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Long-Term Effects of Tumor Necrosis Factor-α Treatment on Insulin Signaling Pathway in HepG2 Cells and HepG2 Cells Overexpressing Constitutively Active Akt/PKB

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Abstract Tumor necrosis factor-α (TNF-α) mediated attenuation of insulin signaling pathway is an important cause in several disorders like obesity, obesity linked diabetes mellitus. TNF-α actions vary depending upon concentration and time of exposure in various cells. In the present study, the effects of long-term TNF-α (1 ng/ml) exposure on the components of insulin signaling pathway in HepG2 and HepG2 cells overexpressing constitutively active Akt1/PKB-α (HepG2-CA-Akt/PKB) have been investigated. In parental HepG2 cells, TNF-α treatment for 24 h reduced the phosphorylation of Akt1/PKB-α and GSK-3β and under these conditions cells also showed reduced insulin responsiveness in terms of Akt1/PKB-α and GSK-3β phosphorylation. TNF-α pre-incubated HepG2-CA-Akt/PKB cells showed lower reduction in Akt1/PKB-α and GSK-3β phosphorylation and insulin responsiveness after 24 h as compared to parental HepG2 cells. We report that the long-term TNF-α pre-incubation in both parental HepG2 and HepG2-CA-Akt/PKB-α cells leads to the reduction in the levels of IRS-1 without altering the levels of IRS-2. In order to understand the reason for the differential insulin resistance in both the cell types, the effect of long-term TNF-α treatment on the proteins upstream to Akt/PKB was investigated. TNF-α pre-incubation also showed reduced insulin-stimulated Tyr phosphorylation of insulin receptor (IR-β) in both the cell types, moreover hyperphosphorylation of IRS-1 at Ser 312 residue was observed in TNF-α pre-incubated cells. As hyperphosphorylation of IRS-1 at Ser 312 can induce its degradation, it is possible that reduced insulin responsiveness after long-term TNF-α pre-incubation observed in this study is due to the decrease in IRS-1 levels. J. Cell. Biochem. 100: 593–607, 2007.

Key words: TNF-α; IRS; Akt1/PKB-α overexpression; HepG2

Insulin resistance is an important contributor to the pathogenesis of type 2 diabetes. Recently, a close link between tumor necrosis factor-α (TNF-α), obesity, and insulin resistance in type 2 diabetes has been suggested [Hotamisligil et al., 1993, 1996; Uysal et al., 1997]. TNF-α is primarily synthesized by macrophages in response to invasive stimuli and it elicits the signaling cascade upon binding with two ubiquitously expressed tumor necrosis factor-α receptors (TNFR), TNFR1 (55 kDa), and TNFR2 (75 kDa) [Nophar et al., 1990; Carpenter et al., 1995]. Virtually all animal models of obesity and insulin resistance have been reported to produce significantly higher levels of circulating TNF-α and in adipose tissue, elevated TNF-α mRNA expression has been observed. TNF-α-mediated attenuation of insulin signaling cascade has been reported in adipocytes, hepatoma cells, fibroblasts, and muscle cells [Hotamisligil et al., 1994; Cheung et al., 1998; Liu et al., 1998]. Although the mechanism of action of TNF-α in the attenuation of insulin signaling cascade is not fully understood, it is known that the major components involved are upstream of proximal insulin signaling cascade. Numerous studies have shown that TNF-α reduces insulin-mediated tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) as well as inhibits the activity of phosphatidyl

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inositol-3 kinase (PI-3 kinase) [Kanety et al., 1995; Kroder et al., 1996]. Phosphorylation of IRS-1 at serine residue (312 in humans and 307 in rats) is an emerging prime target of TNF-α-mediated impaired PI-3 kinase dependent signaling pathway [Aguirre et al., 2000, 2002; Sykiotis and Papavassiliou, 2001; Gao et al., 2002].

It is a well known fact that TNF-α phosphorylates and activates Akt/PKB in a PI-3 kinase-dependent pathway although the nature and extent of activation varies with the cell type and experimental conditions [Hanna et al., 1999; Ozes et al., 1999; O'Toole et al., 2001]. Akt1/PKB-α (57 kDa) is an important Ser/Thr kinase downstream of PI-3 kinase, possessing Pleckstrin Homology (PH) domain [Jones et al., 1991; Coffer and Woodgett, 1992; Andjelkovic et al., 1996; Kohn et al., 1996]. Stimulation of receptor tyrosine kinase activity (RTK) by insulin, IGF, or PDGF elicits the autophosphorylation of IR. This leads to the phosphorylation of docking proteins insulin receptor substrate (IRS). IRS-1 phosphorylated at tyrosine binds to PI-3 kinase (p85 subunit) to activate it. PI-3 kinase catalyzes the generation of second messenger—phosphatidyl inositol 3,4,5-triphosphate (PIP3) by the phosphorylation of phosphatidyl inositol 4,5-biphosphate (PIP2). Generation of PIP3 causes the translocation of Akt/PKB from cytoplasm to plasma membrane through its PH domain. Once bound to the plasma membrane Akt/PKB is activated by series of phosphorylation at Thr 308, located in the activation loop of kinase domain and Ser 473 residues, located in the C-terminus regulatory domain of the protein [Kohn et al., 1995; Andjelkovic et al., 1996; Alessi et al., 1996]. The phosphorylation of Thr 308 is catalyzed by PDK-1 (3′-phosphoinositide-regulated kinase) whereas the phosphorylation at Ser 473 is by uncharacterized PDK-2 [Alessi et al., 1997; Stephens et al., 1998; Hresko et al., 2003]. Recent studies reveal protein complex of mammalian target of rapamycin (mTOR) in complex with rictor catalyzes the phosphorylation of Akt/PKB at Ser 473 residue [Hresko and Mueckler, 2005; Sarbassov et al., 2005]. The Akt/PKB phosphorylation (activation) is a key event in the insulin signal transduction cascade and has been used as the surrogate marker for assessing the activation level of the proximal insulin signal cascade. We have previously reported that chronic long-term exposure of HepG2 cells with TNF-α induces attenuation of insulin signal cascade [Gupta and Khandelwal, 2004]. This was determined by examining the phosphorylation levels of Akt1/PKB-α and its downstream target GSK-3β along with parallel changes in glycogen synthesis. Therefore, our interest was to examine whether this attenuation is due to downregulation of any of the components of insulin signal cascade. In the present study, we have examined the effects of chronic/long-term exposure of TNF-α on parental HepG2 and HepG2 cells overexpressing Akt1/PKB-α (HepG2-CA-Akt/PKB) on the phosphorylation of Akt/PKB (Ser 473) and GSK-3β (Ser 9). Insulin responsiveness upon TNF-α pre-incubated in these cells was also been investigated. It was observed that upon long-term TNF-α exposure, the insulin responsiveness, in terms of Akt1 (Ser 473) and GSK-3β (Ser 9) phosphorylation was more prominently affected in parental HepG2 cells as compared to the HepG2-CA-Akt/PKB cells. In order to further understand the interaction of TNF-α and Akt1/PKB-α, the upstream protein levels were investigated. The long-term exposure of both HepG2 and HepG2-CA-Akt/PKB cells to TNF-α resulted in downregulation of IRS-1 without affecting the levels of IRS-2. IRS-1 was found to be hyperphosphorylated at Ser 312 in the TNF-α pre-incubated cells, indicates that IRS-1 degradation might be responsible for the insulin resistance in downstream proteins Akt/PKB and GSK-3β.

MATERIALS AND METHODS

Cell Culture

HepG2 cells were cultured in D-MEM/F12 (pH 7.4) supplemented with fetal bovine serum (FBS, 10%) and antibiotic-antimycotic (1%). D-MEM/F-12, FBS, and antibiotic-antimycotic (containing penicillin G sodium, streptomycin sulfate, and amphotericin B), geneticine were procured from Gibco, Invitrogen, Ontario, Canada. Cells were incubated in a CO_2 incubator (Fischer Scientific, Ottawa, Ontario, Canada) maintained at 37°C with humidified air and CO_2 (5%). HepG2 cells overexpressing constitutively active Akt1/PKB-α (HepG2-CA-Akt/PKB) were prepared and characterized as described elsewhere [Gupta and Khandelwal, 2004]. The pooled positive clones expressing constitutively active Akt1/PKB-α were maintained in D-MEM/F12 (pH 7.4) supplemented with FBS

(10%), antibiotic-antimycotic (1%), and a selection pressure of geneticin (0.1 mg/ml).

Western Blot Analysis

HepG2 and HepG2-CA-Akt/PKB cells were grown in 100 mm plates up to ~80–85% confluence prior to any treatments. Cells were washed twice with PBS and treated with TNF-α and/or human recombinant insulin (Sigma-Aldrich, Ontario, Canada) in serum-free media. The incubation conditions and concentrations of TNF-α and insulin are indicated in figure legends. After the treatments, cells were washed with ice-cold PBS and immediately frozen in liquid nitrogen. Cells were lysed on ice using lysis buffer (1 ml) containing HEPES (pH 7.4, 50 mM), sucrose (150 mM), sodium orthovanadate (2 mM), β-glycerophosphate (80 mM), sodium fluoride (10 mM), sodium pyrophosphate (10 mM), sodium EGTA (2 mM), sodium EDTA (2 mM), Triton X-100 (1%), SDS (0.1%), phenyl methyl sulfonyl fluoride (1 mM), 100 μL of protease inhibitor cocktail for mammalian cell culture (Sigma-Aldrich). Cells were scrapped, collected, and kept on ice bath for 30 min. Lysates were then centrifuged at 13,000*g* for 2 min at 4°C and supernatants were stored at −80°C for future use. Western blot analysis was performed as described by Towbin et al. [1992]. Aliquots of proteins corresponding to 20 μg protein were mixed with SDS–PAGE sample buffer and heated on boiling hot water bath for 3 min. The proteins were resolved on SDS–PAGE (6.5–10%) and transferred on blotting grade PVDF membrane (Bio-RAD Lab, Ontario, Canada) using mini transblot apparatus, visualization was done using Ponceau S reagent. For IRS-2 blots, the amount of SDS in transfer buffer was increased from 0.1% to 0.2% and transfer time was increased from 1 to 2 h. The blots were blocked with PBST-milk (non-fat dry milk (5%), PBS with Tween 20 (0.02%)) for 1 h at room temperature followed by 2 h at 4°C. The blots were probed with antibodies against Akt1/PKB-α, p-Akt (Ser 473) (BD Biosciences, Mississauga, Ontario, Canada), GSK-3β, p-GSK-3β (Ser 9) (Santa Cruz Biotech., Santa Cruz, CA) and IRS-1, -2, and IR-β (Upstate Biotech., Lake placid, NY) (1:1,000 dilution) overnight at 4°C. The membranes were washed and incubated with appropriate horseradish peroxidase conjugated secondary antibody (1:10,000 dilutions) for 1 h at room temperature. After further washing, visualization of hybridization was carried out using chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA).

Other Methods

Bradford's method of protein estimation was used in order to quantify the protein value, bovine serum albumin (Sigma-Aldrich) was used as standard. NIH image software (version 1.62) was used to determine the relative band intensities of the Western blot. Statistical analyses were performed by Student's *t*-test.

RESULTS

Akt/PKB phosphorylation (activation) is a key proximal event in PI-3 kinase-mediated insulin response. Therefore, as a first step, we determined the phosphorylation of Akt/PKB at Ser 473 by varying the concentration of insulin (0.1–10 nM) in parental HepG2 and HepG2-CA-Akt/PKB cells. As expected, a dose-dependent increase in Akt/PKB phosphorylation at Ser 473 due to insulin treatment was observed. In parental HepG2 cells an eightfold increase at 10 nM insulin concentration was observed (Fig. 1A,B). To begin with, HepG2-CA-Akt/PKB cells already have enhanced basal levels of phosphorylated Akt/PKB (Ser 473), this was further increased by 3.5-fold with 10 nM insulin (Fig. 1A,C). Hence, an optimal concentration of insulin (10 nM) was used in our further studies. In our previous studies, we have shown that an optimal phosphorylation of Akt (Ser 473) occurs at TNF-α (1 ng/ml) and therefore, this concentration of TNF-α was used in the present study [Gupta and Khandelwal, 2004]. In order to understand the interactions between Akt/PKB and TNF-α, we pre-incubated parental HepG2 and HepG2-CA-Akt/PKB cells with TNF-α for 0, 4, 8, and 24 h followed by absence or the presence of insulin (10 nM) treatment for 5 min and determined the phosphorylation of Akt/PKB at Ser 473 (Fig. 2). Parental HepG2 cells showed an increase in the phosphorylation of Akt/PKB (Ser 473), with a maximal peak at 8 h that declined at 24 h of TNF-α pre-incubation (Fig. 2A,B). As expected, insulin stimulated the phosphorylation of Akt (Ser 473) in parental HepG2 cells without TNF-α pre-incubation (Fig. 2A,C). However, there was a gradual time-dependent decline in the phosphorylation of Akt (Ser 473) by insulin in TNF-α

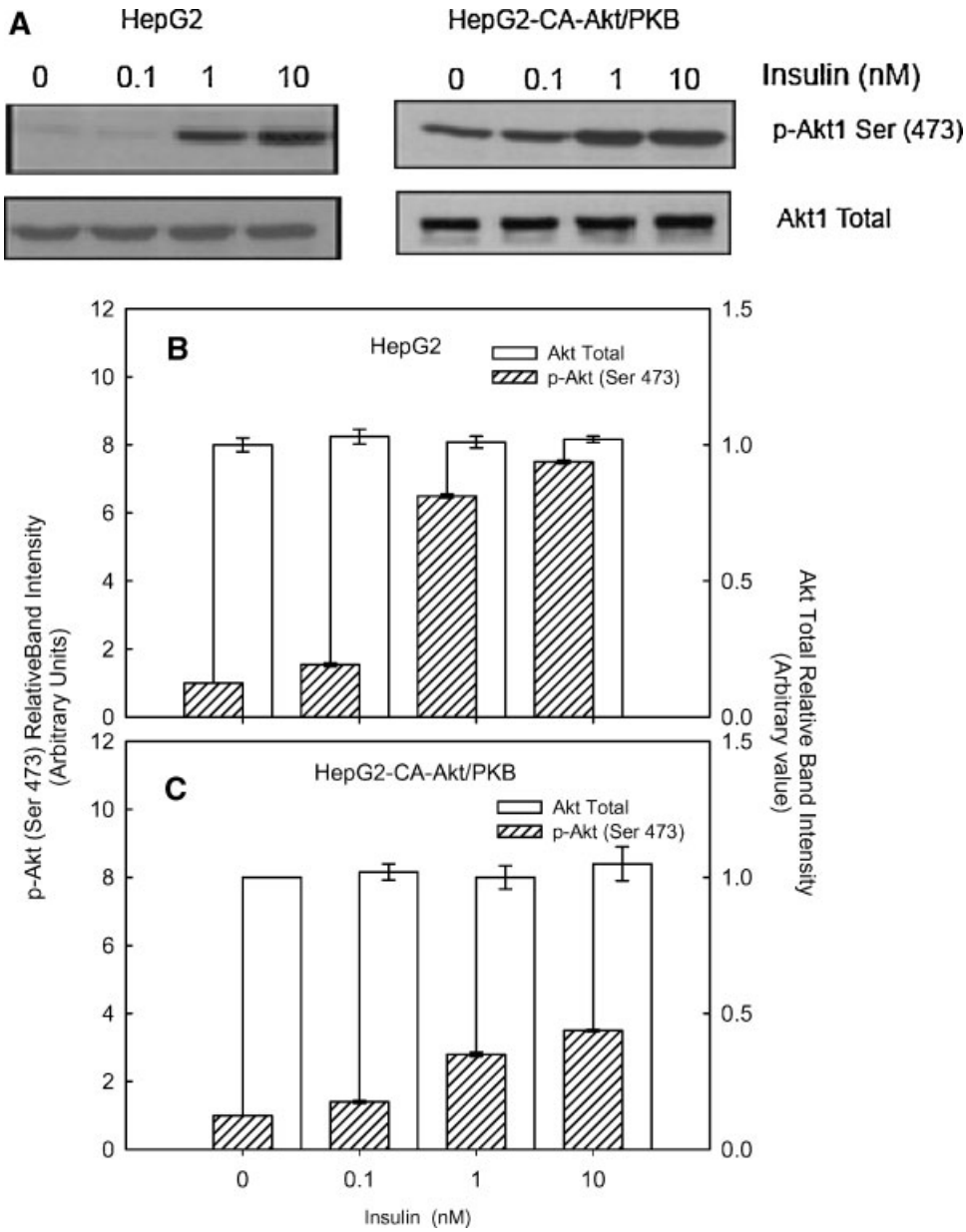

Fig. 1. The effect of varying concentrations of insulin on the phosphorylation of Akt/PKB-α (Ser 473) in parental HepG2 and HepG2-CA-Akt/PKB cells. Cells were starved overnight followed by incubation with varying concentrations of insulin (0–10 nM) in fresh serum-free media for 5 min at 37°C. The cells were lysed as described in Materials and Methods section. Whole cell lysates were subjected to SDS–PAGE and Western blot analysis was performed using antibodies against p-Akt (Ser 473) or Akt1. A typical representative Western blot is shown in subpart (**A**). The relative band intensities were determined using NIH-image software and the control band was given an arbitrary value of 1. The results shown in subparts (**B**) and (**C**) are the average ± SEM of three experiments.

pre-incubated cells (Fig. 2A,C). Insulin treatment of parental HepG2 cells pre-incubated with TNF-α for 24 h showed a 1.88-fold decrease compared to controls (Fig. 2C). Previously, we have reported that HepG2 cells overexpressing constitutively active Akt1/PKB-α (HepG2-CA-Akt/PKB) have characteristics similar to insulin-stimulated HepG2 cells [Gupta et al., 2004]. Therefore, our interest was to examine the effect of insulin on the phosphorylation of Akt/PKB (Ser 473) in these cells pre-incubated with TNF-α. A gradual time-dependent decline in the Akt/PKB phosphorylation was observed. A threefold decrease in the phosphorylation of Akt (Ser 473) in 24 h pre-incubated cells with TNF-α was observed (Fig. 2D,E). Despite the

decline in Akt/PKB phosphorylation after 24 h of pre-incubation with TNF-α, no significant change (1.3-fold decrease) in its insulin-mediated phosphorylation of Akt/PKB (Ser 473) in HepG2-CA-Akt/PKB cells was observed (Fig. 2F). The decrease in Akt/PKB phosphorylation after 24 h of TNF-α pre-incubation in both parental HepG2 and HepG2-CA-Akt/PKB cells was not accompanied by any changes in the total protein levels (Fig. 2).

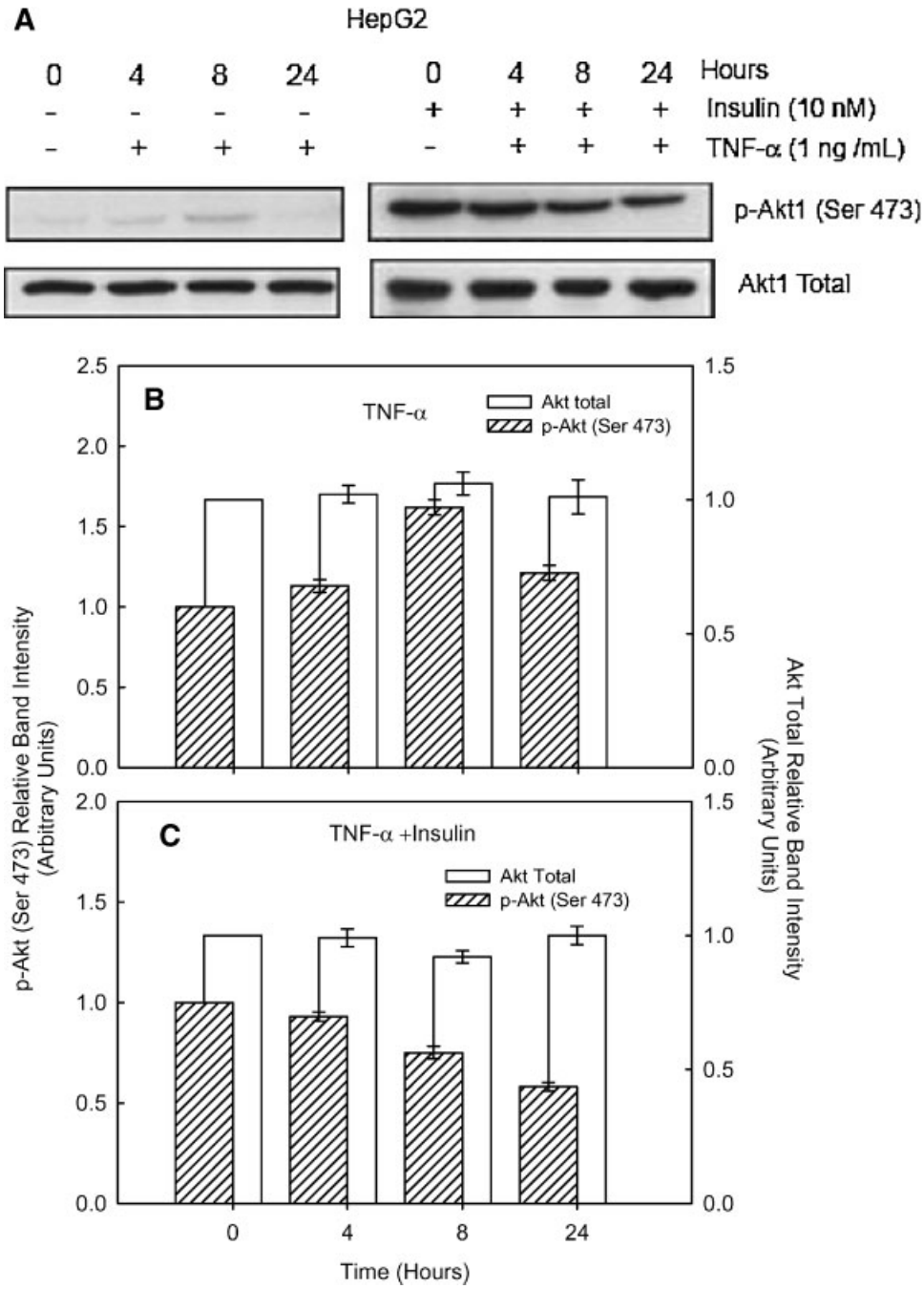

Fig. 2. The effects of insulin treatment on the phosphorylation of Akt1/PKB-α in HepG2 and HepG2-CA-Akt/PKB cells with long-term pre-incubated TNF-α. Cells starved overnight and treated with TNF-α (1 ng/ml) for 0, 4, 8, and 24 h at 37°C followed by the treatment of insulin (10 nM) or PBS for 5 min. Cells were lysed and subjected to Western blot analysis as described in Materials and Methods section. Subparts (**A**) and (**D**) represent the Western blots for HepG2 cells and HepG2-CA-Akt/PKB, respectively. The relative band intensities of HepG2 are shown in subparts (**B**) and (**C**) and HepG2-CA-Akt/PKB are shown in subparts (**E**) and (**F**) in the absence and presence of insulin, respectively. Control (no treatment, 0 h) for p-Akt and total Akt was taken as onefold. The relative band intensities shown are the average ± SEM of three experiments.

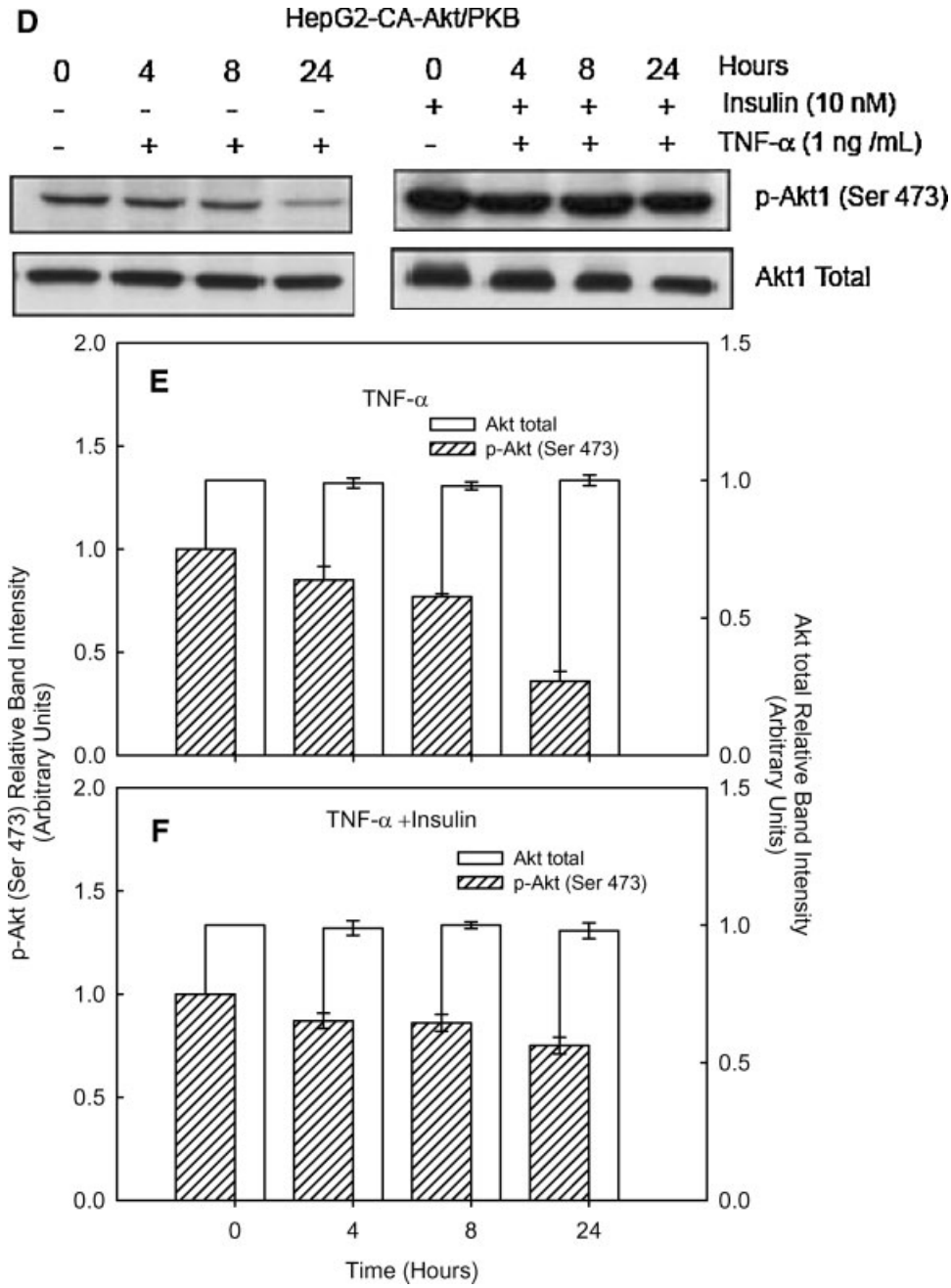

Fig. 2. (*Continued*)

GSK-3β is a direct downstream high-affinity effector of Akt/PKB that is inactivated after being phosphorylated at Ser 9 upon insulin treatment [Cross et al., 1995; Alessi et al., 1996]. Since there was a decline in Akt/PKB phosphorylation by insulin in both HepG2 and HepG2-CA-Akt/PKB cells pre-incubated with TNF-α, the effect of long-term TNF-α pre-incubation on GSK-3β (Ser 9) phosphorylation in these cells was also studied (Fig. 3). In parental HepG2 cells, upon 24 h pre-incubation with TNF-α, a 3.7-fold decrease in phosphorylation of GSK-3β (Ser 9) without any change in total GSK-3β protein levels was observed (Fig. 3A,B). HepG2-CA-Akt/PKB cells pre-incubated with TNF-α for 24 h also showed a decrease in the phosphorylation of GSK-3β at Ser 9 (Fig. 3D,E). Insulin-mediated

phosphorylation of GSK-3β (Ser 9) upon TNF-α pre-incubation also followed the same trend as Akt phosphorylation (Fig. 2), in both parental HepG2 (Fig. 3A,C) and HepG2-CA-Akt/PKB cells (Fig. 3D,F). There was no significant change in the insulin-mediated phosphorylation of GSK-3β in HepG2-CA-Akt/PKB cells whereas a decline in parental HepG2 cells.

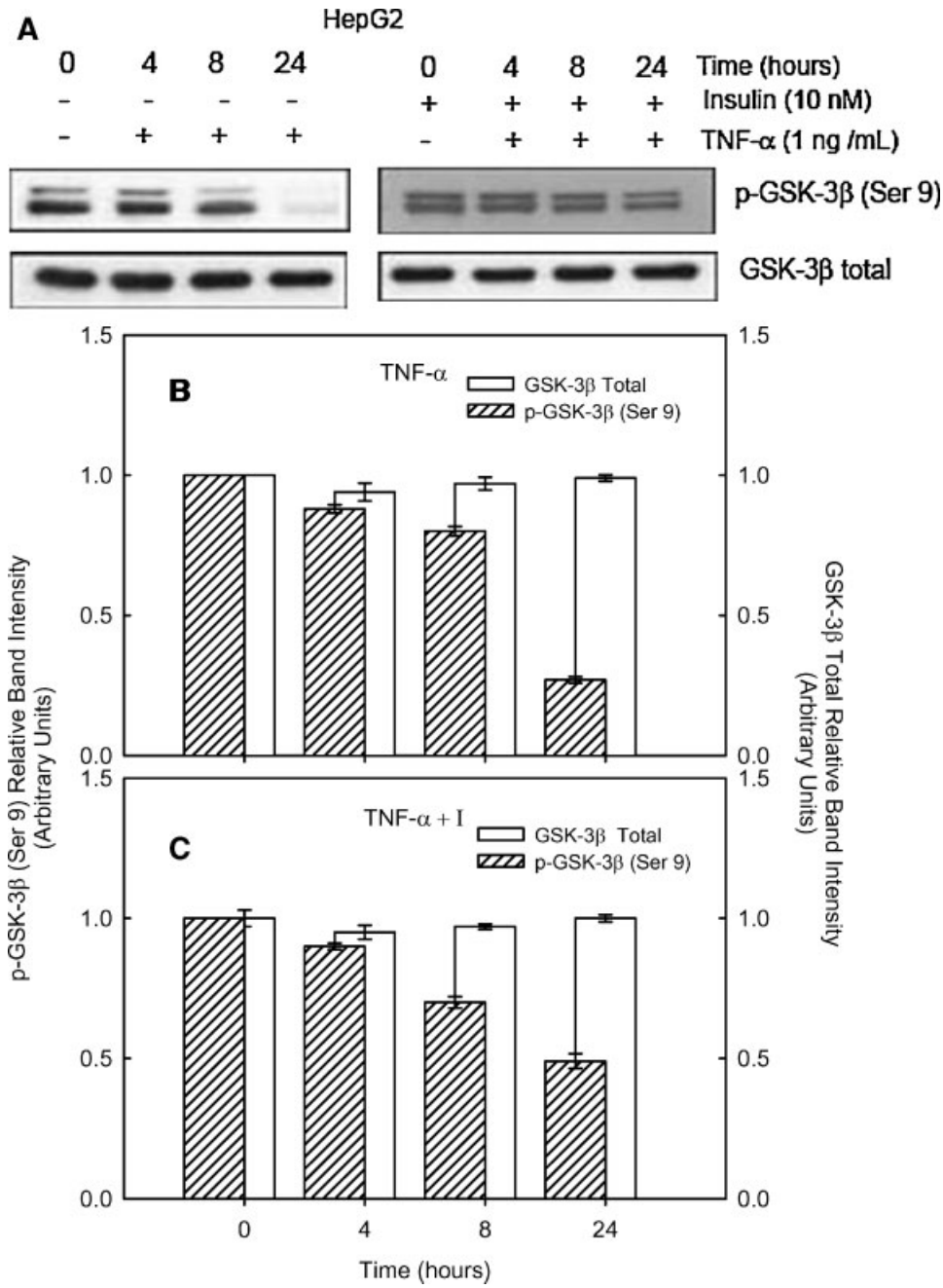

Fig. 3. The effects of insulin treatment on the phosphorylation of glycogen synthase kinase-3β (Ser 9) in HepG2 and HepG2-CA-Akt/PKB cells pre-incubated with TNF-α. Cells were starved overnight and treated with TNF-α (1 ng/ml) for 0, 4, 8, and 24 h at 37°C followed by the treatment of insulin (10 nM) or PBS for 5 min. These cells were lysed and subjected to Western blot analysis as described in the Materials and Methods section. The blots were probed with antibodies against p-GSK-3β (Ser 9) and GSK-3β. Subparts (**A**) and (**D**) represent the Western blots obtained in case of parental HepG2 and HepG2-CA-Akt/PKB cells, respectively. The relative band intensities, of HepG2 are shown in subparts (**B**) and (**C**) and of HepG2-CA-Akt/PKB are shown in subparts (**E**) and (**F**) in the absence and presence of insulin, respectively. Control (no treatment, 0 h) for p-GSK-3β and GSK-3β total was given an arbitrary value of 1. The results shown are the average ± SEM of three experiments.

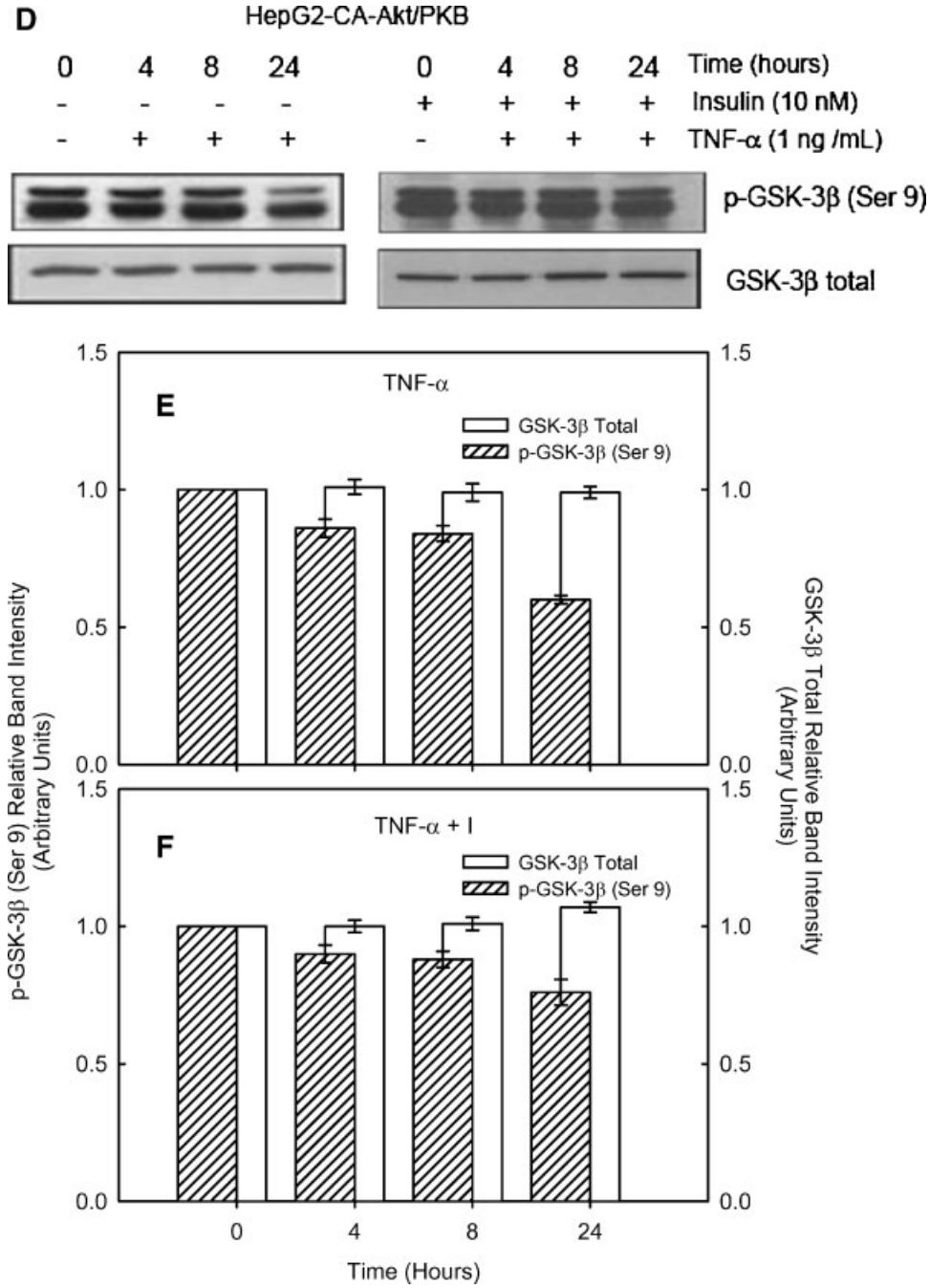

Fig. 3. (*Continued*)

It was further examined whether higher insulin resistance upon long-term pre-incubation with TNF-α, in terms of Akt and GSK-3β phosphorylation in parental HepG2 cells, was due to any changes in the levels of upstream proteins in the insulin signaling cascade. Therefore, we examined the protein levels of insulin receptor (IR-β subunit) subunit as well as IRS-1 and -2. A 1.5- and 1.2-fold decrease in the total protein levels of IR-β subunit in comparison to control (no treatment) in both parental HepG2 as well as HepG2-CA-Akt/PKB cells, respectively, pre-incubated with TNF-α was observed (Fig. 4A,B). We further quantified the levels of IRS-1 and -2 docking proteins in both the cell types (Fig. 5A). IRS proteins are key components of proximal insulin signal transduction cascade [White, 2002]. Long-term pre-incubation of cells

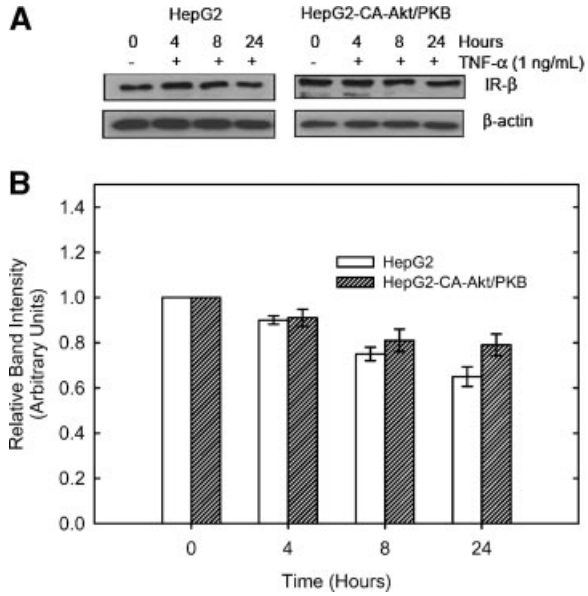

Fig. 4. The effect of long-term TNF-α pre-incubation on insulin receptor-β subunit (IR-β) in HepG2 and HepG2-CA-Akt/PKB cells. Overnight starved HepG2 and HepG2-CA-Akt/PKB cells were pre-incubated for 0, 4, 8, and 24 h at 37°C with TNF-α (1 ng/ml). These cells were washed, lysed, and subjected to Western blot analysis using antibody against IR-β subunit and β-actin. Subpart (**A**) represents the Western blot analysis and subpart (**B**) represents the relative band intensities. Control (no treatment, 0 h) was given an arbitrary value of 1. The results shown are the average ± SEM of three experiments.

with TNF-α for 24 h resulted in a 3.2- and 3.5-fold decrease in the IRS-1 protein levels in HepG2 and HepG2-CA-Akt/PKB cells, respectively, without any significant alterations in the levels of IRS-2 in both the cases (Fig. 5A–C). Although the decrease in the levels of IRS-1 was observed in both parental HepG2 as well as HepG2-CA-Akt/PKB cells, insulin resistance in terms of Akt (Ser 473) and GSK-3β (Ser 9) phosphorylation were more prominent in parental HepG2 cells rather than HepG2-CA-Akt/PKB cells (Figs. 2 and 3). This behavior could be due to overexpression of constitutively active Akt1/PKB-α in HepG2-CA-Akt/PKB cells.

The TNF-α-mediated attenuation of insulin signaling cascade might also involve the phosphorylation of IRS-1 at Ser (312 residue in human or 307 residue in rat) leading to impaired activation of PI-3 kinase-dependent pathways [Aguirre et al., 2000, 2002; Sykiotis and Papavassiliou, 2001; Gao et al., 2002]. In order to further explore these effects with the long-term TNF-α pre-incubation in presence or absence of insulin in both the cell lines, we examined the phosphorylation of IRS-1 (Ser 312) and the total Tyr phosphorylation of IRS-1 and IR-β subunit (Fig. 6). In parental HepG2 cells, TNF-α pre-incubation induced an enhanced phosphorylation of IRS-1 (Ser 312) after 4 h followed by decrease upon further incubation (Fig. 6A,B). The treatment of insulin resulted in a 4.9-fold increase in total Tyr phosphorylation of IRS-1 which was decreased after pre-incubation with TNF-α. A similar pattern in total Tyr phosphorylation of IR-β subunit, 4.2-fold increase upon insulin treatment was observed. These effects were abolished gradually upon TNF-α pre-incubation in parental HepG2 cells. In HepG2-CA-Akt/PKB cells, TNF-α pre-incubation induced an enhanced phosphorylation of IRS-1 (Ser 312) after 4 h of treatment followed by a gradual decrease at 8 h (Fig. 6A,C). The levels of Tyr phosphorylation at IRS-1 and IR-β subunit increased up to 2.9- and 3.3-fold, respectively, with insulin treatments that gradually decreased in TNF-α pre-incubated cells (Fig. 6C). It should be noted that the basal levels of Tyr phosphorylation of IRS-1 and IR-β subunits in HepG2-CA-Akt/PKB was higher as compared to parental HepG2 cells (Fig. 6A) and this was further increased with TNF-α pre-incubation. From the data obtained (Figs. 5 and 6), it is possible to extrapolate the phosphorylation of IRS-1 (Ser 312) and Tyr residues per arbitrary unit (amount). In parental HepG2 cells upon 24 h of TNF-α pre-incubation, the phosphorylation of IRS-1 at Ser 312 and pY20 residues are 3.0- and 1.6-fold higher compared to controls, respectively. Similarly, in HepG2-CA-Akt/PKB cells, phosphorylation of IRS-1 at Ser 312 and pY20 are, respectively, 3.0- and 1.2-fold higher than control. Upon the pre-incubation with TNF-α, a hyperphosphorylation of IRS-1 (Ser 312) in both parental HepG2 as well as HepG2-CA-Akt/PKB is observed.

DISCUSSION

Insulin resistance is a major defect frequently associated with chronic obesity and obesity-linked type 2 diabetes [Ferrannini, 1995; Walker, 1995]. A close link between enhanced expression of TNF-α in adipose tissues and obesity has been demonstrated [Hotamisligil et al., 1993, 1996]. Moreover, animal model of knockouts indicates that the absence of TNF-α or the TNF-receptor improved insulin

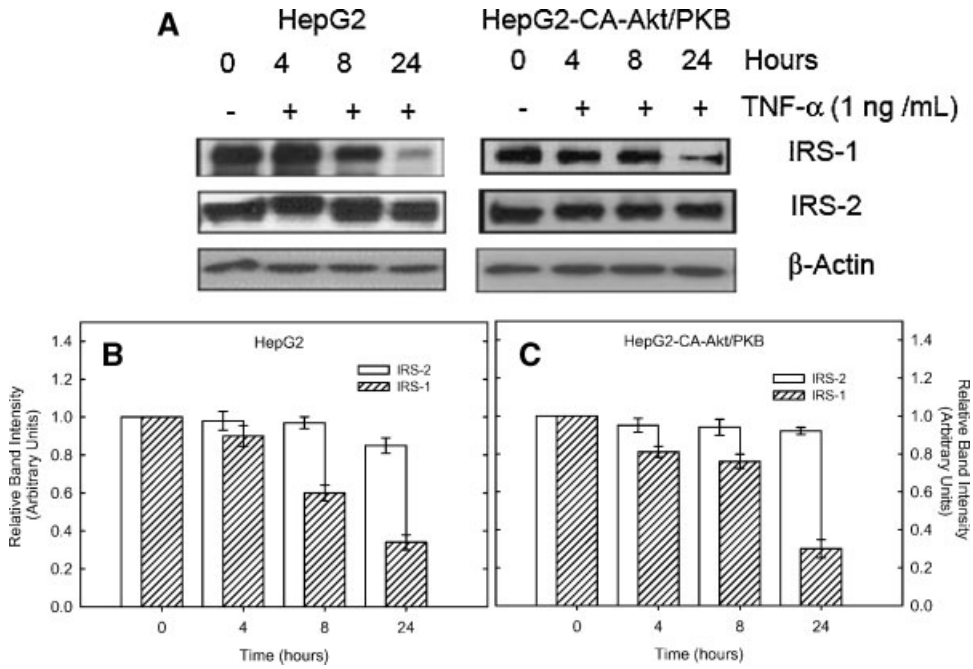

Fig. 5. The effect of long-term TNF-α pre-incubation on the levels of insulin receptor substrate (IRS-1 and -2) proteins in parental HepG2 and HepG2-CA-Akt/PKB cells. Overnight starved HepG2 and HepG2-CA-Akt/PKB cells were pre-incubated for 0, 4, 8, and 24 h at 37°C with TNF-α (1 ng/ml). These cells were washed, lysed, and subjected to Western blots as described in the Materials and Methods section. The blots were probed with antibody against IRS-1, -2, and β-actin. Subpart (**A**) represents Western blots and subparts (**B**) and (**C**) represent the relative band intensities. Control (no treatment, 0 h) was given an arbitrary value of 1. The band intensities are an average ± SEM of three experiments.

sensitivity in genetic obese (ob/ob) mice [Uysal et al., 1997, 1998]. In the present study, the long-term effects of TNF-α exposure on insulin signaling pathway in parental HepG2 and HepG2 cells overexpressing constitutively active Akt1/PKB-α (HepG2-CA-Akt/PKB) were studied. Akt1 phosphorylation (activation) is a key event in the insulin signaling cascade and has been used as a surrogate marker for activation of proximal insulin signal cascade [Shepherd et al., 1998]. Akt is activated by several hormones, growth factors like serum, insulin, IGF, and PDGF through a cascade of protein phosphorylation [Kohn et al., 1995; Andjelkovic et al., 1996; Alessi et al., 1996]. In HepG2 cells, a dose-dependent increase in the phosphorylation Akt/PKB at Ser 473 upon increasing the concentration of insulin was observed (Fig. 1A,B). In HepG2-CA-Akt/PKB cells, the constitutive activation of Akt1/PKB-α is reflected by higher basal levels of phosphorylated Akt1 (Ser 473) that was further increased up to 3.5-fold upon the treatment of insulin (Fig. 1A,C). Kohn et al. [1996] reported that the constitutive expression of Akt1/PKB-α in 3T3-L1 fibroblast cells led to its activation even in the absence of insulin, however, there was no further stimulation by insulin. The constitutive expression of membrane targeted Akt1/PKB-α in L6 muscle cells also showed a 15-fold higher activity. In spite of this, further stimulation upon insulin treatment by 3.4-fold was observed [Hajduch et al., 1998]. In our case, HepG2 cells with stable transfection of Akt1/PKB-α construct (pc DNA3-myr-flag-PKB-α) showed increase in the active form of Akt1/PKB-α and the phosphorylation level was further stimulated by insulin. A possible explanation for this further increase in the phosphorylation of Akt/PKB (Ser 473) upon insulin treatment could be due to activation of endogenous inactive Akt1/PKB-α in these cells.

TNF-α has been reported to interact with Akt/PKB through a PI-3 kinase-dependent pathway [Ozes et al., 1999; O'Toole et al., 2001]. Its effect on insulin signaling appears to be related to cell type and duration of TNF-α treatment. Reddy et al. [2000] suggested that TNF-α-mediated activation of PI-3 kinase in HepG2 and U 937 cells is rapid but transient involving IRS-1. Ruan et al. [2002] reported that long-term exposure of TNF-α in 3T3-L1 adipocytes

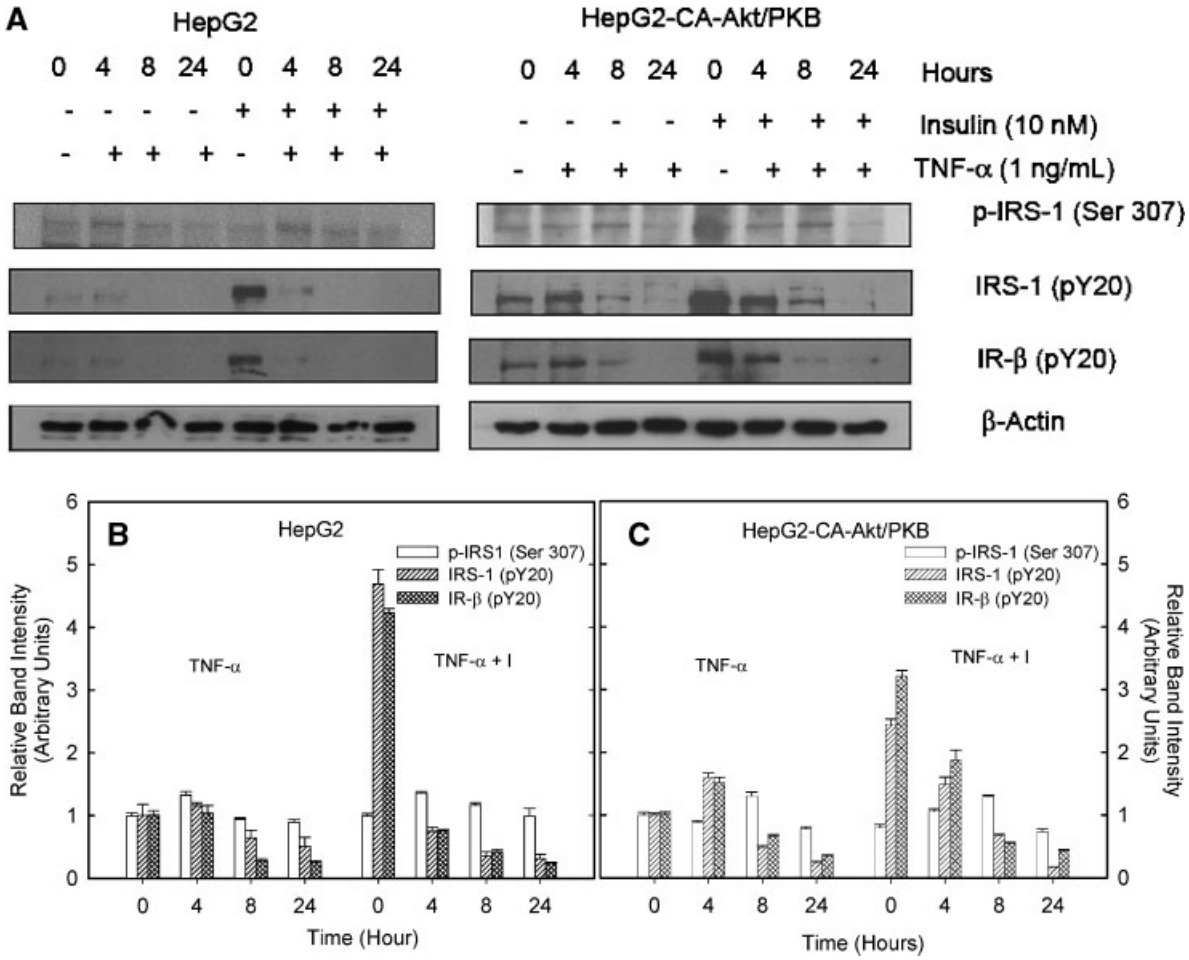

Fig. 6. The effect of long-term TNF-α pre-incubation in the presence or absence of insulin on the phosphorylation of IRS-1 (pY20) and IR-β subunit (pY20) in HepG2 and HepG2-CA-Akt/PKB cells. Cells were starved overnight and pre-incubated with TNF-α (1 ng/ml) for 0, 4, 8, and 24 h at 37°C followed by with or without insulin (10 nM) treatment for 5 min. The cells were washed, lysed, and subjected to Western blot analysis as described in Materials and Methods section. The blots were probed with antibody against p-IRS-1 (307), IRS-1 (pY20), p-IR-β, and β actin. Western blots are shown in subpart (**A**) and the relative band intensities of parental HepG2 and HepG2-CA-Akt/PKB cells are shown in subparts (**B**) and (**C**), respectively, the subparts (B) and (C) also show the effect of TNF-α (1 ng/ml) pre-incubation for 0, 4, 8, and 24 h in the absence and presence of insulin (10 nM) treatment for 5 min. Control cells (no treatment, 0 h) were given an arbitrary value of 1. The results shown in subparts (B) and (C) are the average ± SEM of three independent experiments.

has pronounced effect on insulin signal cascade leading to insulin resistance. Our observation suggests that in both parental HepG2 (Fig. 2A,B) and HepG2-CA-Akt/PKB (Fig. 2D,E) cells, a decrease in the phosphorylation of Akt1/PKB-α without any change in total protein levels was observed upon 24 h of pre-incubation with TNF-α. In parental HepG2 cells, insulin-mediated stimulation of Akt (Ser 473) upon TNF-α pre-incubation was lesser (Fig. 2A,C). In contrast, under identical experimental conditions, cells overexpressing Akt1/PKB-α, HepG2-CA-Akt/PKB, did not exhibit insulin resistance (Fig. 2D,F). GSK-3β is a downstream effector of Akt/PKB that is inactivated upon phosphorylation at Ser 9 [Cross et al., 1995]. The long-term pre-incubation of TNF-α in both parental HepG2 (Fig. 3A,C) and HepG2-CA-Akt/PKB (Fig. 3D,E) cells showed a decrease in the phosphorylation of GSK-3β (Ser 9) similar to Akt/PKB phosphorylation (Fig. 2). Although the decrease in the phosphorylation of Akt1 (Ser 473) followed a decrease in the phosphorylation of its downstream target, GSK-3β (Ser 9) upon long-term TNF-α pre-incubation (Figs. 2 and 3), the HepG2-CA-Akt/PKB cells (Fig. 3D,F) did not show decreased insulin responsiveness in terms of GSK-3β

phosphorylation at Ser 9 also compared to parental HepG2 cells (Fig. 3A,C). The reason for the decrease in the phosphorylation of Akt/PKB (Ser 473) (Fig. 2D,E) accompanied with no significant changes in the insulin response in HepG2-CA-Akt/PKB could be due to overexpression of the constitutively active Akt1/PKB-α (Fig. 2D,F). The level of inactive Akt/PKB after 24 h is high enough to respond to insulin treatment in these cells.

The cause of peripheral insulin resistance has been attributed to the impaired tyrosine kinase activity of IR based on biochemical studies performed on skeletal muscle biopsies from type 2 diabetes patients [Arner et al., 1987; Maegawa et al., 1991; Nolan et al., 1994]. Feinstein et al. [1993] reported that TNF-α (5 nM) treatment, for 1 h suppresses tyrosine phosphorylation of IR and its substrates, while Guo and Donner [1996] showed that in short-term exposure of TNF-α in 3T3 adipocytes promotes phosphorylation of IR and binding of IRS-1 to PI-3 kinase. Moreover, normal IR functions have been reported in spite of downstream abnormalities associated with insulin signaling cascade [Caro et al., 1987; Klein et al., 1995; Krook et al., 2000]. Our observation suggests that levels of IR-β subunit do not change significantly in comparison to control cells upon long-term pre-incubation of TNF-α in both parental HepG2 as well as HepG2-CA-Akt/PKB cells (Fig. 4A,B) ruling out the possibility of down-regulation of IR as the reason for reduced insulin resistance in parental HepG2 cells.

As a next step, we determined the levels of IRS-1 and -2 in order to understand the insulin resistance in parental HepG2 cells upon long-term pre-incubation of TNF-α. A sharp decrease in levels of IRS-1 with no alteration in the levels of IRS-2 was observed (Fig. 5). Four types of insulin receptor substrate (IRS) proteins have been reported IRS-1, -2, -3, and -4, of which IRS-1 and -2 are two major proteins involved in the insulin resistance [Sun et al., 1991]. Knockdown mouse models of IRS-1 and -2 confirm their indispensable roles in insulin action [Araki et al., 1994; Withers et al., 1998]. The glucose metabolism and growth rates were reduced by 50–60% in IRS-1 deficient mice. However, IRS-2-deficient mice develop the phenotype of type-2 diabetes due to insulin resistance and β-cell failure [Kubota et al., 2000; Masaki et al., 2004]. IRS-1 and -2 deficient mice are insulin resistant and hyperinsulinemic [Miki et al., 2001]. Dual knockdown of IRS-1 and -2 in mice were associated with decrease in the Akt and FOXO1 phosphorylation [Taniguchi et al., 2005]. Rondinone et al. [1997] showed that adipocytes from type 2 diabetic patients showed marked reduction in IRS-1 protein without any change in IRS-2 levels suggesting alternate docking roles for IRS-2. Moreover, it was suggested that binding of PI-3 kinase to IRS-2 required higher insulin concentrations than that needed for a similar binding to insulin and this may be key to resistance to insulin action [Rondinone et al., 1997]. HepG2-CA-Akt/PKB cells also showed reduced IRS-1 protein levels without affecting IRS-2 levels (Fig. 5A,C). However, these cells did not exhibit insulin resistance as parental cell line, the reason for this behavior could be that these cells overexpressing constitutively active Akt1/PKB-α show characteristics similar to parental HepG2 cells stimulated with insulin [Gupta et al., 2004] and certainly a possibility that IRS-2 could act as an alternative docking protein to IRS-1 remains.

The attenuation of insulin signaling in response to TNF-α has been attributed to enhanced phosphorylation of IRS at Ser residue (312 in human or 307 in rat) leading to impaired activation of PI-3 kinase-dependent insulin signaling pathway [Aguirre et al., 2000, 2002; Rui et al., 2001; Gao et al., 2002]. IRS proteins are activated upon the phosphorylation at Tyr residues. The other regulation is provided by the phosphorylation of Ser residues which results in the prevention of Tyr phosphorylation in IRS proteins [Hemi et al., 2002; Hers et al., 2002]. The data presented in Figure 6 indicate that the long-term pre-incubation of TNF-α indeed reduces the insulin responsiveness in terms of Tyr phosphorylation of IRS-1 and IR-β subunit. It was observed that the HepG2-CA-Akt/PKB cells show higher basal Tyr phosphorylation of IR-β and IRS-1 compared to parental HepG2 cells. Moreover, hyperphosphorylation of IRS-1 (Ser 312) at 24 h of TNF-α pre-incubation was observed. Greene et al. [2003] have suggested that Ser 312 phosphorylation could lead to IRS-1 degradation. Some of the same molecules involved in metabolic actions of insulin have been proposed to play a feed back inhibition of the insulin signaling and cellular insulin resistance [Birnbaum, 2001; White, 2002]. It is possible that in long-term pre-incubation, TNF-α induces hyperphosphorylation of IRS-1 at Ser 312 and this in turn is

responsible for degradation of IRS-1, therefore reduced insulin responsiveness. However, HepG2-CA-Akt/PKB cells overexpressing constitutively active form of Akt/PKB can overcome this hyperphosphorylation of IRS-1 (Ser312) which is not the case in parental HepG2 cells. This could be the reason for higher insulin resistance in parental HepG2 cells.

The present observation supports the fact that TNF-α-mediated attenuation of insulin signaling involves downregulation of IRS-1, a key component of insulin signal transduction cascade. The downregulation and hyperphosphorylation of IRS-1 (Ser 312) along with reduced phosphorylation of Tyr in IR-β subunit affect the phosphorylation of its downstream targets Akt1/PKB-α followed by GSK-3β in parental HepG2 cells. The overexpression of constitutively active Akt1/PKB-α in HepG2 cells resists this TNF-α-mediated attenuation but could not overcome the downregulation of IRS-1 or its hyperphosphorylation at Ser 312. The unaltered levels of IRS-2 upon long-term TNF-α pre-incubation and its role as alternate docking proteins for insulin action remain a possibility to be further explored.

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