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Platelet-derived growth factor negatively regulates the insulin-like growth factor signaling pathway through the coordinated action of phosphatidylinositol 3-kinase and protein kinase C beta I

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ABSTRACT

We recently described that epidermal and fibroblast growth factors (EGF and FGF) regulate the IGF-I signaling pathway at the level of IRS-1 through the cooperative action of two independent signaling pathways; one dependent on phosphatidylinositol 3-kinase (PI 3-kinase) and the other on protein kinase D1 (PKD1) (Karam et al. [22]). To determine whether this mechanism could be generalized to another tyrosine kinase receptor-dependent growth factor, the effect of platelet-derived growth factor (PDGF) on the IGF-I signaling pathway was studied. PDGF inhibited IGF-I-stimulated IRS-1 tyrosine phosphorylation and subsequent IGF-I-induced PI 3-kinase activity, and stimulated IRS-1 serine 307 phosphorylation. These effects were mediated through a PI 3-kinase-dependent but extracellular signal-regulated kinase (ERK)-independent signaling pathway. However, PDGF-induced IRS-1 serine 307 phosphorylation was not sufficient *per se* to inhibit the IGF-I signaling but required another independent pathway. Noteworthy, although acutely stimulated by PDGF, and contrary to what we previously described (Karam et al. [22]), PKD1 did not associate with IRS-1and did not inhibit the IGF-I signaling in response to PDGF. However, we identified PKCβI as a new regulatory partner of PI 3-kinase for PDGF-induced inhibition of the IGF-I signaling pathway. Therefore, our results reinforce the idea that a coordinated action of two independent pathways seems absolutely necessary to negatively regulate IRS-1. Moreover, they also demonstrated that, depending of the cross-talk considered, subtle and specific regulatory mechanisms occur at the level of IRS-1 and that a unique regulatory model is not conceivable.

1. Introduction

Insulin-like growth factors (IGF-I and -II) are two mitogenic polypeptides secreted by most tissues that regulate cell proliferation, differentiation and survival by autocrine, paracrine and/or endocrine pathways (for review [21]). Their biological effects are transmitted through their binding to the extracellular α-subunits of the type I IGF receptor (IGF-IR), a widely expressed transmembrane protein with strong structural homology with the insulin receptor (IR) [34]. Binding of IGFs to IGF-IR leads to the conformational change of the receptor and to the autophosphorylation of the intracellular domain of the transmembrane β-subunits. This stimulates the tyrosine kinase activity of the β-subunits which phosphorylate onto tyrosine residues several intracellular targets such as insulin receptor substrate-1 (IRS-1) [31].

As a docking protein, IRS-1 represents a crucial crossroad in the IGF-I signaling pathway. In fact, after being phosphorylated onto 18 potential tyrosine residues [13,36] by the IGF-IR, IRS-1 associates with Src homology 2 (SH2) domain-containing proteins like Shc, Nck, Grb2, Gbr10 and the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). This initiates specific intracellular signaling pathways resulting in the biological effects of the IGFs (for review [35]). Therefore, any modulation of the amount or of the tyrosine phosphorylation state of IRS-1 modulates the IGF-I signaling pathway. Increased IRS-1 expression stimulates cell transformation [19] whereas decreased IRS-1 amount reduces cell viability [27]. Moreover, tyrosine phosphorylation level of IRS-1 can be reduced by tyrosine phosphatase activities [10,26] or modulated by additional serine/threonine phosphorylations mediated by kinases like MAPK, protein kinase C (PKC), protein kinase B, casein kinase II, and PI 3-kinase (for review [12]). Among the 50 potential serine phosphorylation sites of IRS-1, serine 307 residue was more largely studied since it represents an inhibitory signal of IGF-IR- and IR-induced IRS-1 tyrosine phosphorylation and was implicated in insulin resistance [2,5,28].

Abbreviations: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IGF-IR, type 1 IGF receptor; IR, insulin receptor; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PKD1, protein kinase D1

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The crucial role of IGFs in tumor progression [15,17] makes them as attractive targets for the development of innovative therapeutic strategies [30]. However, the efficacy of these approaches requires having a wide understanding of the regulatory mechanisms that occur *in vivo* in a context where each cell is simultaneously subjected to multiple stimulatory and inhibitory signals. With this in view, we recently demonstrated that two growth factors [epidermal growth factor (EGF) and fibroblast growth factor (FGF)] inhibited the IGF-I signaling at the level of IRS-1 through the coordinated action of two independent signaling pathways implicating PI-3 kinase and protein kinase D1 (PKD1) [22]. Due to the increasing role of PKD1 in numerous cell functions (for review [8]), such cross-talk identification leads us to determine whether this regulatory process was generalizable to another tyrosine kinase receptor-dependent signaling pathway or specific to that of EGF and FGF.

We demonstrated that platelet-derived growth factor (PDGF) inhibits the IGF-I-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity. Similarly to EGF and FGF, this inhibition requires the concomitant action of at least two independent partners including PI 3-kinase. However, contrary to EGF or FGF [22], PDGF-activated PKD1 had no role in this regulatory process which involves, however, PKCβI. Therefore, by describing a new regulatory process that specifically occurs at the level of IRS-1 in response of PDGF and which is distinct from that induced by EGF or FGF, our results highlight the specific and complex regulatory networks that take place at the level of IRS-1 in order to modulate the IGF-I signaling pathway. Moreover, we identified PKCβI as a crucial partner that acts in concert with PI 3-kinase to regulate the IGF-I signal transduction efficiency in response to PDGF treatment.

2. Materials and methods

2.1. Antibodies and materials

Anti-phosphotyrosine, anti-Akt1, anti-IRS-1, anti-phospho-ERK1/2 and anti-phospho-IRS-1 (Ser307) antibodies used for immunoblotting were purchased from Upstate Biotechnology-Millipore (Lake Placid, NY). Anti-IRS-1 and anti-p85-subunit of PI 3-kinase antibodies used for PI 3-kinase assay and immunoprecipitation were a kind gift from J-F Tanti (INSERM, Nice, France) or were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) like anti-PKD1, anti-PKCα, anti-PKCβI, anti-α-actinin and anti-actin antibodies. Anti-phospho-PKD1 ($Ser^{744/748}$) and anti-phospho-Akt (Thr^{308} and Ser^{473}) antibodies were from Cell Signaling (Danvers, MA). EGF, bFGF and PDGF were from PeproTech (Rocky Hill, NJ), and IGF-I was from GroPep (Adelaide, Australia). PKD1-targeting (sc-36245), PKCα-targeting (sc-36243), PKCβI-targeting (sc-29450) and control non-targeting (sc-37007) siRNAs were purchased from Santa Cruz Biotechnology. Gö6976, Gö6983 and GF109203X were from Calbiochem (La Jolla, CA) and wortmannin was from Sigma-Aldrich (Saint-Quentin Fallavier, France). Protein A-Sepharose was from GE Healthcare Life Sciences (Orsay, France). All other biochemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France) or ICN (Orsay, France).

2.2. Cell culture

The MCF-7 cell line was grown to 80–85% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Before each experiment, cells were starved in serum free media for 16–24 h.

2.3. Western immunoblotting

MCF-7 cells were cultured as described and treated for different periods of time with or without EGF, FGF or PDGF before being acutely stimulated or not for 5 min with IGF-I. Cells were then solubilized in buffer A (20 mM Tris, pH 7.4, 137 mM NaCl, 100 mM NaF, 10 mM EDTA, 2 mM Na_3VO_4, 10 mM pyrophosphate, 1 mM PMSF, 100 U/mL aprotinin) containing 1% Nonidet P-40 (NP-40), and proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) or nitrocellulose sheets. These were incubated with the specific antibodies for 1 h at room temperature or overnight at 4 °C and revealed by chemiluminescence (ECL, Amersham).

2.4. Immunoprecipitation

Cells were washed with ice-cold buffer A and solubilized as described above. Supernatants from solubilized cells were then immunoprecipitated for 2 h at 4 °C with anti-IRS-1 antibodies preadsorbed to protein A-Sepharose. The immune pellets were washed three times with buffer A, treated with Laemmli buffer, boiled for 10 min, and separated by 7.5% acrylamide SDS-PAGE. Proteins were then transferred to nitrocellulose sheets and immunoblotted with anti-PKD1 and anti-IRS-1 antibodies as described above.

2.5. Determination of PI 3-kinase activity

Lysates from cells treated as above were incubated for 2 h at 4 °C with anti-IRS-1 or anti-p85 antibodies coupled to protein A-Sepharose beads. Thereafter, immune pellets were successively washed twice with each of the three following buffers: (a) PBS containing 1% Nonidet P-40 and 200 μM Na_3VO_4; (b) 100 mM Tris pH 7.4, 0.5 M LiCl, and 200 μM Na_3VO_4; and (c) 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, and 200 μM Na_3VO_4. Immunoprecipitated PI 3-kinase activity was measured in the immune pellets by *in vitro* phosphorylation of phosphatidylinositol (PI) [9,16]. The reaction products were separated by thin layer chromatography on silica plates in methanol/chloroform/ammoniac buffer. After autoradiography, PI 3-kinase activity was quantified by Cerenkov analysis of the spots corresponding to PI 3-P.

2.6. siRNA transfection

siRNA transfection was done according to manufacturer's protocol. Briefly, 2.5–3 × 10^5 cells were seeded per well in 2 mL antibiotic-free DMEM supplemented with 10% FBS and incubated for 24 h. For each well, 1 μg of siRNA (siPKD1, siPKCα or siPKCβI) and 8 μL of siRNA transfection reagent, each diluted in 100 μL siRNA transfection medium, were combined, incubated for 45 min at room temperature and then applied to the cells in a final volume of 1 mL siRNA transfection medium. After 7 h of incubation at 37 °C, DMEM supplemented with 10% FBS was added and cells were cultured for an additional 18–24 h at 37 °C before analysis.

3. Results

3.1. PDGF inhibits the IGF-I-induced tyrosine phosphorylation of IRS-1 in MCF-7 cells

We previously showed that EGF and FGF modulate the IGF-I signaling pathway through the concomitant action of a PKD1- and a PI 3-kinase-dependent signaling pathways [22]. Therefore, we searched to determine whether this mechanism could be generalized to other tyrosine kinase receptor dependent-growth factors such as PDGF. Thus, MCF-7 cells were pretreated or not with PDGF for different periods of time (5 to 60 min) before being acutely stimulated for 5 min with IGF-I. The cells were lysed and the tyrosine phosphorylation state of IRS-1 was analyzed. In unstimulated cells, tyrosine phosphorylation of IRS-1 was not detectable but increased dramatically after 5 min of IGF-I stimulation (Fig. 1A). Whatever the preincubation time, PDGF pretreatment markedly inhibited the IGF-I-induced IRS-1 tyrosine phosphorylation. This inhibition occurred

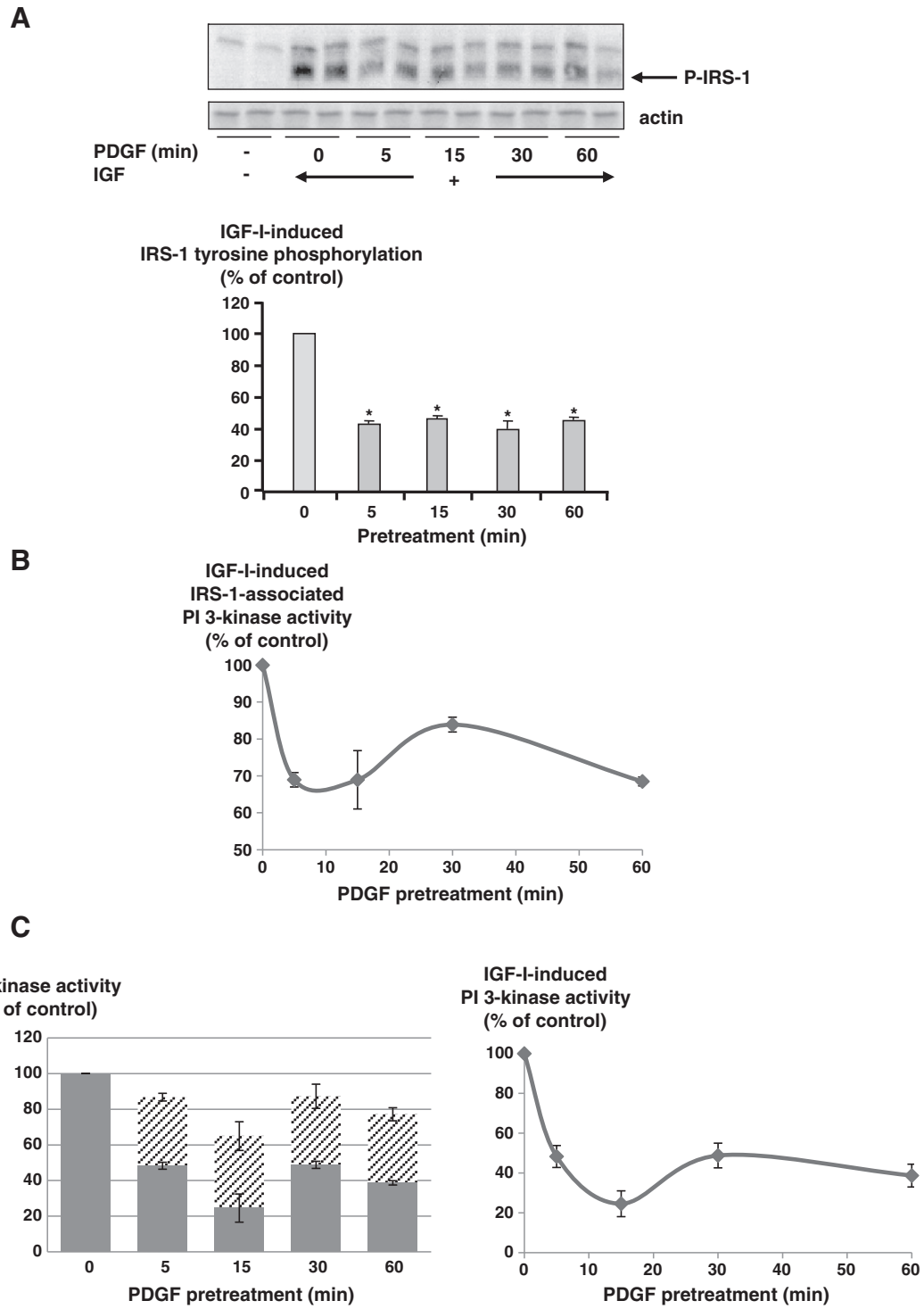

Fig. 1. PDGF pretreatment inhibits IGF-I-induced IRS-1 tyrosine phosphorylation and IGF-I-stimulated PI 3-kinase activity. Cells were pre-incubated for different periods of time (5–60 min) with PDGF (50 ng/mL) and then acutely stimulated for 5 min with IGF-I (22.5 ng/mL). A. Cells were lysed and proteins were separated by SDS-PAGE, transferred to PVDF sheets and immunoblotted with anti-phosphotyrosine or anti-actin antibodies as described in Materials and methods. The autoradiograms presented are those of typical experiments conducted in duplicate. Histogram represents quantitative analysis of total tyrosine phosphorylated IRS-1 under each set of conditions, corrected for background, and expressed as percentages of phosphotyrosylated IRS-1 measured after 5 min of IGF-I stimulation without any pretreatment with PDGF (control). Results are the means ± SEM for three independent experiments (*, $P < 0.01$). B. Cells were lysed and proteins were immunoprecipitated with anti-IRS-1 antibodies. The immune pellets were used to measure PI 3-kinase activity as described under Materials and methods. Results are the means ± SEM for four independent experiments. The results are expressed as percentages of the activity measured after 5 min of IGF-I stimulation without any pretreatment with PDGF (control). C. Same experiment as before except that PI 3-kinase activity was measured in immunoprecipitates from anti-p85 subunit of PI-3 kinase antibody. Histogram (left) presents the respective part of PDGF- (hatched symbol) and IGF-I-stimulated (plain symbol) PI 3-kinase activity. Graph (right) presents only the latter. Results are the means ± SEM for four independent experiments. The results are expressed as percentages of the activity measured after 5 min of IGF-I stimulation without any pretreatment with PDGF (control).

rapidly (as soon as 5 min of PDGF pretreatment) and remained mostly stable (around 55–60%) for at least 60 min. PDGF-mediated inhibition of IGF-I-induced IRS-1 tyrosine phosphorylation was not a consequence of a decreased IRS-1 protein content [23] nor of a reduced IGF-I-induced autophosphorylation of the IGF-IR β-subunits (data not shown).

3.2. PDGF modulates the IGF-I-induced PI 3-kinase activity in MCF-7 cells

Since IGF-I-stimulated PI 3-kinase activity is dependent upon the tyrosine phosphorylation state of IRS-1, we asked whether PDGF pretreatment affects subsequent IGF-I-induced PI 3-kinase activity. MCF-7 cells were pretreated or not with PDGF for different periods of time (5 to 60 min) and then acutely stimulated for 5 min with IGF-I. Cells were lysed, proteins were solubilized and PI 3-kinase activity was measured in immunoprecipitates obtained with antibodies directed against either IRS-1 (Fig. 1B) or the p85 regulatory subunit of PI 3-kinase (Fig. 1C). IGF-I strongly stimulated IRS-1-associated (52.6 ± 7.02-fold increase; n = 4) and total (5.27 ± 0.42-fold increase; n = 4) PI 3-kinase activities. PDGF pretreatment modulated with characteristic and very similar triphasic time courses IRS-1-associated (Fig. 1B) and total (Fig. 1C) PI 3-kinase activities stimulated by IGF-I. Indeed, these activities were first rapidly inhibited by PDGF pretreatment, with maximal effect (approximately 33% and 75% inhibition for IRS-1-associated and total PI 3-kinase activities, respectively) obtained after 5–15 min of pretreatment, then this inhibition decreased after 30 min of PDGF pretreatment before increasing again after 60 min (30% and 60% inhibition, respectively).

3.3. PDGF inhibits the IGF-I-induced IRS-1 tyrosine phosphorylation by a PI 3-kinase dependent but ERK-independent signaling pathway

In MCF-7 cells, two major signaling pathways (*i.e.* PI 3-kinase and extracellular-regulated kinase (ERK) signaling pathways) are commonly activated after tyrosine kinase receptor stimulation. However, in these cells, although PDGF strongly stimulated PI 3-kinase activity (maximal effect, 2.15-fold, observed after 5 min and remained stable for at least 60 min) (Fig. 2A), this growth factor had, as previously shown [22,23], and contrary to EGF and FGF, no effect on ERK activity (Fig. 2B). This last result was confirmed whatever the PDGF incubation period considered (data not shown).

Since the PI 3-kinase signaling pathway remains the main pathway to be activated in response to PDGF, we determine whether it could mediate the PDGF-induced inhibition of the IGF-I-stimulated IRS-1 tyrosine phosphorylation. Thus, cells were incubated with or without wortmannin, a specific inhibitor of PI 3-kinase, then pretreated or not with PDGF for different periods of time before being acutely stimulated for 5 min with IGF-I and analyzed for the tyrosine phosphorylation state of IRS-1. Tyrosine phosphorylation of IRS-1, that was barely detectable in basal conditions (untreated cells), was strongly stimulated by IGF-I and not affected by wortmannin preincubation (Fig. 3A). Noteworthy, PDGF-induced inhibition of the IGF-I-stimulated IRS-1 tyrosine phosphorylation was strongly impaired by wortmannin (Fig. 3A). The phosphorylation state analysis of Akt (a downstream target of PI 3-kinase) onto two activating phosphorylated residues (T308 and S473) confirmed the specific inhibitory action of wortmannin on the PI 3-kinase signaling pathway (Fig. 3B).

Therefore, taken all together, our results demonstrate that PDGF inhibits the IGF-I-induced tyrosine phosphorylation of IRS-1 by a PI 3-kinase-dependent and ERK-independent signaling pathway.

3.4. PDGF stimulates serine 307 phosphorylation of IRS-1 by a PI 3-kinase-dependent signaling pathway

To further characterize how PDGF modulates the IGF-I signaling pathway at the level of IRS-1, MCF-7 cells were stimulated with PDGF for different periods of time (5–60 min) before analysis of IRS-1 phosphorylation onto S307 residue. As shown in Fig. 4A, PDGF stimulated the phosphorylation of IRS-1 onto serine 307. This phosphorylation was rapid (significantly occurring after 5 min stimulation), reached maximal values after 15 min of treatment, and remained elevated for at least 1 h. PDGF-stimulated serine 307 phosphorylation of IRS-1 was totally impaired by wortmannin (Fig. 4B) demonstrating

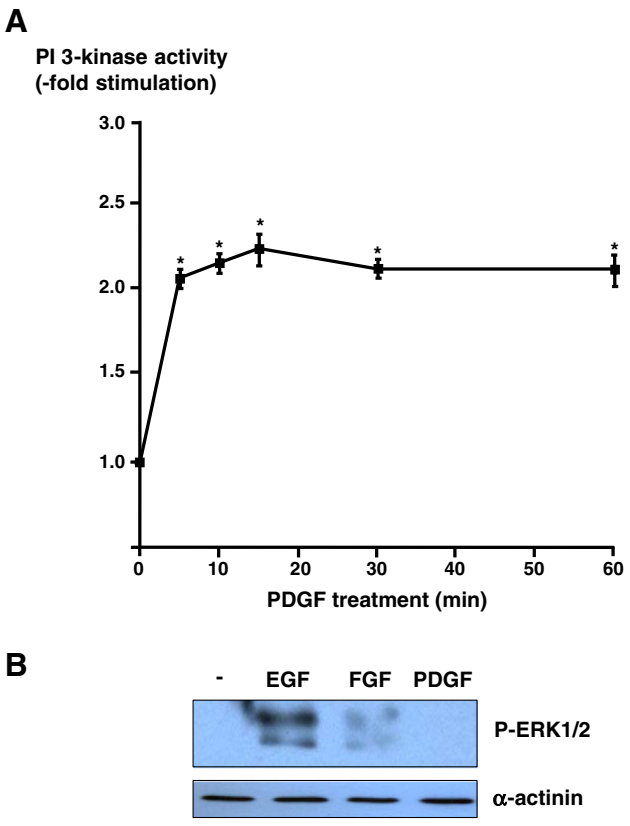

Fig. 2. PDGF stimulates PI 3-kinase activity but not ERK in MCF-7 cells. A. Cells were stimulated or not with PDGF (50 ng/mL) for different periods of time (5–60 min), then homogenized and proteins were immunoprecipitated with an antibody directed against the p85 subunit of PI 3-kinase. PI 3-kinase assay was performed on the immune pellets as described under Materials and methods. Results are expressed relative to basal activity measured in non-stimulated cells and are the means ± SEM for three independent experiments (*, $P < 0.01$). B. Cells were stimulated or not with EGF (40 ng/mL), FGF (5 ng/mL) or PDGF (50 ng/mL) for 15 min. Then, cells were lysed and proteins were separated by SDS-PAGE, transferred to PVDF sheets and immunoblotted with anti-phospho-ERK1/2 (P-ERK1/2) or anti-α-actinin antibodies as described in Materials and methods. The autoradiograms presented are those of typical experiments.

that PDGF-induced serine 307 phosphorylation of IRS-1 is mediated by a PI 3-kinase-dependent signaling pathway.

3.5. PDGF activates PKD1 by a PI 3-kinase-independent signaling pathway

In response to EGF or FGF, PI 3-kinase acts in concert with PKD1 which associates with IRS-1 to negatively regulate this docking protein [22]. We therefore asked whether PDGF may regulate the IGF-I signaling by the same mechanism. PDGF induced the activating phosphorylation of PKD1 in MCF-7 cells (Fig. 5A and B). This occurred, as previously shown for EGF and FGF [22], by a PI 3-kinase independent signaling pathway since PDGF-induced PKD1 phosphorylation was not affected by wortmannin (Fig. 5A). Maximal phosphorylation occurred after 15 min of PDGF treatment and remained stable up to 60 min (Fig. 5A). As expected, PDGF-stimulated PKD1 phosphorylation was totally impaired by Gö6976 but not by GF109203X and Gö6983 (Fig. 5B), confirming the inhibitory spectra of these three compounds, Gö6976 (inhibitor of PKCα, βI, and μ/PKD1), Gö6983 (inhibitor of PKCα, β, γ, δ, and ζ), and GF109203X (inhibitor of PKCα, βI, βII, γ, δ, and ε) [11,24].

3.6. PDGF does not induce the association of PKD1 with IRS-1 and inhibits the IGF-I signaling pathway independently of PKD1

Since PDGF activated PKD1 similarly to EGF and FGF [22], we asked whether this kinase may be implicated in the PDGF-induced

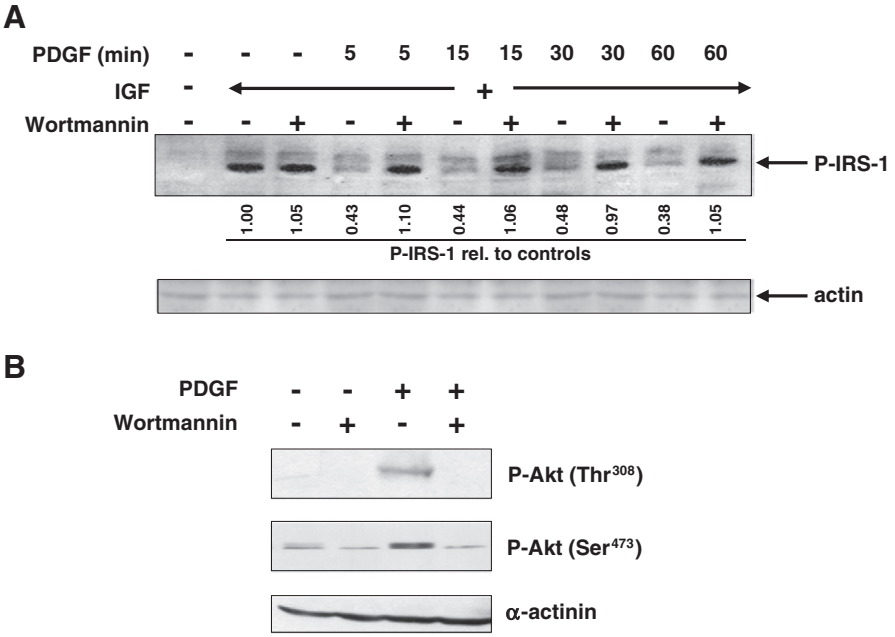

Fig. 3. PDGF inhibits the IGF-I-induced IRS-1 tyrosine phosphorylation by a PI 3-kinase-dependent signaling pathway. A. MCF-7 cells were pre-incubated with or without wortmannin (100 nM) for 20 min as indicated and then pretreated or not with PDGF (50 ng/mL) for different periods of time (5–60 min) prior to being stimulated with IGF-I (22.5 ng/mL) for 5 min. Cells were lysed and proteins were separated by SDS-PAGE, transferred to PVDF sheets and immunoblotted with anti-phosphotyrosine or anti-actin antibodies as described in Materials and methods. The autoradiograms presented are those of typical experiments. Values, presented under the autoradiogram, represent quantitative analysis from three independent experiments of total tyrosine phosphorylated IRS-1 under each set of conditions, corrected for background, and expressed relative to phosphotyrosylated IRS-1 measured after 5 min of IGF-I stimulation without any pretreatment with PDGF (1.00). B. MCF-7 cells were pre-incubated with or without wortmannin (100 nM) for 20 min as indicated and then stimulated or not with PDGF (50 ng/mL) for 30 min. Cells were lysed, and proteins were separated by SDS-PAGE, transferred to nitrocellulose sheets and immunoblotted with two distinct anti-phospho-Akt (P-Akt (Thr308) and P-Akt (Ser473)) or anti-α-actinin antibodies. The autoradiograms presented are those of typical experiments.

inhibition of the IGF-I signaling pathway. Interestingly, unlike EGF and FGF and whatever the incubation time tested (5–60 min), PDGF did not induce PKD1 association with IRS-1 (Fig. 5C). Moreover, specific inhibition of PKD1, performed by transfecting cells with specific PKD1-targeting (siPKD1) siRNA, showed that although PKD1 expression was strongly impaired compared to control non-targeting (siControl) siRNA transfected cells (Fig. 5D, left panel), PDGF-induced inhibition of the IGF-I-stimulated phosphorylation of IRS-1 (Fig. 5D, middle panel)

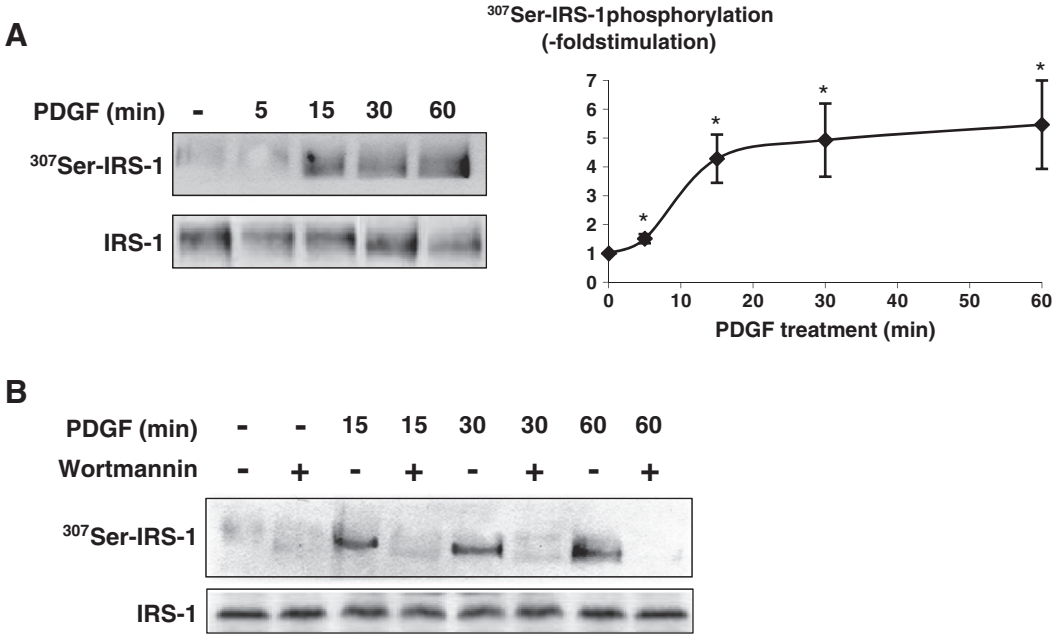

Fig. 4. PDGF induces Ser307-phosphorylation of IRS-1 by a PI 3-kinase-dependent signaling pathway. A. MCF-7 cells were stimulated or not for different periods of time (5–60 min) with PDGF (50 ng/mL) as indicated. Cells were lysed and proteins were immunoprecipitated with anti-IRS-1 antibodies. Immunoprecipitated proteins were then separated by SDS-PAGE, transferred to nitrocellulose and immunodetected with anti-IRS-1 or anti-phospho-IRS-1 (Ser307) antibody as described in Materials and methods. Autoradiograms presented are those of typical experiments. Graphs represent quantitative analysis of Ser307-phosphorylated IRS-1 under each set of conditions, corrected for background, and expressed relative to untreated cells (control). Results are the means ± SEM for four independent experiments (*, $P < 0.01$). B. Same experiment as described above except that in some conditions cells were preincubated with wortmannin (100 nM) for 20 min prior to being stimulated with PDGF (50 ng/mL) for different periods of time as indicated. Autoradiograms presented are those of typical experiments.

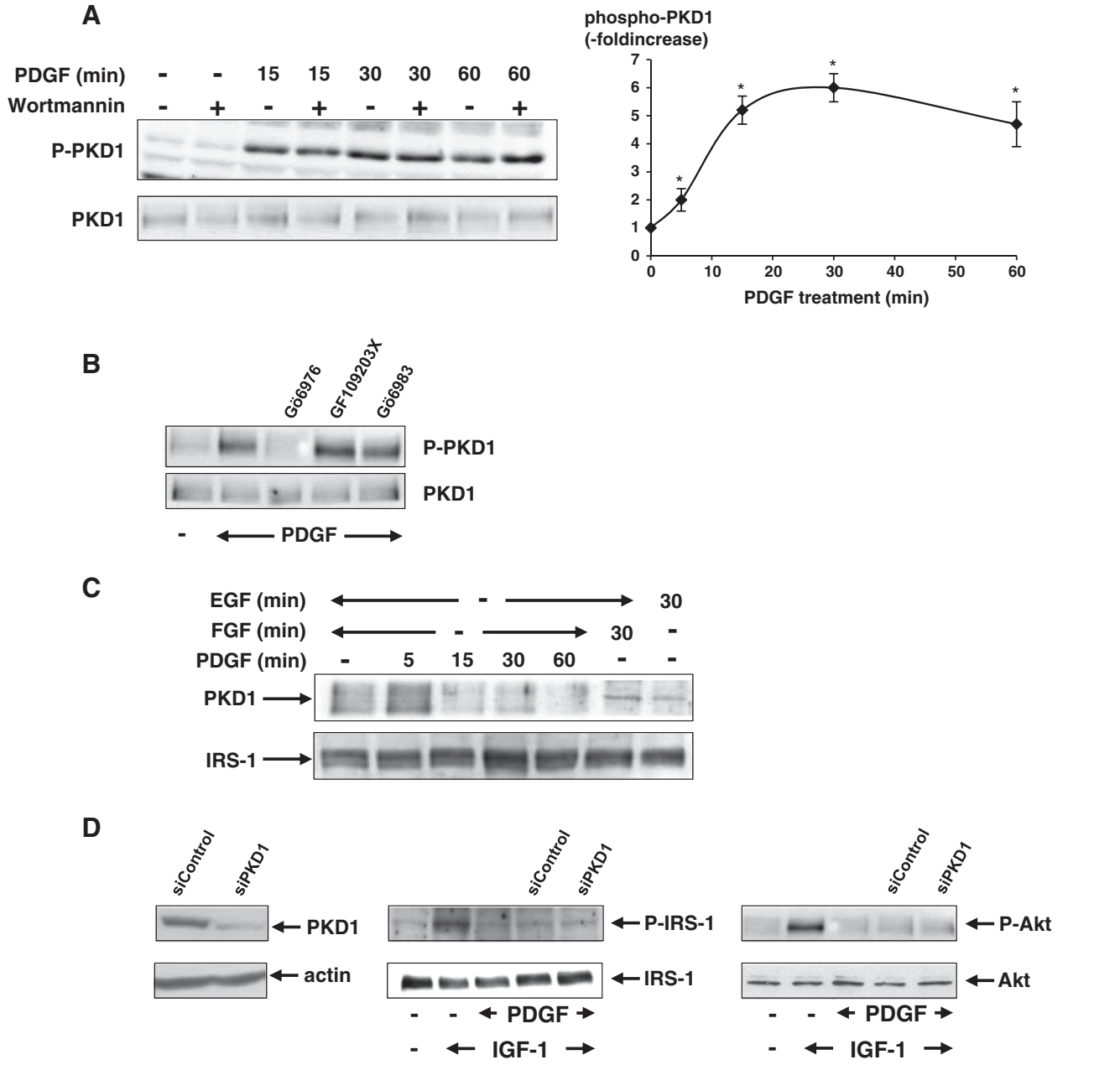

Fig. 5. PKD1 is activated by PDGF through a PI 3-kinase-independent signaling pathway but is not implicated in the PDGF-induced negative regulation of the IGF-I signaling pathway. A. MCF-7 cells were pre-incubated with or without wortmannin (100 nM) for 20 min and then stimulated or not for different periods of time (15–60 min) with PDGF (50 ng/mL). Cells were lysed and proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phospho-PKD1 or anti-PKD1 antibodies. Autoradiograms presented are those of typical experiments. Graph represents quantitative analysis of phospho-PKD1 under each set of conditions, corrected for background, and expressed relative to untreated cells (control). Results are the means ± SEM for four independent experiments (*, $P < 0.01$). B. MCF-7 cells were pre-incubated with or without Gö6983 (5 μM), Gö6976 (2.5 μM) or GF109203X (2.5 μM) for 1 h. Then, cells were incubated for 30 min with or without PDGF (50 ng/mL). Cells were lysed and proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phospho-PKD1 or anti-PKD1 antibodies. Autoradiograms presented are those of typical experiments. C. MCF-7 cells were incubated for different periods of time (5–60 min) with or without PDGF (50 ng/mL) or for 30 min with EGF (40 ng/mL) or FGF (5 ng/mL). Cells were lysed and proteins immunoprecipitated with anti-IRS-1 antibodies were separated by SDS-PAGE, transferred to nitrocellulose and then immunodetected with anti-PKD1 or anti-IRS-1 antibodies. Autoradiograms presented are those of typical experiments. D. MCF-7 cells were transfected with either PKD1-targeting (siPKD1) or control non-targeting (siControl) siRNA. Three days after transfection, cells were pretreated for 15 min with or without PDGF (50 ng/mL) prior to being stimulated with IGF-I (22.5 ng/mL) for 5 min. Then, cells were lysed and proteins were separated by SDS-PAGE, transferred to PVDF sheets and immunoblotted with anti PKD1, anti-actin, anti-phosphotyrosine, anti-IRS-1, anti-phospho-Akt (Thr308) or anti-Akt antibodies as described in Materials and methods. Autoradiograms presented are those of typical experiments conducted in triplicate.

and Akt (Fig. 5D, right panel) remained unchanged. This result indicates that PDGF inhibited the IGF-I signaling independently of PKD1. Taken together, these results demonstrated that, although activated by PDGF, a tyrosine kinase receptor-dependent ligand such as EGF and FGF, PKD1 does not systematically associate with IRS-1 and regulate the IGF-I signaling pathway, which therefore puts in evidence the existence of subtle and specific regulatory mechanisms.

3.7. PDGF inhibits the IGF-I-stimulated IRS-1 tyrosine phosphorylation and the IGF-I-stimulated PI 3-kinase activity by PKC-dependent signaling pathway

To further address and determine the molecular mechanisms involved in the regulation of the IGF-I signaling in response to PDGF and since PKC family is usually implicated in the regulation of tyrosine

kinase receptor-dependent signaling, the inhibitory effect of PDGF on IGF-I-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity was analyzed in the presence of PKC inhibitors. Cells were incubated for 1 h with or without one of the three PKC inhibitors, then pretreated with PDGF for 15 min before being acutely stimulated for 5 min with IGF-I. As previously described (Fig. 1), IGF-I-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity were inhibited by PDGF pretreatment (Fig. 6A and B). Such inhibitions were totally impaired by all three inhibitors used (Fig. 6A and B), and were specific since none of the three inhibitors affected basal and IGF-I-stimulated IRS-1 tyrosine phosphorylation state [22] or IGF-I-stimulated PI 3-kinase activity (Fig. 6B) [22]. Similar results were obtained for total PI 3-kinase activity measured in anti-p85 immunoprecipitates (data not shown). Noteworthy, whatever the PKC inhibitor used, PDGF-induced serine 307 phosphorylation of IRS-1 was unchanged (Fig. 6C). Taken together, these results demonstrated that PDGF-induced inhibition of the IGF-I signaling occurred by a PKC-dependent signaling pathway but that the serine 307

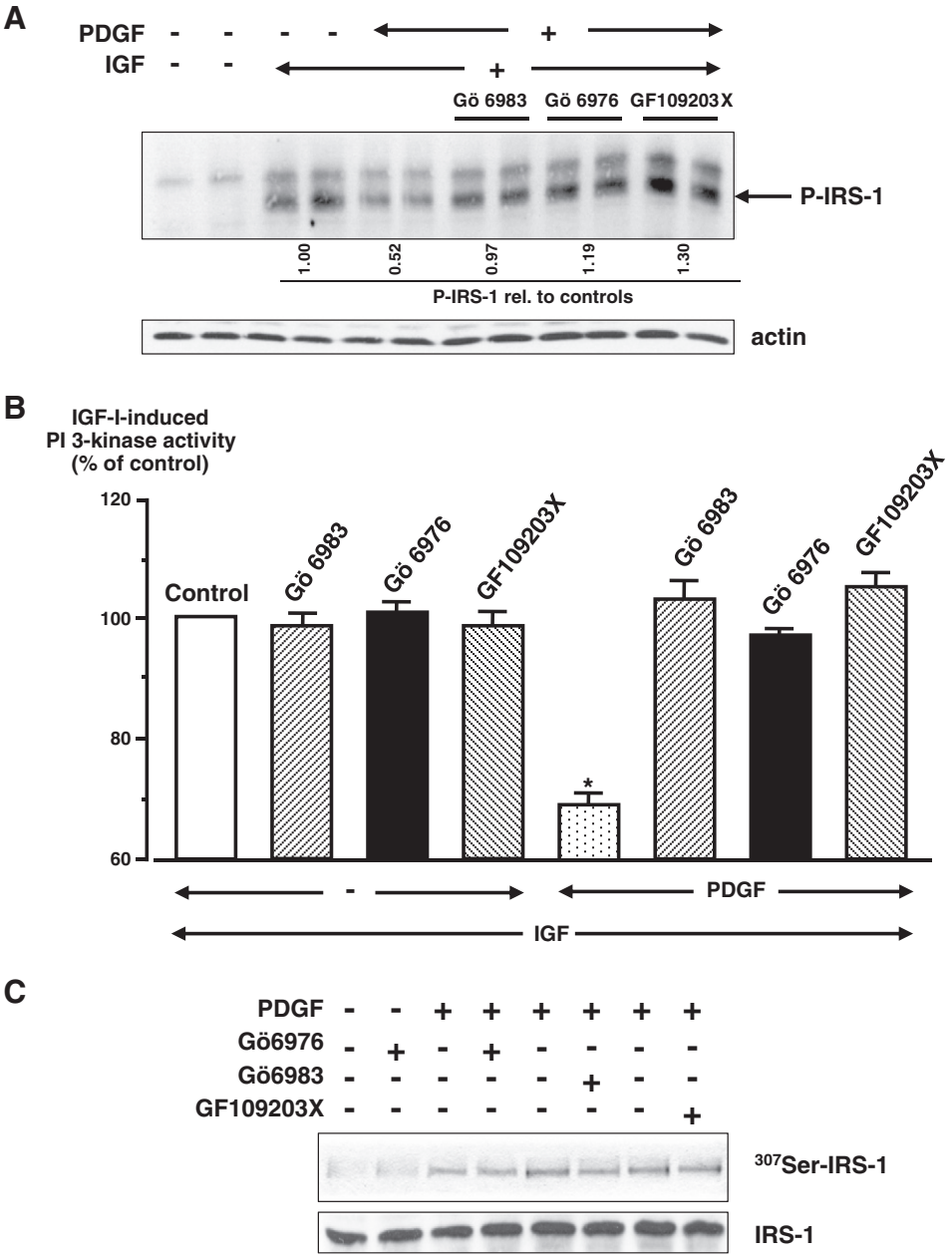

Fig. 6. PDGF inhibits the IGF-I-induced IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity by a PKC-dependent signaling pathway. A. MCF-7 cells were pre-incubated with or without Gö6983 (5 μM), Gö6976 (2.5 μM) or GF109203X (2.5 μM) for 1 h. Cells were then pretreated for 15 min with or without PDGF (50 ng/mL) prior to being stimulated with IGF-I (22.5 ng/mL) for 5 min. Cells were then lysed and proteins were separated by SDS-PAGE, transferred to PVDF sheets and immunoblotted with anti-phosphotyrosine or anti-actin antibodies as described in Materials and methods. Autoradiograms presented are those of typical experiments conducted in duplicate. Values, presented under the autoradiogram, represent quantitative analysis from three independent experiments of total tyrosine phosphorylated IRS-1 under each set of conditions, corrected for background, and expressed relative to phosphotyrosylated IRS-1 measured after 5 min of IGF-I stimulation without any pretreatment with PDGF. B. Same experimental conditions as in A. After cell lysis, proteins were immunoprecipitated with antibodies directed against IRS-1. PI 3-kinase assay was performed on the immune pellets as described under Materials and methods. Results are expressed as percentages of the activity measured after 5 min of IGF-I stimulation without any pretreatment (control). Results are the means ± SEM for three independent experiments (*, $P < 0.01$). C. After 1 h incubation with or without inhibitors as indicated in A, cells were stimulated for 30 min with or without PDGF (50 ng/mL) before being lysed and proteins were immunoprecipitated with anti-IRS-1 antibodies. Immunoprecipited proteins were then separated by SDS-PAGE, transferred to nitrocellulose and immunodetected with anti-IRS-1 or anti-phospho-IRS-1 (Ser307) antibody as described in Materials and methods. Autoradiograms presented are those of typical experiments.

phosphorylation of IRS-1 occurred by a PI 3-kinase-dependent (Fig. 4B) but a PKC-independent signaling pathway.

3.8. PDGF inhibits the IGF-I-signaling by a PKC beta I-dependent signaling pathway

According to the inhibitory spectra of the three inhibitors used, two PKC isoforms, PKCα and PKCβI, were suspected to be implicated in the PDGF-induced inhibition of the IGF-I signaling pathway. In order to determine whether one or both of these two proteins played a role in the regulation of the IGF-I signaling, inhibition of PKCα and PKCβI was performed by transfecting cells with specific PKCα-(siPKCα) and PKCβI-(siPKCβI) targeting or control non-targeting (siControl) siRNAs. As shown in Fig. 7, PKCα and PKCβI expression was unchanged after transfection of MCF-7 cells with siControl but very efficiently and specifically inhibited with siPKCα and siPKCβI (about 85% and 81%, respectively, n = 3). Whereas siControl and siPKCα had no significant effect, siPKCβI transfection profoundly reduced PDGF-induced inhibition of the IGF-I-stimulated IRS-1 tyrosine phosphorylation and Akt serine 473 phosphorylation. Noteworthy, and as may be expected, whatever the siRNA transfection condition, PDGF-induced IRS-1 serine 307 phosphorylation was unchanged (data not shown). Taken together, these results demonstrated that PDGF inhibits the IGF-I signaling pathway through PKCβI.

4. Discussion

By regulating fundamental biological processes such as proliferation, survival and metabolism, RTK signaling pathways are attractive targets for therapies against major diseases such as cancer and diabetes. However, each receptor-dependent signaling pathway is submitted to a plethora of regulatory mechanisms that play a crucial role in signal transduction efficiency and specificity. Therefore, the identification of these regulatory mechanisms appears essential to further develop more pertinent and safer pharmaceutical strategies. In the present study, we identify a new regulatory cross-talk between two tyrosine kinase receptor-dependent signaling pathways, PDGF and IGF-I. We showed that PDGF negatively modulated the IGF-I signaling pathway by inhibiting the IGF-I-induced IRS-1 tyrosine phosphorylation through PI 3-kinase- and PKCβI-dependent and PKD1-independent signaling pathways. PI 3-kinase was previously demonstrated as a negative regulator of several tyrosine kinase receptor-dependent pathways such as IGF-I [22] or insulin [29] thus appearing as a major regulatory actor in signal transduction. In the *in vitro* cross-talk context, this enzyme acts in a dual way, either as a positive mediator of its upstream tyrosine kinase receptor-dependent stimulatory pathways [20] or as a negative regulator of its target tyrosine kinase receptor-dependent signaling pathways [6]. Therefore, this dual role may allow PI 3-kinase to promote its activating (*i.e.* first in time) pathway to the disadvantage of the others. Nevertheless, in an *in vivo* context, the initiator signal concept is more difficult to assess since a cell is constantly subjected to multiple afferents. In this case, the predominant pathway could be determined by the local ligand concentration or the receptor expression level. Since MCF-7 cells express different PI 3-kinase isoforms, another hypothesis would be that this dual role could be fulfilled by the specific action of different members of this family. However, this hypothesis seems unlikely since similar results as those obtained

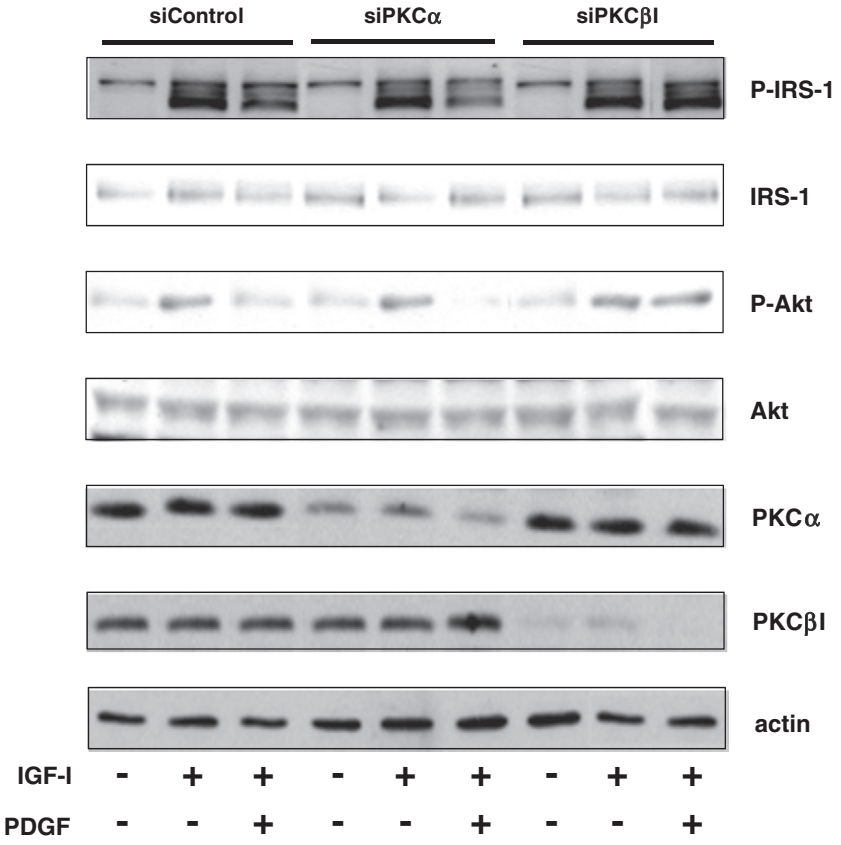

Fig. 7. PKCβI mediates the PDGF-induced inhibition of the IGF-I signaling pathway. MCF-7 cells were transfected with either PKCα- (siPKCα) or PKCβI- (siPKCβI) targeting siRNAs or control non-targeting siRNAs (siControl). Three days after transfection, cells were pretreated for 15 min with or without PDGF (50 ng/mL) prior to being stimulated with IGF-I (22.5 ng/mL) for 5 min. Then, cells were lysed and proteins were separated by SDS-PAGE, transferred to PVDF sheets and immunoblotted with anti-phosphotyrosine, anti-IRS-1, anti-phospho-Akt (Ser473), anti-Akt, anti-PKCα, anti-PKCβI or anti-actin antibodies as described in Materials and methods. Autoradiograms presented are those of typical experiments conducted in triplicate.

with wortmannin were achieved with inhibitors that specifically target PI 3-kinase isoforms (data not shown).

PDGF induced serine 307 phosphorylation of IRS-1 by a PI 3-kinase-dependent signaling pathway (Fig. 4). Once phosphorylated, this residue was described to exert an inhibitory action on IGF-I- or insulin-induced IRS-1 tyrosine phosphorylation therefore playing a role in insulin resistant states [1–3,28]. Nevertheless, we showed that this PDGF-induced IRS-1 serine 307 phosphorylation is not sufficient *per se* to block the IGF-I signaling since IGF-I-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity were not affected by PDGF treatment when cells were cultured in the presence of PKC inhibitors (Fig. 6). This indicates that PI 3-kinase, which mediates IRS-1 serine 307 phosphorylation, needs the cooperative action of at least another independent pathway to inhibit the IGF-I one at the level of IRS-1. We previously showed that, when stimulated with EGF or FGF, PI 3-kinase cooperates with PKD1 to inhibit the IGF-I signaling pathway [22]. However, a feature of our work was to show that, although being strongly activated by PDGF in MCF-7 cells, and contrary to what might be suspected, PKD1 is not involved in PDGF-induced inhibition of the IGF-I signaling and therefore is not, in this specific case, the cooperative partner of PI 3-kinase. This result could be due to the lack of association between IRS-1 and PKD1 in response to PDGF thereby further strengthening our hypothesis that an association between PKD1 and IRS-1 is crucial for EGF- and FGF-induced IRS-1 inhibition [22]. Another argument would reside in the kinetics of activation of PKD1 which are not absolutely identical in response to these three growth factors. In fact, PKD1 was more rapidly activated (maximal effect observed after 15 min) by PDGF than by EGF or FGF (maximal PKD1 activity measured after 30 min) [22]. Such small but significant difference may allow PKD1 to interact with distinct intracellular partners permitting or not, according to its steric environment or its subcellular sequestration, its association with IRS-1. Therefore, taken all together, these new results, obtained in the context of the cross-talk between the PDGF and the IGF-I signaling pathways, highlight the complexity and specificity of the regulations that take place between signaling pathways and demonstrate that a general regulatory model is not always systematically applicable.

Instead of PKD1, PKCβI was identified to cooperate with PI 3-kinase and inhibit the IGF-I signaling in response to PDGF. PKCβ was recently described as a main regulator of physiological processes such as proliferation, apoptosis and metabolism induced by several factors [7,14,32]. Moreover, this PKC isoform seems to play a crucial role in obesity and insulin resistance [18,25,33]. Given the strong similarities between the insulin and the IGF-I signaling, it is therefore conceivable that PKCβI modulates, by a similar way, key proteins of these two pathways, such as IRS-1, leading to a blockade of downstream signals. In the context of the IGF-I signaling, PKCβI would therefore exert a negative control of cell proliferation. Contrary to PKD1 that associates with IRS-1 in response to EGF and FGF [22], we observed no association between PKCβI and IRS-1 (data not shown). This suggests that either PKCβI acts indirectly on the IGF-I signaling pathway at the level of IRS-1 through one of its downstream targets or that the association between PKCβI and IRS-1 is too fleeting or occurs in too small amount to be detected by a co-immunoprecipitation approach.

The specificity of the recruited proteins that inhibit the IGF-I signaling pathway together with PI 3-kinase (*i.e.* PKD1 or PKCβI) may be the consequence of the specific pathways activated downstream of each tyrosine kinase receptor. Thus, whereas EGF and FGF activated both the PI 3-kinase and the MEK/ERK signaling pathways in MCF-7 cells, PDGF only activated the first one (Fig. 2B and [23]). Moreover, as discussed above for PKD1, peculiar enzyme activation time courses may also participate to this specificity. Thus, while EGF and FGF transiently stimulated PI 3-kinase [22], PDGF ensured its prolonged and sustained activation. Such differences may be determinant for the recruitment, stimulation and subcellular localization of specific intracellular partners and may implement particular signaling pathways.

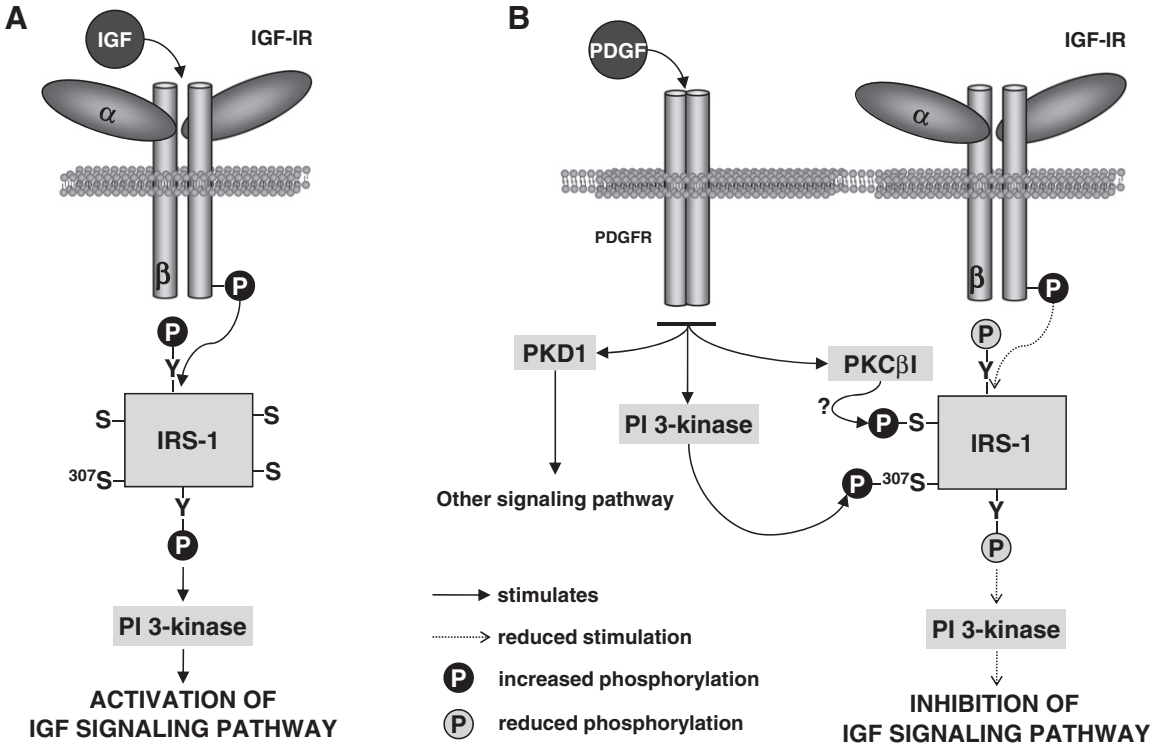

Fig. 8. Schematic model of the regulation of the IGF signaling pathway induced by PDGF. Panel A: Upon IGF binding, IGF-IR is activated and phosphorylates IRS-1 onto tyrosine (Y) residues generating binding sites for docking proteins as PI 3-kinase. Active PI 3-kinase mediates the IGF signaling by stimulating its downstream targets such as Akt/PKB. Panel B: PDGF stimulates two independent signaling pathways that lead to the respective activation of PI 3-kinase and PKCβI. Whereas PI 3-kinase induces the phosphorylation of IRS-1 onto serine (S) 307, PKCβI potentially (?) phosphorylates IRS-1 onto distinct serine residues. These two events concomitantly prevent the further IGF-I-induced tyrosine phosphorylation of IRS-1 and the subsequent IGF signaling pathway. Moreover, PDGF stimulates PKD1 which does not play a role in the regulation of IRS-1 in response to this growth factor. See text for further details.

Consistent with this, it is of interest to note that, in 3T3-L1 adipocytes, PI 3-kinase activation time courses were different in response to EGF or PDGF (sustained and transient respectively) and that only PDGF down-regulated the insulin-induced IRS-1 tyrosine phosphorylation [29].

Taking these results into account, the following model can be proposed to explain how PI 3-kinase and PKCβI might regulate the IGF-I signaling in response to PDGF (Fig. 8). In cells only stimulated by IGF-I (Fig. 8A), the IGF-IR induces the tyrosine phosphorylation of IRS-1 which recruits docking proteins such as PI 3-kinase and initiates specific IGF-I downstream signaling pathways. When cells were pretreated with PDGF, the PI 3-kinase and PKCβI signaling pathways were activated (Fig. 8B). On the one hand, the PI 3-kinase pathway leads to serine 307 phosphorylation of IRS-1 whereas, on the other hand, PKCβI would negatively regulate IRS-1 perhaps by phosphorylating it, directly or not, on another residue. The concerted action of these two pathways would therefore reduce the affinity of IRS-1 towards the IGF-IR tyrosine kinase activity and its subsequent tyrosine phosphorylation in response to IGF-I. Moreover, PDGF also stimulates PKD1 which does not interfere with the IGF-I signaling pathway at the level of IRS-1.

PDGF-induced serine 307 phosphorylation of IRS-1 is mediated by a PI 3-kinase-dependent but Gö6976-, Gö6983- or GF109203X-insensitive signaling pathway. This excludes most of PKC isoforms, among them is PKCβI, and indicates that another serine/threonine kinase, distinct from PKCs and localized downstream of PI 3-kinase, such as mTOR or p70/S6 kinase (for review [4]), would be implicated in this mechanism. Such results may seem surprising since some PKC isoforms have been described to phosphorylate IRS-1 and in particular its serine 307 residue (for review [12]). Such discrepancy may be due to cell context and ligand response specificity since other studies have been conducted neither in MCF-7 nor in response to PDGF.

Noteworthy, we noticed once again a high similarity between the inhibitory time courses of IGF-I- or insulin-stimulated IRS-1-associated PI 3-kinase activity measured after growth factor pretreatment in MCF-7 (Fig. 1B and [22]) or in 3T3-L1 adipocytes [29], respectively. These characteristic triphasic curves further strengthen the hypothesis that a subtle common regulatory mechanism exists for the insulin and the IGF-I signaling pathways whose physiological significance remains to be determined.

In conclusion, our results confirm the notion that a coordinated action of at least two independent pathways, including that of PI 3-kinase, is necessary to regulate the IGF-I signaling at the level of IRS-1. Moreover, by highlighting the role of PKCβI in this regulatory process, we showed that the negative regulatory partners that cooperate with PI 3-kinase are highly specific upon the cell activating ligand illustrating the complexity of the cellular processes that need to be fully considered to develop efficient therapeutic strategies against pathologies such as cancers and diabetes.

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References

[1] V. Aguirre, T. Uchida, L. Yenush, R. Davis, M. White, The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307), J. Biol. Chem. 275 (2000) 9047–9054.
[2] V. Aguirre, E.D. Werner, J. Giraud, Y.H. Lee, S.E. Shoelson, M.F. White, Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action, J. Biol. Chem. 277 (2002) 1531–1537.
[3] E. Arvisais, X. Hou, T.A. Wyatt, K. Shirasuna, H. Bollwein, A. Miyamoto, T.R. Hansen, B.R. Rueda, J.S. Davis, Prostaglandin F2alpha represses IGF-I-stimulated IRS1/phosphatidylinositol-3-kinase/AKT signaling in the corpus luteum: role of ERK and P70 ribosomal S6 kinase, Mol. Endocrinol. 24 (2010) 632–643.
[4] S. Boura-Halfon, Y. Zick, Serine kinases of insulin receptor substrate proteins, Vitam. Horm. 80 (2009) 313–349.
[5] K.D. Copps, M.F. White, Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2, Diabetologia 55 (2012) 2565–2582.
[6] K. De Fea, R. Roth, Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase, J. Biol. Chem. 272 (1997) 31400–31406.
[7] M.R. Farren, L.M. Carlson, K.P. Lee, Tumor-mediated inhibition of dendritic cell differentiation is mediated by down regulation of protein kinase C beta II expression, Immunol. Res. 46 (2010) 165–176.
[8] Y. Fu, C.S. Rubin, Protein kinase D: coupling extracellular stimuli to the regulation of cell physiology, EMBO Rep. 12 (2011) 785–796.
[9] S. Giorgetti, R. Ballotti, A. Kowalski-Chauvel, M. Cormont, E. Van Obberghen, Insulin stimulates phosphatidylinositol-3-kinase activity in rat adipocytes, Eur. J. Biochem. 207 (1992) 599–606.
[10] B. Goldstein, Regulation of insulin receptor signaling by protein-tyrosine dephosphorylation, Receptor 3 (1993) 1–15.
[11] M. Gschwendt, S. Dieterich, J. Rennecke, W. Kittstein, H.J. Mueller, F.J. Johannes, Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase C isoenzymes, FEBS Lett. 392 (1996) 77–80.
[12] P. Gual, Y. Le Marchand-Brustel, J.F. Tanti, Positive and negative regulation of insulin signaling through IRS-1 phosphorylation, Biochimie 87 (2005) 99–109.
[13] T. Gustafson, W. He, A. Craparo, C. Schaub, T. O'Neill, Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain, Mol. Cell. Biol. 15 (1995) 2500–2508.
[14] S.N. Gustafsson, N. Heldring, K. Dahlman-Wright, Estrogen receptor-alpha, RBCK1, and protein kinase C beta 1 cooperate to regulate estrogen receptor-alpha gene expression, J. Mol. Endocrinol. 49 (2012) 277–287.
[15] S.E. Hankinson, W.C. Willett, G.A. Colditz, D.J. Hunter, D.S. Michaud, B. Deroo, B. Rosner, F.E. Speizer, M. Pollak, Circulating concentrations of insulin-like growth factor-I and risk of breast cancer, Lancet 351 (1998) 1393–1396.
[16] S.J. Heydrick, D. Jullien, N. Gautier, E. Van Obberghen, Y. Le Marchand-Brustel, Defect in skeletal muscle phosphatidylinositol-3-kinase in obese insulin-resistant mice, J. Clin. Invest. 91 (1993) 1358–1366.
[17] J.M. Holly, D.J. Gunnell, S.G. Davey, Growth hormone, IGF-I and cancer. Less intervention to avoid cancer? More intervention to prevent cancer? J. Endocrinol. 162 (1999) 321–330.
[18] W. Huang, R.R. Bansode, N.C. Bal, M. Mehta, K.D. Mehta, Protein kinase Cbeta deficiency attenuates obesity syndrome of ob/ob mice by promoting white adipose tissue remodeling, J. Lipid Res. 53 (2012) 368–378.
[19] T. Ito, Y. Sasaki, J. Wands, Overexpression of human insulin receptor substrate 1 induces cellular transformation with activation of mitogen-activated protein kinases, Mol. Cell. Biol. 16 (1996) 943–951.
[20] J.G. Jackson, M.F. White, D. Yee, Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells, J. Biol. Chem. 273 (1998) 9994–10003.
[21] J.I. Jones, D.R. Clemmons, Insulin-like growth factors and their binding proteins: biological actions, Endocr. Rev. 16 (1995) 3–34.
[22] M. Karam, C. Lassarre, C. Legay, J.M. Ricort, Phosphatidylinositol 3-kinase and protein kinase D1 specifically cooperate to negatively regulate the insulin-like growth factor signaling pathway, Biochim. Biophys. Acta 1823 (2012) 558–569.
[23] C. Lassarre, J.M. Ricort, Growth factor-specific regulation of insulin receptor substrate-1 expression in MCF-7 breast carcinoma cells: effects on the insulin-like growth factor signaling pathway, Endocrinology 144 (2003) 4811–4819.
[24] G. Martiny-Baron, M.G. Kazanietz, H. Mischak, P.M. Blumberg, G. Kochs, H. Hug, D. Marme, C. Schachtele, Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976, J. Biol. Chem. 268 (1993) 9194–9197.
[25] A. Mima, Y. Ohshiro, M. Kitada, M. Matsumoto, P. Geraldes, C. Li, Q. Li, G.S. White, C. Cahill, C. Rask-Madsen, G.L. King, Glomerular-specific protein kinase C-beta-induced insulin receptor substrate-1 dysfunction and insulin resistance in rat models of diabetes and obesity, Kidney Int. 79 (2011) 883–896.
[26] T. Noguchi, T. Matozaki, K. Horita, Y. Fujioka, M. Kasuga, Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation, Mol. Cell. Biol. 14 (1994) 6674–6682.
[27] Y.I. Oh, J.H. Kim, C.W. Kang, Involvement of insulin-like growth factor-I secretion and all-trans-retinoic acid-induced decrement in viability in MCF-7 cells, Chemotherapy 57 (2011) 17–26.
[28] K. Paz, R. Hemi, D. LeRoith, A. Karasik, E. Elhanany, H. Kanety, Y. Zick, A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation, J. Biol. Chem. 272 (1997) 29911–29918.
[29] J.-M. Ricort, J.-F. Tanti, E. Van Obberghen, Y. Le Marchand-Brustel, Cross-talk between the platelet-derived growth factor and the insulin signaling pathways in 3T3-L1 adipocytes, J. Biol. Chem. 272 (1997) 19814–19818.
[30] P.D. Ryan, P.E. Goss, The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer, Oncologist 13 (2008) 16–24.
[31] X.J. Sun, P. Rothenberg, C.R. Kahn, J.M. Backer, E. Araki, P.A. Wilden, D.A. Cahill, B.J. Goldstein, M.F. White, Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein, Nature 352 (1991) 73–77.

[32] D. Surdez, M. Benetkiewicz, V. Perrin, Z.Y. Han, G. Pierron, S. Ballet, F. Lamoureux, F. Redini, A.V. Decouvelaere, E. Daudigeos-Dubus, B. Geoerger, P.G. de, O. Delattre, F. Tirode, Targeting the EWSR1-FLI1 oncogene-induced protein kinase PKC-beta abolishes Ewing sarcoma growth, Cancer Res. 72 (2012) 4494–4503.
[33] C.E. Tabit, S.M. Shenouda, M. Holbrook, J.L. Fetterman, S. Kiani, A.A. Frame, M.A. Kluge, A. Held, M.M. Dohadwala, N. Gokce, M.G. Farb, J. Rosenzweig, N. Ruderman, J.A. Vita, N.M. Hamburg, Protein kinase C-beta contributes to impaired endothelial insulin signaling in humans with diabetes mellitus, Circulation 127 (2013) 86–95.
[34] A. Ullrich, A. Gray, A.W. Tam, T. Yang-Feng, M. Tsubokawa, C. Collins, W. Henzel, T. Le Bon, S. Kathuria, E. Chen, S. Jacobs, U. Francke, J. Ramachandran, Y. Fujita-Yamaguchi, Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity, EMBO J. 5 (1986) 2503–2512.
[35] M. White, The insulin signalling system and the IRS proteins, Diabetologia 40 (Suppl. 2) (1997) 2–17.
[36] L. Yenush, K. Makati, J. Smith-Hall, O. Ishibashi, M.J. Myers, M. White, The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1, J. Biol. Chem. 271 (1996) 24300–24306.