Phosphorylation of Ser\textsuperscript{357} of Rat Insulin Receptor Substrate-1 Mediates Adverse Effects of Protein Kinase C-\(\delta\) on Insulin Action in Skeletal Muscle Cells*

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The activation of the protein kinase C (PKC) family of serine/threonine kinases contributes to the modulation of insulin signaling, and the PKC-dependent phosphorylation of insulin receptor substrate (IRS) -1 has been implicated in the development of insulin resistance. Here we demonstrate Ser\textsuperscript{357} of rat IRS-1 as a novel PKC-\(\delta\)-dependent phosphorylation site in skeletal muscle cells upon stimulation with insulin and phorbol ester using Ser(P)\textsuperscript{357} antibodies and active and kinase dead mutants of PKC-\(\delta\). Phosphorylation of this site was simulated using IRS-1 Glu\textsuperscript{357} and shown to reduce insulin-induced tyrosine phosphorylation of IRS-1, to decrease activation of Akt, and to subsequently diminish phosphorylation of glycogen synthase kinase-3. When the phosphorylation was prevented by mutation of Ser\textsuperscript{357} to alanine, these effects of insulin were enhanced. When the adjacent Ser\textsuperscript{358}, present in mouse and rat IRS-1, was mutated to alanine, which is homologous to the human sequence, the insulin-induced phosphorylation of glycogen synthase kinase-3 or tyrosine phosphorylation of IRS-1 was not increased. Moreover, both active PKC-\(\delta\) and phosphorylation of Ser\textsuperscript{357} were shown to be necessary for the attenuation of insulin-stimulated Akt phosphorylation. The phosphorylation of Ser\textsuperscript{357} could lead to increased association of PKC-\(\delta\) to IRS-1 upon insulin stimulation, which was demonstrated with IRS-1 Glu\textsuperscript{357}. Together, these data suggest that phosphorylation of Ser\textsuperscript{357} mediates at least in part the adverse effects of PKC-\(\delta\) activation on insulin action.

To accomplish its fundamental role in maintaining in vivo metabolic homeostasis, insulin binds and activates insulin receptors, which in turn recruit insulin-receptor substrate (IRS)\textsuperscript{3} proteins (1). By disruption of their respective genes in mice, IRS-1 and IRS-2 have been assigned a central role in mediating the normal actions of insulin. Defects at the level of the IRS proteins also comprise a major locus for the development of metabolic disorders, including insulin resistance and type 2 diabetes (2, 3). IRS proteins are regulated at several steps, including gene expression, protein degradation, and phosphorylation (4, 5). Although the phosphorylation of IRS-1 on tyrosine residues is mandatory for insulin-stimulated responses, serine/threonine phosphorylation appears to be the mechanism for the precise regulation and could either enhance or attenuate the insulin effects (6, 7). However, in most studies serine phosphorylation of IRS-1 has been implicated as a negative regulator of insulin signaling (8–12). It can induce the dissociation of IRS-1 from its receptor and hinder the phosphorylation of tyrosine residues (8), release the IRS-1 from intracellular complexes that maintain them in close proximity to the receptor (13), or induce its protein degradation (14). These multiple effects suggest that the serine residues subjected to phosphorylation play a pivotal role in regulating IRS-1 function. Of note, the unbalanced chronic stimulation of IRS-1 serine kinases leads to hyperphosphorylation of IRS-1 and is a major pathophysiological mechanism in the development of insulin resistance.

Among these IRS-1 kinases, members of the protein kinase C (PKC) family of serine/threonine kinases have received considerable attention for their regulatory role in insulin signaling. Although particularly the activation of atypical PKCs has been reported as positive modulation of insulin signaling (15, 16), the majority of studies has demonstrated that PKCs are implicated in impaired insulin signaling including classical (17–19), novel (20), and more recently also atypical PKC isoforms (21). Activation of PKC isoforms by insulin (11, 21, 22), hyperglycemia (18, 23), and lipids (24–27) leads to reduced insulin action and insulin resistance. The knock-out of PKC-\(\alpha\) in mice or inhibition of PKC-\(\epsilon\) in rats enhances insulin signaling (28, 29), and PKC-\(\theta\)-deficient mice are protected from fat-induced insulin resistance (30). Serine/threonine phosphorylation of IRS-1 has been implicated as important mechanism in the PKC-mediated regulation of insulin action (31), and a few PKC-dependent phosphorylation sites have been identified so far, among them (the serine residues corresponding to rat IRS-1 sequence) Ser\textsuperscript{24} (32), Ser\textsuperscript{318} (11), and Ser\textsuperscript{1101} (33).

Given the major role of the serine kinases of the PKC family in the regulation of insulin signaling, the knowledge about the
PKC-dependent phosphorylation of IRS-1 appeared fragmentary. In the present study we focused on PKC-δ-mediated serine phosphorylation of IRS-1. This isoform has also been involved in the regulation of insulin signaling (34–38). Upon activation PKC-δ could associate to and regulate the function of the insulin receptor (39) and also interact with IRS-1 (40), thereby regulating its tyrosine phosphorylation (41). An in vitro approach identified several PKC-δ phosphorylation sites in human IRS-1 (41), but only the phosphorylation of Ser357 could be shown to be functional in cells, thereby modulating insulin action in a negative manner (32). We aimed to investigate novel, functional active PKC-δ phosphorylation sites of IRS-1 and could demonstrate the phosphorylation of Ser357 by PKC-δ and its adverse effects on insulin signal transduction.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Reagents, and Antibodies**—C2C12 cells were from ATCC (Wesel, Germany). Baby hamster kidney cells stably expressing the human insulin receptor (BHKIR) were kindly provided by R. Lammers (Department of Internal Medicine, Tuebingen, Germany). The oligonucleotides were synthesized by Invitrogen. Cell culture media, supplements, and fetal calf serum were purchased from Invitrogen; protease inhibitor mixture was from Roche Applied Science; phosphatase inhibitors was from Sigma; α protein phosphatase was purchased from New England Biolabs (Beverly MA); antibodies against phospho-Akt Ser473, Thr308, and Tyr(P)100, and phospho-GSK 3α/β (Ser21/9) were from Cell Signaling (Frankfurt, Germany); antibodies against Akt and PKC-δ were from BD Biosciences (San Diego, CA); the antibodies against IRS-1 (C terminus) were from Upstate Biotechnology (Lake Placid, NY); and the antibodies against GSK-3α/β were from Santa Cruz (Heidelberg, Germany). The cytomegalovirus promoter-based expression vector for rat IRS-1 was described in Ref. 34, and the murine IRS-1 expression vector serving as template. Positive clones were verified by sequencing.

**Generation of Site-specific Ser(P)357 Antibodies**—Polyclonal anti-Ser(P)357 antiserum was raised against a synthetic peptide AHRHRGpSSRLPPLNHSRSI flanking Ser357 in IRS-1 (see Fig. 1A). The antiserum was purified by immunoaffinity chromatography. In the first step, antiserum was applied to an affinity column containing the unphosphorylated peptide. To eliminate cross-reactivity with the adjacent Ser358, the eluate was purified using Ser(P)358 peptide coupled to an affinity column.

**Site-directed Mutagenesis**—Mutagen of Ser357 of IRS-1 to alanine or glutamate, mutation of Ser358 to alanine, and mutation of Ser357 and Ser358 to alanine were made by oligonucleotide-mediated mutagenesis. The mutagenic upstream primers used were IRS-1-ala357, cc cac ggc cat cag cat cga ggc gcc tcc agg tgt cac ccc cca ctc aac cac; IRS-1-ala358, cc cac cgt cat cag cgc gag tcc agg tgt cac ccc cca ctc aac cac; IRS-1-ala357/358, cc cac ggc cat cag cat cga ggc gag gcc agg tgt cac ccc cca ctc aac cac; IRS-1-ala358, cc cac ggc cat cag cat cga ggc gag gcc agg tgt cac ccc cca ctc aac cac; and IRS-1-ala357/358, c cat cag cat cga ggc gag gcc agg tgt cac ccc cca ctc ctc, with the wild type IRS-1 expression vector serving as template. Positive clones were verified by sequencing.

**Identification of Ser357 as a Novel Phosphorylation Site in IRS-1**—PKC-δ was found to phosphorylate Ser357 of IRS-1 in vitro by Greene et al. (41) and our group using the previously described mass spectrometry-based identification procedure (43). The sequence homology of rodent and human IRS-1 is shown in Fig. 1A. The human IRS-1 contains alanine instead of serine at position 358 (position 363 in humans), i.e. whenever Ser358 is mutated to alanine, this represents the human wild type (WT) sequence.

To study the regulation and function of this site in vivo, we generated polyclonal phospho-site-specific antibodies using a phosphopeptide sequence corresponding to the region of IRS-1 surrounding Ser357. The antisera were immunopurified as described under “Experimental Procedures” to eliminate cross-reactivity with the adjacent Ser358 (Fig. 1A). The specificity of the immunopurified antibodies was tested in BHKIR cells transiently transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358 and IRS-1 Ala357/358 and stimulated with insulin or phorbol ester TPA, a pharmacological activator of classical and novel PKC isoforms for 30 min (Fig. 1B). The phospho-site-specific Ser357 antibody clearly detected IRS-1 upon stimulation with insulin and TPA in the cells overexpressing IRS-1 WT and IRS-1 Ala358, indicating induction of the phosphorylation of Ser357 by insulin and activation of PKC isoforms. We detected
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To further establish the specificity of the phospo-site-specific Ser\(^{357}\) antibodies, C2C12 cells were transfected with IRS-1 WT and stimulated with TPA. Immunoprecipitated IRS-1 was incubated with λ phosphatase, which led to a complete loss of the immunoreactivity of the antibody, indicating that dephosphorylated IRS-1 was not recognized (Fig. 1C). Analyzing non-transfected C2C12 cells, we could also detect the phosphorylation of Ser\(^{357}\) of endogenous IRS-1 after stimulation with insulin or TPA (Fig. 1D). Moreover, phosphorylation of Ser\(^{357}\) was significantly induced in muscle tissue of insulin-treated mice, suggesting a physiological relevance for this site (Fig. 1E).

Additionally, we observed in insulin or TPA-treated human myotubes enhanced phosphorylation of Ser\(^{357}\) of the endogenous human IRS-1 protein, which contain alanine instead of serine at position 358 (Fig. 1F).

**FIGURE 1. Specificity of Ser(P)\(^{357}\) antibodies in cultured cells.** A, amino acid sequence surrounding Ser\(^{357}\) of rodent and human IRS-1 (amino acid numbers are indicated), B, baby hamster kidney cells stably expressing the human insulin (ins) receptor (BH-1 cells) were transfected with IRS-1 WT, IRS-1 Ala\(^{357}\), IRS-1 Ala\(^{358}\), and IRS-1 Ala\(^{357/358}\) and treated with insulin (10 nM, 30 min) or TPA (0.5 μM, 30 min). After stimulation the cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with phospho-site-specific Ser\(^{357}\) antibody. The blot was reprobed with a polyclonal IRS-1 antibody. The arrow indicates endogenous IRS-1 visible after long exposure. Representative results from three experiments are shown. con, control; pSer357, Ser\(^{357}\). C, C2C12 cells were transfected with IRS-1 WT and were stimulated for 30 min with TPA (0.5 μM). IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody, and immunoprecipitates (IP) were incubated with buffer alone or with buffer and λ protein phosphatase for 30 min at 30°C, and immunoblotted (IB) with the phospho-site-specific Ser\(^{357}\) antibody. The addition of buffer without incubation at 30°C served as another control. The blot was reprobed with a polyclonal IRS-1 antibody. D, C2C12 cells were stimulated with insulin (10 nM, 30 min) or TPA (0.5 μM, 30 min). IRS-1 was immunoprecipitated and immunoblotted with site-specific Ser\(^{357}\) antibody and reprobed with a polyclonal IRS-1 antibody. E, male CH3 mice were fasted overnight and subsequently intravenously injected with 4 IU of insulin into the inferior vena cava. Muscle samples were obtained after 5 min of insulin treatment. Shown are immunoblots of muscle tissue of three control and three insulin-treated mice. Ser\(^{357}\) phosphorylation intensity was quantified based on scanning densitometry of the immunoblots (means ± S.E., n = 7, * p < 0.05 insulin-treated mice versus control). F, human myotubes were stimulated with 100 nM insulin or 0.5 μM TPA.

almost no signal in IRS-1 Ala\(^{357}\) and IRS-1 Ala\(^{357/358}\) transfected cells, similar to the immunoblots obtained with control transfected cells (Fig. 1B). These data clearly show that the immunopurified antibody recognizes IRS-1 solely when it is phosphorylated on Ser\(^{357}\) without any cross-reactivity with the adjacent putative phosphorylation residue Ser\(^{358}\). Similar results have been obtained using C2C12 skeletal muscle cells (data not shown).

**FIGURE 2. PKC-δ-mediated IRS-1 Ser\(^{357}\) phosphorylation in C2C12 cells.** A, C2C12 cells were transfected with IRS-1 WT or co-transfected with PKC-δ or kinase-dead mutant of PKC-δ (PKC-δ KN). The cells were stimulated with either 10 nM insulin (ins) or 0.5 μM TPA for 30 min. A representative immunoblot with the phospho-site-specific Ser\(^{357}\) antibody and PKC-δ and reprobe of the blot with the polyclonal IRS-1 antibody is shown. B, Ser\(^{357}\) phosphorylation intensity was quantified based on scanning densitometry of the immunoblots (means ± S.E., n = 7, * p < 0.05 PKC-δ co-transfected cells versus IRS-1 WT alone; #, p < 0.05 PKC-δ KN co-transfected cells versus PKC-δ co-transfected cells).
mutant was used (Fig. 2). These findings indicate that the insulin- and TPA-induced phosphorylation of Ser357 required PKC-δ activity.

The Phosphorylation of Ser357 of IRS-1 Leads to Reduced Phosphorylation of Akt in Skeletal Muscle Cells—We next investigated the functional role of phosphorylation of Ser357 in insulin signal transduction. The kinase Akt is an important insulin signaling molecule, shown to transduce the metabolic actions of insulin, including increases in transport and storage of glucose in muscle, and its activation is largely controlled by IRS-1 during insulin action (44). Therefore, we studied the influence of Ser357 of IRS-1 on the insulin-induced time-dependent phosphorylation of Akt. To examine a possible influence of the adjacent Ser358 residue, C2C12 cells were transiently transfected with IRS-1 WT, IRS-1 Ala357, IRS-1 Ala358, and IRS-1 Ala357/358 and stimulated for various time points with 10 nM insulin (Fig. 3A). The insulin-dependent increase in Ser473 phosphorylation of Akt was clearly enhanced in IRS-1 Ala357, IRS-1 Ala358, and IRS-1 Ala357/358 expressing cells after 10 and 60 min of insulin stimulation compared with wild type IRS-1 (Fig. 3A). The possible negative role of Ser357 phosphorylation in insulin action was further demonstrated in IRS-1 Glu357-expressing cells, which exhibited a significantly diminished Ser473 phosphorylation of Akt after 10 and 60 min of insulin stimulation compared with wild type-expressing cells (Fig. 3, B and C). Because we did not observe any difference of single IRS-1 Ala357 and the double alanine mutant, we used in further experiments IRS-1 Ala357/358 as loss-of-function and IRS-1 Glu357 as gain-of-function mutant.

Insulin-stimulated Phosphorylation of GSK-3α in Skeletal Muscle Cells Is Modulated by Ser357 Phosphorylation of IRS-1—The sustained negative effect of Ser(P)357 on Akt suggests a physiological function of this phosphorylation in insulin signaling. Thus, we asked whether the regulation of Akt activity was reflected by a modulation of its downstream effector GSK-3. C2C12 cells were transiently transfected with IRS-1 WT, IRS-1 Ala357/358, or IRS-1 Glu357 and stimulated for various time points with 10 nM insulin (Fig. 4, A and B). The increase in Ser21 phosphorylation of GSK-3α was more pronounced in IRS-1 Ala357/358-expressing cells after 10 and 60 min of insulin stimulation compared with cells expressing IRS-1 WT, whereas expression of IRS-1 Glu357 resulted in a clearly decreased phosphorylation (Fig. 4, B and C). Thus, the negative effect of Ser357 phosphorylation on insulin signaling could also be demonstrated at the level of GSK-3. Although both isoforms α and β of GSK-3 were present in C2C12 cells (data not shown), we observed insulin-induced phosphorylation only at Ser21 of GSK-3α.
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**Effect of Ser\(^{357}\) phosphorylation on insulin-stimulated Tyr phosphorylation of IRS-1**

A and B: C2C12 cells were transfected with IRS-1 WT, IRS-1 Ala\(^{357/358}\), or IRS-1 Glu\(^{357}\). The cells were incubated with 10 nM insulin (ins) for the indicated times (0, 5, 10, and 60 min). IRS-1 was immunoprecipitated with a polyclonal IRS-1 antibody (IP) and immunoblotted (IB) with the phosphotyrosine antibody. The blot was reprobed with a polyclonal IRS-1 antibody. The phosphotyrosine antibody was quantified based on scanning densitometry of immunoblots normalized for IRS-1 (mean ± S.E., n = 3; *p < 0.05 IRS-1 Ala\(^{357/358}\) versus IRS-1 WT; #p < 0.05 IRS-1 Glu\(^{357}\) versus IRS-1 WT).

C: Phosphorylation by IRS-1 Ala357 and Ala357/358 enhanced the phosphorylation of this site after insulin stimulation (Fig. 6C). These data suggest that phosphorylation of Ser\(^{357}\), but not of Ser\(^{358}\), is involved in the attenuation of insulin signaling. To provide further evidence for this interpretation, the effect of IRS-1 Glu\(^{357}\) and IRS-1 Glu\(^{357/358}\)-Ala\(^{358}\) were compared. The IRS-1 mutant Glu\(^{357}\).Ala\(^{358}\) excludes the possible influence of a phosphorylated Ser\(^{358}\). The expression of both IRS-1 Glu\(^{357}\) and IRS-Glu\(^{357/358}\)-Ala\(^{358}\) clearly reduced the insulin-stimulated phosphorylation of Ser\(^{21}\) of GSK-3, the tyrosine phosphorylation of IRS-1, and of Ser\(^{308}\) of Akt compared with IRS-1 WT-expressing cells without significant differences of the mutants (Fig. 7). These data clearly underline the functional relevance of phosphorylation of Ser\(^{357}\) of IRS-1 on insulin action.

Inhibition of the PKC-δ-induced Down-regulation of Akt Phosphorylation by IRS-1 Ala\(^{357/358}\) — Activation of PKC isoforms leads to enhanced Ser/Thr phosphorylation of IRS-1 and thus is implicated in impaired insulin signal transduction. To illustrate this concept further, we focused on the inhibitory effect of PKC-δ in downstream insulin signaling, i.e. on Akt phosphorylation and the potential role of Ser\(^{357}\) herein. TPA pretreatment of C2C12 cells expressing PKC-δ and IRS-1 WT led to a clear down-regulation of the insulin-induced phosphorylation of Ser\(^{473}\) of Akt, an effect prevented by co-transfection of PKC-δ KN indicating the inhibitory effect of PKC-δ on insulin action (Fig. 8A). Moreover, co-transfection of IRS-1 Ala\(^{357/358}\) instead of IRS-1 WT could clearly reduce the inhibitory action of PKC-δ on insulin-induced Akt phosphorylation (Fig. 8A). Thus, we conclude that phosphorylation of Ser\(^{357}\) by PKC-δ at least partially mediates the adverse effects of PKC-δ on insulin signaling. We studied also whether the phosphorylation of Ser\(^{357}\) could regulate the association of IRS-1 and PKC-δ. We observed in IRS-1 WT-expressing cells an insulin-stimulated increase in the association of both proteins after 5 and 10 min of stimulation (Fig. 8B), similar to the results obtained in primary mouse skeletal muscle cells (40). The insulin-dependent recruitment of PKC-δ to IRS-1 was significantly enhanced in IRS-1 Glu\(^{357}\) expressing cells after 5 and 10 min of insulin stimulation, whereas the mutation of Ser\(^{357}\) to alanine showed no influence (Fig. 8B). These data suggest that the phosphorylation of Ser\(^{357}\) could be involved in the insulin-me-
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DISSUCION

The family of PKC isoforms represents important modulators of signaling molecules that regulate among other cellular functions the metabolic and mitogenic properties of insulin. All classes of PKCs have been involved in this regulation, and the effects described so far cover the transduction of the positive effect of insulin on glucose uptake and insulin secretion by atypical and novel PKCs (16, 36), the participation in the self-induced attenuation of insulin action by atypical PKCs (21), and the implication in lipid- and hyperglycemia-induced insulin resistance by classical and novel PKCs (17, 24, 33, 46). Data of the novel PKC isoform PKC-δ mirrored this positive and negative modulation of insulin signaling; PKC-δ is shown to be important for insulin-stimulated glucose uptake (36), and it participates in the insulin-dependent activation of Akt (38). On the other hand, we and others have shown that activation of PKC-δ by lipids and leptin is involved in the impairment of insulin signaling (35, 47) and in the induction of apoptosis of insulin-secreting cells (48). Serine phosphorylation of IRS-1 appears to be a major mechanism for the adverse effects of PKC-δ on insulin action (32, 41, 47). Although in vitro at least 18 PKC-δ-dependent phosphorylation sites in IRS-1 have been identified (41), so far only two sites could be demonstrated to be phosphorylated in vivo and to be functional, these are Ser^{24} and Ser^{318} (32, 42, 47). Neither of these sites were shown to be phosphorylated upon insulin stimulation by PKC-δ, although insulin could activate PKC-δ and induce its association to IRS-1 (40).

In the present study we could demonstrate Ser^{357} of IRS-1 as a novel, PKC-δ-dependent phosphorylation site, which is phosphorylated upon insulin stimulation. This site was first identified by an in vitro kinase assay and verified in cell cul-
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Moreover, we could clearly show that the phosphorylation of Ser\textsuperscript{357} is functional and resulted in impaired insulin-stimulated tyrosine phosphorylation of IRS-1, reduced activation of Akt, and subsequently reduced phosphorylation of GSK-3. The mutation of Ser\textsuperscript{358} to alanine, thus representing the human sequence, did not modulate the majority of the studied insulin effects, namely the phosphorylation of Thr\textsuperscript{308} of Akt, phosphorylation of GSK-3, and tyrosine phosphorylation of IRS-1. The phosphorylation of Ser\textsuperscript{373} of Akt was regulated differently, because expression of the single IRS-1 Ala\textsuperscript{358} mutant enhanced the stimulating effect of insulin. Akt, however, requires phosphorylation of both sites, Thr\textsuperscript{308} and Ser\textsuperscript{473}, for full activation (49), which could explain that only the phosphorylation of Ser\textsuperscript{357}, but not of Ser\textsuperscript{358}, attenuates the downstream signaling to GSK-3. Although insulin activates the phosphorylation of both sites of Akt, different kinases are responsible for these phosphorylations, which might be regulated differently. Moreover, the effect of the single IRS-1 Ala\textsuperscript{358} mutant on the phosphorylation of Ser\textsuperscript{473} of Akt is not supported by the gain-of-function mutants IRS-1 Glu\textsuperscript{357} and Glu\textsuperscript{357}-Ala\textsuperscript{358}, because this mutation of Ser\textsuperscript{358} to alanine did not significantly influence the phosphorylation of Ser\textsuperscript{473}. Thus, the data strongly suggest a substantial role of Ser\textsuperscript{357} and indicate that our results are not restricted to rodent IRS-1, because in human IRS-1 Ser\textsuperscript{358} is replaced by alanine.

The results also indicate that the adverse effects of PKC-δ activation on insulin action could be mediated at least partially via phosphorylation of Ser\textsuperscript{357}. This was further demonstrated when we induced the negative effect of PKC-δ by pretreatment of IRS-1 WT- and PKC-δ-overexpressing cells with phorbol ester. We found a strong reduction in insulin-induced phosphorylation of Akt, which was abrogated to the same extend by expression of the kinase dead mutant of PKC-δ or expression of the IRS-1 Ala\textsuperscript{357/358} mutant. Thus, both active PKC-δ and IRS-1 phosphorable on serine 357 are necessary for the observed adverse effects of phorbol ester-mediated PKC activation on insulin signaling.

The mechanism behind this negative modulation could involve the phosphorylation of other serine sites of IRS-1, e.g., the previously published PKC-δ-dependent sites Ser\textsuperscript{24} and Ser\textsuperscript{318}. The phosphorylation of Ser\textsuperscript{357} did not result in the dissociation of PKC-δ and IRS-1, as was reported for PKC-ζ and IRS-1 after phosphorylation of Ser\textsuperscript{318} (22), but even appeared to result in an enhanced recruitment. This could facilitate other serine phosphorylation events on IRS-1 or its receptor, leading to the described reduced tyrosine phosphorylation and attenuation of insulin signaling (41).

Interestingly, the insulin-induced activation of PKC-δ has also been implicated in a positive modulation of insulin action. Overexpression of PKC-δ in mouse skeletal myotubes led to a very rapid increase in tyrosine phosphorylation of the insulin receptors without insulin stimulation, and overexpression of a dominant-negative PKC-δ prevented the insulin-dependent tyrosine phosphorylation of the insulin receptors (39, 40). Moreover, the insulin-dependent activation of PKC-δ was shown to be important for a maximum stimulation of Akt in skeletal muscle cells (38). Thus, insulin alone might not be sufficient to induce the adverse effects of PKC-δ on insulin action.
These effects of PKC-δ had been reported after pharmacological activation using phorbol ester (32), stimulation with leptin (47), or lipid infusion (35). Following this aspect, it must be noted that we observed an insulin-dependent phosphorylation of Ser357 (37), but this effect was weak compared with the phosphorylation intensity after stimulation with phorbol ester. It could be speculated that in the pathophysiological situation of metabolic disturbance with hyperinsulinemia, hyperglycemia, and hyperlipidemia, a more pronounced and sustained activation of PKC-δ occurs that then leads to a significant phosphorylation of Ser357. This phosphorylation could then mediate the desensitizing effect on insulin action in combination with other phosphorylation events. In conclusion we demonstrated here a novel, functional relevant serine residue of IRS-1 that could be involved upon PKC-δ-dependent phosphorylation in the attenuation of insulin signaling in the insulin-resistant state.

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