR autophosphorylation and blocked the inhibitory effect of TNFα. Moreover, the fact that PKCα associates with IRS-1 and not with IR further indicates that IRS-1 is required for the inhibitory effect of PKCα and that it plays a role in reduction of insulin signaling, as previously suggested (27,29).

As we have shown, PKCδ is induced by insulin to associate with both the IR and its first downstream element, IRS-1. This association, upstream in insulin signal transduction, is consistent with the suggested role for PKCδ in regulating the function of IR itself (25,26). Our results, showing that an important component of the ability of TNF-α to interfere with IR signaling involves disturbance of the insulin-induced physical association between PKCδ and IR/IRS-1, further strengthen this idea. Additional support for an upstream location of PKCδ in insulin signaling is provided by results obtained in rat skeletal muscle showing that specific inhibitors of PI3K fail to inhibit insulin-induced activation of PKCδ (15).

We found that overexpression of PKCα reduced both basal and insulin-induced tyrosine phosphorylation of IR, whereas inhibition of PKCα increased IR tyrosine phosphorylation in the basal state. These results are in agreement with a previous study (27) in which it was shown that PKC isoforms α, δ, and θ, when coexpressed with HIR (human insulin receptor) and IRS-1 in HEK293 cells, inhibited HIR tyrosine phosphorylation, probably through serine/threonine phosphorylation of IRS-1. PKCδ, on the other hand, appears to have a dual effect on IR phosphorylation. In addition to serine phosphorylation, which was observed in cells overexpressing wild-type PKCδ even without insulin stimulation (data not shown; see also 26), overexpression of PKCδ resulted in IR tyrosine phosphorylation. This was not reported by Kellerer et al. (27). Because PKCs are serine/threonine kinases, the effect of PKCδ to increase IR tyrosine phosphorylation must be mediated by some other factor or factors. One possibility is that PKCδ, by its physical interaction with IR, may have a permissive effect on the autophosphorylation mechanism. Blockade of IR autophosphorylation by overexpression of DMPKCδ is consistent with this possibility. Alternatively, or additionally, PKCδ may affect other elements that mediate this phosphorylation. Another possibility may be related to the reported effects of PKCδ on IR routing (26,43). Recent results from our laboratory suggest that PKCδ increases the routing of IR by modulating the activity of specific proteins involved in the internalization. Internalized IR is relatively highly phosphorylated on tyrosine residues (44).

The results regarding the opposing effects of both insulin and TNF-α on PKCs α and δ suggest that the two isoforms may play opposite roles in insulin signaling. Opposing effects of these isoforms have been reported in other systems and in signaling cascades other than the insulin signaling pathway, such as induction or inhibition of apoptosis, regulation of proliferation and differentiation, and mediation of cell transformation (45–47). Our additional finding that blockade of PKCα abrogated TNF-α inhibition of IR signaling further demonstrates that both PKC isoforms are required for its effect.

The results of this study indicate that TNF-α inhibition of insulin-induced IR and IRS-1 phosphorylation, IRS-1/PI3 K association, and glucose transport occurs, in part, via modulation of the activity of PKC isoforms α and δ and their association with upstream elements in the insulin signaling cascade. We, therefore, propose that TNF-α activates PKCα and -δ and influences their association with IR and IRS-1 in a manner that interferes with the ability of insulin to regulate these isoforms. We further suggest that tyrosine phosphorylation, which may occur at distinct and separate sites in response to insulin than to TNF-α, is a key element in this phenomenon. This would be consistent with studies that indicate the importance of tyrosine phosphorylation in directing the substrate specificity of PKC, in particular PKCδ (16). The mechanisms and specific sites of tyrosine phosphorylation of PKCs α and δ in response to insulin and TNF-α are currently being investigated. The apparently mutually antagonistic effects of insulin and TNF-α on major elements upstream in the insulin signaling cascade indicate that there may be a delicate balance among different substrates for the satisfactory propagation of the insulin signal. Moreover, the effects of this cytokine on specific PKC isoform interactions with these upstream elements support a role for TNF-α in insulin resistance of skeletal muscle.

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