Orjinal Araştırma Makalesi/Original Paper

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p38 MAPK Attenuates Insulin Signaling by Inhibiting IRS1 Tyrosine Phosphorylation in IRS1 Overexpressed 293T Cells

p38 MAPK, IRS1 Ekspresyonu Arttırılmış 293T Hücrelerinde IRS1 Tirozin Fosforilasyonunu İnhibe Ederek İnsülin Sinyalini Yavaşlatmaktadır

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ÖZET

Amaç: Bu çalışmanın amacı IRS1 ekspresyonu arttırılmış 293T hücrelerinde p38 MAPK' ın insülin sinyali üzerindeki etkisinin belirlenmesidir.
Materyal Metot: 293T hücreleri pcDNA3.1flag-tagged-insan-IRS1 ekspresyon vektörü ile transfekte edilmiştir. p38 MAPK inhibitörü ve aktivatörü olarak sırasıyla anisomisin ve SB203580 kullanılmıştır. IRS1, AKT ve ERK1/2 ekspresyon ve aktivasyon profilleri western blot ile belirlenmiştir.
Bulgular: Anisomisin muamelesi IRS1 Ser/Thr fosforilasyonu aracılığıyla insülin indüklü IRS1 tirozin fosforilasyonunu azalmıştır. Anisomisin muamele grupta kontrol grubuna oranla insülin indüklü AKT ve ERK1/2 fosforilasyonlarında da azalma olduğu belirlenmiştir. Anisomisinin insülin sinyali üzerindeki negatif etkilerinin SB203580 muamelesi ile geri döndürüldüğü gözlemlenmiştir.
Sonuç: p38 MAPK, IRS1, AKT ve ERK1/2 fosforilasyonlarını inhibe etmektedir. p38 MAPK, insülin direncinde terapötik hedef olarak kullanılabilir ve spesifik p38 MAPK inhibitörleri, terapötik yaklaşımlar için potansiyel ve umut verici ajanlar olabilir.
Anahtar kelimeler: p38 MAPK, IRS1, İnsülin sinyali, İnsülin direnci

ABSTRACT

Objective: The aim of this study was to investigate the effect of p38 MAPK on insulin signaling in IRS1 overexpressed 293T cells.
Materials and Methods: 293T cells were transfected with pcDNA3.1flag-tagged-human-IRS1 expression vector. Anisomycin and SB203580 were used as p38 MAPK activator and inhibitor respectively. Expression and phosphorylation profiles of IRS1, AKT and ERK1/2 were detected by western blotting.
Results: Anisomycin treatment led to decrease in insulin induced tyrosine phosphorylation of IRS1 through Ser/Thr phosphorylations. We also detected insulin induced AKT and ERK1/2 phosphorylations reduced by anisomycin compared to insulin treated group. We observed that negative effects of anisomycin on insulin signaling reversed by the treatment of SB203580.
Conclusion: p38 MAPK inhibits the phosphorylations of IRS1, AKT and ERK1/2. p38 MAPK may serve as a therapeutic target for insulin resistance and specific p38 MAPK inhibitors might be potential and promising agents for the therapeutic approaches
Key words: p38 MAPK, IRS1, Insulin signaling, Insulin resistance

INTRODUCTION

The p38 MAPK kinases are stress-activated serine/threonine protein kinases and belongs to the mitogen-activated protein kinase (MAPK) family, which also includes JNK, ERK5 and ERK1/2 (Zhang et al., 2015). The p38 MAPK family consists of four isoforms known as p38α, p38β, p38γ and p38δ. Although, p38α and p38β are widely expressed in human tissues, p38γ and p38δ show tissue restricted pattern (Schindler et al., 2007). The p38 members are mainly activated by stress-inducing conditions such as UV irradiation, proinflammatory cytokines, reactive oxygen species, free fatty acids and high

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glucose level in the cells. Activated p38 members regulates several cellular process such apoptosis, proliferation, survival, inflammation and stress responses (Wang et al., 2016). Recent studies have shown that, the members of p38 MAPKs also plays an important role in glucose metabolism (Geier et al., 2005; McGee et al., 2006; Liu et al., 2014; Pereira et al., 2016). The p38α regulates the hepatic glucose production and may contribute to uncontrolled hepatic gluconeogenesis in diabetes (Cao et al., 2005; Collins et al., 2006). Kidney is another organ that has a crucial role in glucose production in fasting state and p38α induces the glucose production in kidney (Feifel et al., 2002). Although, the role of p38 in glucose metabolism has been shown, there are still controversial results in the literature on this issue. Some studies have shown that p38α has a positive effect on glucose uptake during exercise in skeletal muscles. On the other hand, its activation decreases glucose uptake in adipose tissue (Liu et al., 2009). Therefore, understanding the tissue specific effect of p38α is crucial to clarify the molecular pathogenesis of metabolic disorders such as insulin resistance, Type 2 diabetes and obesity.

The IRS1 is the main docking protein which regulates the glucose homeostasis and is activated by insulin receptor or insulin-like growth factor receptor (IGFR) through the tyrosine phosphorylation at multiple residues at its C-terminal (Gorgisen et al., 2017). By in large, activity of IRS1 is regulated by posttranslational modifications. Although tyrosine phosphorylation of IRS1 activates the insulin signaling and results in activations of AKT and ERK1/2, serine/threonine phosphorylations generally inhibit the signal transduction depending on the phosphorylation sites of IRS1. Serine/Treonine phosphorylations of IRS1 are induced by downstream targets of IRS1 as a negative feedback mechanism or unrelated pathways. Dysregulation of IRS1 Ser/Thr phosphorylations is the common mechanism in insulin resistance and Type 2 diabetes (Boura-Halfon, 2009; Hancer et al., 2014). Tyrosine phosphorylation level of IRS1 significantly decrease in Type 2 diabetes patients due to increased Ser/Thr phosphorylation level (Gorgisen et al., 2016) Several studies have shown that proinflammatory cytokines TNF-α and stress activated kinase JNK inhibits tyrosine phosphorylation of IRS1 by promoting its serine phosphorylations (Ozes et al., 2001; Tanti et al., 2009). Although p38 MAPK is induced by TNF-α and cellular stress and regulates the glucose metabolism, its role in insulin signaling in kidney is still controversial.

In this study, we aimed to examine the role of p38α on insulin signaling in IRS1 overexpressing 293T cells.

MATERIAL AND METHODS

Material

Anisomycin and SB203580 were purchased from Sigma-Aldrich (Sant Louis MO). Reagents were obtained from the following sources: monoclonal anti-IRS1, antiphosphotyrosine, anti-ERK1/2, anti-pERK1/2, anti-AKT antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-pAKT, anti-phospho-p38 MAPK and anti-p38 MAPK antibodies were from Cell Signaling (Beverly, MA); monoclonal Beta-Actin antibody was from Sigma (Sant Louis MO); anti-rabbit HRP and anti-mouse HRP were purchased from Bio-Rad (Hercules, CA).

Cell Culture, Transfection and Treatments

293T embryonic kidney cells were grown in DMEM supplemented with 10% FBS, 100 mg/ml penicillin, 50 mg/ml streptomycin, and 1 mM glutamine. Sixty to seventy percent confluent 293T in 100-mm tissue culture plates were transfected with 15 µg of flag-tagged pcDNA3.1 or flag-tagged pcDNA3.1 human IRS1 by the calcium phosphate precipitation method. After 16 h, the cells were shocked with medium containing %10 glycerol, and cultured in complete DMEM for 24 h, then cells were serum-starved for 16 h, and treated with anisomycin (10 µg/ml) SB203580 (2 µM) and insulin (100ng/ml) for indicated times. Expression of IRS1 and mutants were verified by western blotting using anti-IRS1antibody.

Western Blotting

Control and transfected cells were treated with insulin and lysed in Triton X-100 buffer containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.15 units/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A. For western blot analysis 100 µg proteins were fractionated by 10% or 12% SDS-PAGE, then transferred to PVDF membranes. The

membranes were blocked in 1% BSA in PBST. The blots were first labeled with phospho-specific antibodies for p38 MAPK, IRS1, AKT and ERK. Labeled blots were then stripped off and re-labeled with antibodies which detect all phospho-forms of these proteins. Beta-actin was used as loading control for these blots. Signal intensity on blots was determined using the enhanced chemiluminescent detection system. Student t-test was used for the statistical analyses.

RESULTS

Effects of anisomycin on insulin signaling in IRS1 overexpressing 293T cells

Anisomycin treatment induced the phosphorylation of p38 MAPK as early as 15 min. post-treatment and reached to maximum after 1 hour treatment in 293T cells (Figure 1).

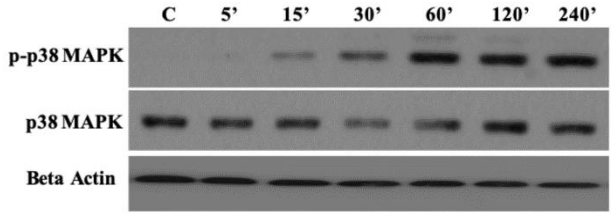

Figure 1: Time curve for the activation of p38 MAPK by anisomycin.

In IRS1 overexpressing 293T cells, insulin induced 11-fold increase in tyrosine phosphorylation of IRS1 and anisomycin treatment decreased tyrosine phosphorylation of IRS1 by 33% and expression level of IRS1 by 60%, compared to control group (Figure 2a). After determining the effects of anisomycin on IRS1, we wanted to determine whether these changes directly affect the expressions and activations of downstream targets of IRS1 such as AKT and ERK1/2. Insulin stimulation increased AKT phosphorylation by 7-fold and anisomycin treatment reduced this phosphorylation by more than 50%. Interestingly, we also found that although anisomycin treatment did not affect the phosphorylation level of AKT in the absence of insulin, we observed 50% increase in the expression level of AKT in anisomycin treated cells compared to control group, (Figure 2b). Since ERK1/2 are also phosphorylated after insulin stimulation, we also determined the effect of anisomysin on insulin induced ERK1/2 phosphorylations. As shown in Figure 2c, the impact of anisomycin on insulin-induced ERK1/2 phosphorylation followed the same pattern. Insulin treatment induced the phosphorylations of ERK1/2 by 2.8-fold, and anisomycin treatment inhibited this more than 50%. However, contrary to what we observed with AKT levels, anisomycin treatment not only increased but decreased the expression level of ERK1/2 nearly 50%, compared to control group (Figure 2c).

Effects of SB203580 on insulin signaling in IRS1 overexpressed 293T cells

Contrary to the negative effects of anisomycin on insulin-induced tyrosine phosphorylation of IRS1, the p38α inhibitor SB203580 showed positive effect. Insulin stimulation induced 20-fold increase in IRS1 tyrosine phosphorylation and pretreatment of cells with SB203580 elevated this to 45-fold (Figure 3a). Although, SB203580 treatment alone did not affect tyrosine phosphorylation of IRS1 it increased level of IRS1 by 60%, and addition of insulin to SB203580-treated cells increased the level of IRS1 by more than 2-fold (Figure 3a).

Contrary to what we observed for IRS1 phosphorylation, pretreatment of 293T cells with SB203580 did not potentiate insulin-induced AKT phosphorylation, in fact, SB203580 treatment slightly reduced phosphorylation of AKT compared to insulin treatment group. (Figure 3b). However, SB203580 and insulin treatment alone increased the level of AKT compared to control group and their combination even further increased AKT level although it was not statistically significant (Figure 3b).

When we looked at the effect of SB203580 on insulin-induced ERK1/2 phosphorylation we observed that SB203580 treatment did not potentiate ERK1/2 phosphorylation after insulin stimulation, in fact, SB203580 treatment caused 2-fold decrease background pERK1/2 level. However, neither insulin nor SB203580 had any effect on the expression level of ERK1/2 (Figure 3c).

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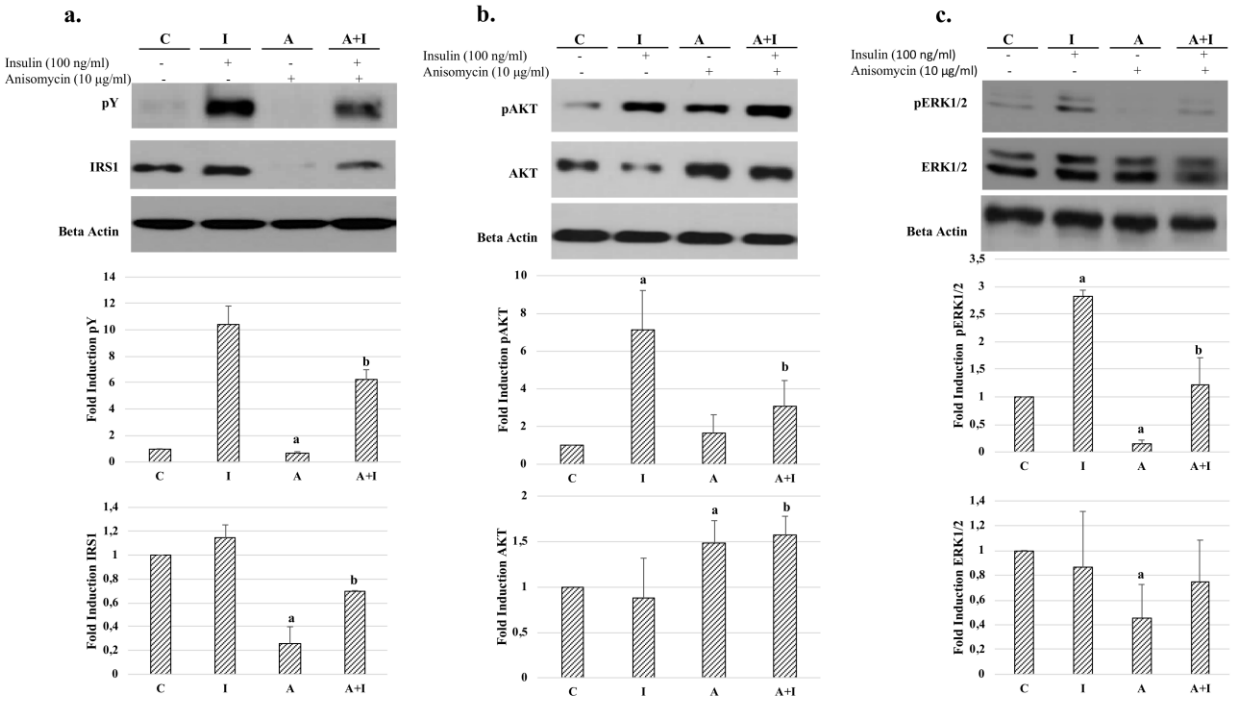

Figure 2: a. Effects of anisomycin on expression and insulin-induced tyrosine phosphorylation of IRS1 and; b. Effects of anisomycin on expression and insulin-induced Ser473 phosphorylation of AKT and; c. Effects of anisomycin on expression and insulin-induced ERK1/2 phosphorylation of ERK1/2.

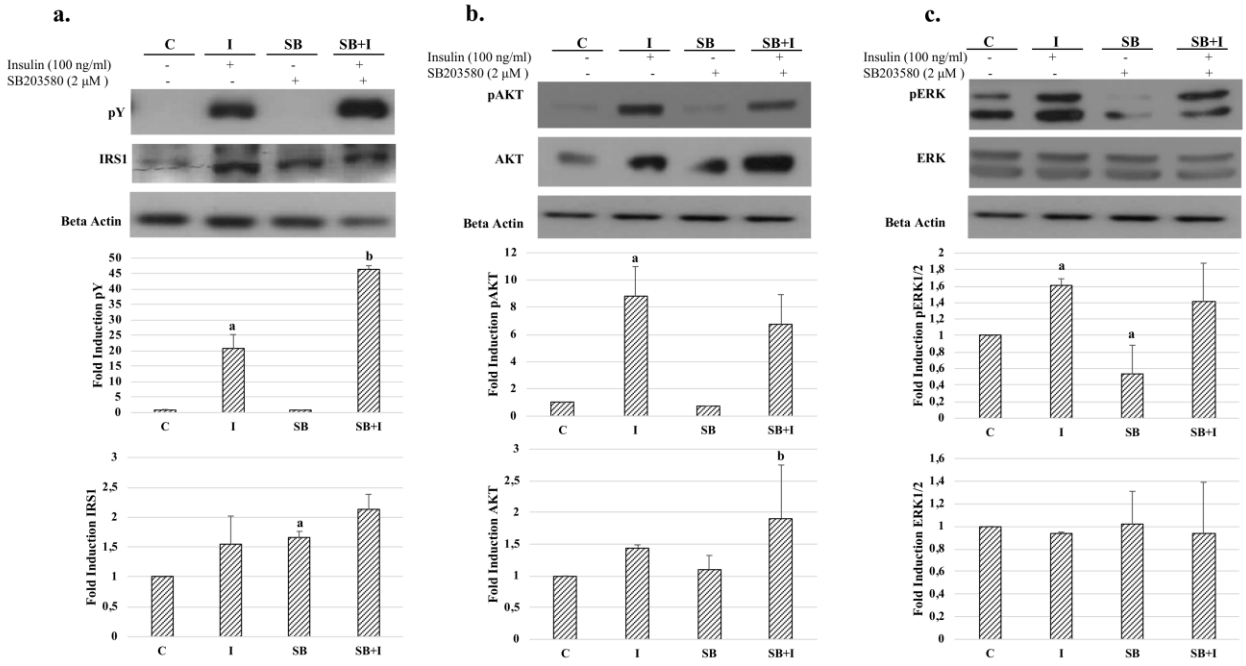

Figure 3: a. Effects of SB203580 on expression and insulin-induced tyrosine phosphorylation of IRS1; b. Effects of SB203580 on expression and insulin-induced Ser473 phosphorylation of AKT; c. Effects of SB203580 on expression and insulin-induced phosphorylation of ERK1/2.

DISCUSSION

Previous studies showed that activation of p38α significantly inhibits the insulin signaling (Fang et al., 2019), but the molecular mechanism behind this effect was not elucidated. In the present study, we investigated the effects of activator and inhibitor of p38α on insulin-induced phosphorylations of IRS1, AKT and ERK1/2 in 293T cells overexpressing IRS1.

In agreement with previous observations, our results showed that anisomycin treatment inhibits insulin-induced tyrosine phosphorylation of IRS1 compared to insulin treated group in 293T cells overexpressing IRS1. In previous studies several groups showed that insulin-induced tyrosine phosphorylation of IRS1 was significantly supressed by various pathological conditions which caused phosphorylations of IRS on Ser/Thr residues (Guo et al., 2014). It is well documented that Anisomycin promotes the activation of p38s, JNKs and in turn JNK stimulates the phosphorylation of IRS1 at Ser307, Ser636 and Ser639 positions and these phosphorylations inhibit the IRS1-dependent signaling (Copps et al., 2012). In addition to direct effect on IRS1, JNK may also induce IRS1 Ser/Thr phosphorylations by activating mTOR, which phosphorylate IRS1(Ozes et al., 2001; Hiratini, 2005). In addition to JNK and mTOR-mediated Ser/Thr phosphorylation, anisomycin-induced activation or ectopic expression p38α also caused significant inhibition of tyrosine phosphorylation of IRS1 through increased the Ser/Thr phosphorylation in HepG2 cells (Hemi et al., 2011).

According to our result, in addition to having significant inhibitory effect on insulin-induced tyrosine phosphorylation, anisomycin treatment significantly decreased baseline level of IRS1. Our result indicates that anisomycin treatment may induce the degradation of IRS1 via Ser/Thr phosphorylation. To further support above findings, we used inhibitor of p38α. As anticipated, SB203580 treatment showed an opposite effect of anisomycin on tyrosine phosphorylation and expression IRS1. Therefore, our results strongly suggest that activation of p38α directly or indirectly attenuates insulin-induced tyrosine phosphorylation IRS1 and this effect is reversed by p38 MAPK inhibitors.

MAPK pathways communicate with other pathways and create a wide network in signal tranduction (Cuenda et al., 2007). AKT is one of the main downstream targets of IRS1 and it is primarly responsible for the metabolic functions of insulin (Wang et al., 2016), therefore, determining the effect of activator/inhibitor of p38α on phosphorylation/expression of AKT was also very important. Indeed, we observed that while Anisomycin increased, insulin decreased the basal level of AKT. It seems that activation of p38α is stabilizing AKT protein, and insulin-mediated T-loop phosphorylations (S473/T308) are destabilizing AKT protein. Indeed, it was shown that activations of p38 and JNKs prevent T-loop phosphorylation of AKT by inducing phosphorylations of AKT at different sites. (Park et al., 2002) In another study showed that anisomycin-induced JNK activation attenuated Ser473 phosphorylation of AKT through p70S6K in retina (Miller et al., 2017). In a sense, our results supports previous findings and demonstrate that stress-inducing agents, such as anisomycin, can inhibit insulin signaling by modulating the phosphorylation/expression of IRS1 and its downstream targets directly.

To better understand as to how anisomycin implicated its effect, we used p38α inhibitor SB203580. As anticipated pretreatment of 293T cells with this inhibitor potentiated insulin-induced tyrosine phosphorylation of IRS, however, it did not only potentiated it decreased insulin-induced phosphorylations AKT and ERK1/2. Moreover, anisomycin-mediated stabilization of basal AKT level was not reverted by SB203580. These results clearly indicate that activations of JNKs also look important.

Under the light of these results, we are suggesting that Anisomycin's effect on insulin signaling is not completely dependent on p38α, but activation of JNK MAPKs also plays important role in suppression of insulin signaling.

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