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Role of PTEN in TNFα induced insulin resistance

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ABSTRACT

Aims/hypothesis: PTEN may play a reversible role in TNFα induced insulin resistance, which has been linked to obesity-associated insulin resistance (IR).
Methods: Western blots for PTEN and p-Akt were performed on H-411E liver cells incubated with insulin, TNFα, and in selected experiments VO-OHpic vanadium complex in the presence and absence of PTEN siRNA. Total PTEN was compared to β-actin loading control and p-Akt was compared to total Akt.
Results: Western blot and Real Time RT-PCR experiments showed increased PTEN after TNFα treatment ($p = 0.04$); slightly decreased PTEN after insulin treatment; and slightly increased PTEN after insulin + TNFα treatment. PTEN siRNA markedly inhibited the TNFα-induced increase in PTEN ($p < 0.01$) without significantly changing the p-Akt levels. The vanadium complex, exhibiting insulin-like effects, also significantly prevented the TNFα-induced increase in PTEN. Combining insulin and VO-OHpic was additive, providing both proof of concept and insight into mechanism.
Discussion: The PTEN increase due to TNFα treatment was reversible by both PTEN siRNA knockdown and VO-OHpic treatment. Thus, PTEN is identified as a potential new therapeutic target for reducing IR in Type 2 DM.

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1. Introduction

In the last 20 years, diabetes has sharply increased in all age, sex, and racial/ethnic groups and is only now beginning to level off according to available Center for Disease Control (CDC) data [1,2]. Most type 2 diabetics are obese and adipose tissue TNFα levels and IR increase in obese animals and man [3–6]. TNFα-null mice show reduced insulin levels reflecting increased insulin sensitivity and reduced fat pad weight despite a high fat diet. In experiments using human tissue(s) in culture, treatment with TNFα produced IR in adipocytes and hepatocytes, but not in primary skeletal myocytes [6–12]. As an extension of this, our group has previously shown that patients with DM2 being treated with anti-TNFα medications (etanercept and infliximab) demonstrated a reduction in fasting blood glucose (FBG), HbA_{1c}, and fasting plasma triglyceride (TG) values [13].

While others have shown that increased TNFα leads to IR, it is still unknown how this change occurs. Some proposed mechanisms include decreased INSR, increased lipolysis, decreased insulin-responsive glucose transporter 4 (GLUT4), decreased peroxisome proliferator-activator receptor gamma (PPARG), and changes in IRS-1 phosphorylation. TNFα phosphorylates the serines in IRS-1 essential for insulin signaling, leading to IR [6]. Our group has previously identified yet another possible mechanism, increased PTEN expression. Using proteomic and genomic assays, our group found two networks that were reciprocally changed by insulin and TNFα: insulin signaling and apoptosis [14]. Additionally, these two networks converge on a single protein, phosphatase and tensin homolog deleted on chromosome 10 (PTEN). These previous

Abbreviations: VO-OHpic, 3- hydroxypicolinate vanadium (IV) complex; CDC, Center for Disease Control; MEM, Eagles minimal essential medium; FBG, fasting blood glucose; TG, fasting plasma triglyceride; IR, insulin resistance; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PPARG, peroxisome proliferator-activator receptor gamma; PMSF, phenylmethylsulfonyl fluoride; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PVDF, polyvinylidene fluoride membrane; SFM, serum free medium.

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experiments strongly suggested that an increase in PTEN levels play a vital and opposing role in each of these major actions of TNFα: termination of insulin signaling and regulation of apoptosis [7,14].

In the current study, we analyzed the effects of various treatments including insulin, TNFα, 3- hydroxypicolinate vanadium (IV) complex (VO-OHpic), and siRNA PTEN on the levels of PTEN and p-Akt. While most phosphatase inhibitors have very general and non-specific inhibitory effects, VO-OHpic is currently the most specific chemical inhibitor of PTEN due to its non-competitive mechanism of inhibition [15,16]. This experiment used siRNA targeting PTEN mRNA to see the effects of a specific knockdown of PTEN on insulin signaling and IR. Our results show a central role for PTEN in TNFα induced IR in H-411E rat hepatoma cells with this form of IR to some extent mimicking IR as seen in obese human DM2.

2. Materials and methods

2.1. Cell culture

Minimally deviant hepatoma cells derived from rat liver (H-411E cells; ATCC, Manassas, Virginia) were cultured in 10 mL Eagles minimal essential medium (MEM) supplemented with 1% glutamine, 10% calf serum, and 5% fetal calf serum (FCS) at 37C under a humidified 5% CO_2/95% O_2 atmosphere using 100-mm dishes. After cells had grown from ~3.0 × 10^6 cells/plate to 80% confluency with ~15 × 10^6 cells/plate, medium was aspirated and cells were rinsed with serum free medium (SFM) before overnight incubation. Next, medium was replaced with 10 mL of fresh SFM 4 h before protein isolation. TNFα treatment involved 10 mL of SFM containing TNFα (1 mg/l) from R&D Systems (Minneapolis, Minnesota) in SFM before and after overnight incubation. After overnight incubation, insulin treatment involved 500 μL of insulin (10 000 mU/l) (Sigma–Aldrich, St Louis, Missouri) and VO-OHpic treatment involved 10 mmol/l VO-OHpic (Sigma–Aldrich, St Louis, Missouri).

2.2. Knock down of PTEN expression with siRNA

After overnight culture as above, H411E cells were incubated with 2 mM siRNA in antibiotic free complete culture media. Dharmafect reagent was used for siRNA transfection (Dharmacon Inc., Lafayette, CO). After 12 h, the transfected cells were treated as above.

2.3. Protein isolation and western blotting

Cells were washed with 5 mL PBS (pH 7.4), aspirated, and scraped with RIPA buffer (0.8 ml/plate) over ice, lysed (21-guage needle), and left on ice with 10 μl phenylmethylsulfonyl fluoride (PMSF) (100 mg/ml) for 45 min before centrifuging at 10,000 g at 4C for 10 min. Supernatant protein was separated by SDS-PAGE, electroblotted onto polyvinylidene fluoride membrane (PVDF), incubated with primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit IgG antibodies according to manufacturer's instructions (Santa Cruz), visualized with ECL Prime kit (Amersham-Pharmacia Biotech, Piscataway, New Jersey), and quantified using Image J [17].

2.4. RNA isolation

Total RNA from control and treated cells was extracted with TRIzol according to the manufacturers protocol (Invitrogen Life Technologies). cDNA synthesis performed according to manufacturer's instructions using 1 μg sample RNA (Superscript III, Invitrogen).

2.5. Real-Time quantitative RT-PCR

The primers for human PTEN were Forward: 5-CAA GAT GAT GTT TGA AAC TAT TCC AAT G-3 and Reverse: 5-CCT TTA GCT GGC AGA CCA CAA3. The mRNA levels were acquired from the value of the threshold cycle (Ct) of PTEN. β-Actin mRNA levels were used as a control.

3. Results

3.1. TNFα increases PTEN protein and mRNA levels in H-411E liver cells

We measured PTEN protein levels in H411E cells treated with TNFα in the presence and absence of insulin (Fig. 1A). These results were confirmed by real time quantitative PCR (Fig. 1B). Both studies are in complete agreement. Compared to control, insulin and insulin + TNFα did not change the expression of PTEN significantly. However, TNFα alone significantly increased PTEN protein and mRNA expression compared to control ($p < 0.05$).

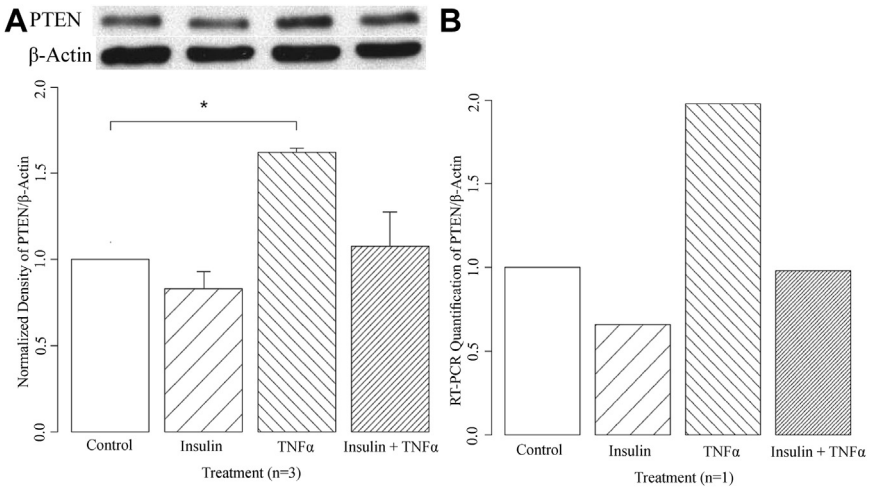

Fig. 1. PTEN expression in H411E cells following insulin and TNFα treatment. A: PTEN protein levels in insulin treated cells in the absence and presence of TNFα. Representative western blots along with a bar graph quantifying the band densities of the three PTEN blots normalized against β-Actin calculated as percentages of control. Data are expressed as mean ± SD (n = 3). B: Relative expression of PTEN mRNA in insulin treated cells with and without TNFα. The steady state levels of mRNA encoding PTEN were analyzed by Real Time RT-PCR (n = 1).

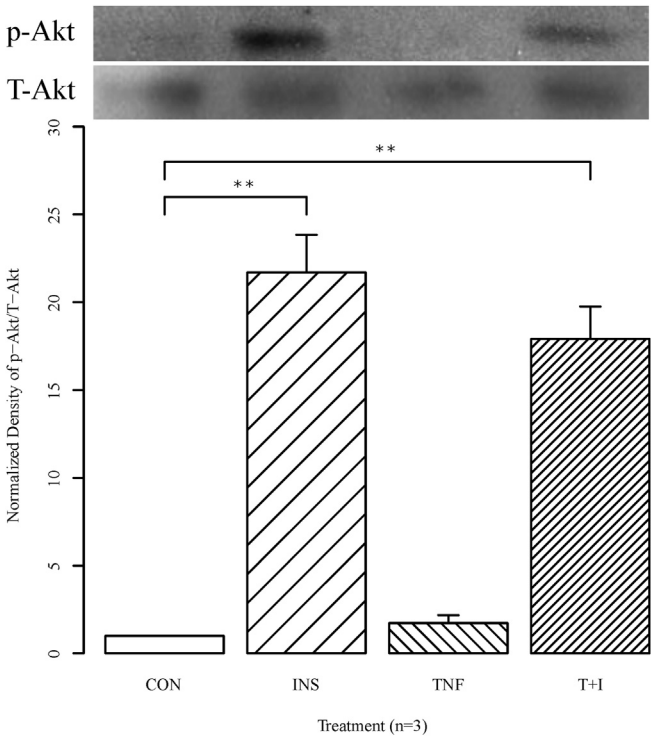

Fig. 2. p-Akt protein levels measured in insulin treated H411E cells in the presence and absence of TNFα. Representative western blots of p-Akt and total Akt (T-Akt) in response to insulin and TNFα are shown. Densitometric quantification of p-Akt expression normalized against T-Akt and calculated as percentages of control. Data are expressed as mean ± SD (n = 3).

3.2. Insulin increases p-Akt protein levels in H-411E liver cells

TNFα did not significantly change p-Akt levels compared to control; insulin + TNFα treatment did not significantly change p-Akt levels compared to insulin alone; however, insulin did significantly increase p-Akt compared to control with ($p = 0.012$) and without TNFα ($p = 0.011$) (Fig. 2). The absence of an inhibitory effect of TNFα on p-Akt in the combination experiment demonstrates the greater potency of insulin over TNFα in these experiments.

3.3. Knockdown of PTEN prevented TNFα induced increase in PTEN in H-411E liver cells

PTEN siRNA knockdown of PTEN by siRNA alone prevented the TNFα-induced increase in PTEN ($p < 0.01$) (Fig. 3A). The p-Akt and T-Akt analysis (Fig. 3B) confirmed that insulin increased p-Akt levels and revealed that neither TNFα nor siRNA knockdown of PTEN significantly changed p-Akt compared to the negative siRNA control.

3.4. VO-OHpic prevented the TNFα induced increase in PTEN in H-411E liver cells

VO-OHpic alone did not significantly change PTEN compared to control. However, VO-OHpic prevented the TNFα induced increase in PTEN with and without additional insulin treatment ($p < 0.05$) (Fig. 4).

4. Discussion

This study has confirmed that TNFα increases PTEN and insulin increases p-Akt in H-411E liver cells at both the mRNA and protein levels (Fig. 1). This TNFα-induced increase in PTEN was prevented by both VO-OHpic and siRNA knockdown of PTEN. Through non-competitive inhibition, VO-OHpic achieves more specific inhibition of PTEN than the general phosphatase inhibitors [15]. In addition to the *in vitro* specificity for PTEN previously reported, herein we show that part of the *in vivo* mechanism of VO-OHpic involves decreasing the amount of PTEN protein. This effect still supports the previous finding that VO-OHpic increased p-Akt signaling in NIH 3T3, L1 fibroblasts, and UmUc-3 epithelial cells [16].

Two recent papers have highlighted the importance of PTEN in obesity and diabetes [18,19]. Both PTEN haploinsufficiency patients and hibernating grizzly bears are overweight with healthy fat and show little evidence of insulin resistance. Before winter hibernation, grizzly bears store up to 60 pounds of fat. Despite being overweight, the bears are sensitive to insulin's antilipolytic effect promoting storage of fat as triglyceride. During hibernation, bears become insulin resistant, and epinephrine and glucagon break down fat for energy utilization. Within a few days of coming out of hibernation, the bears' metabolism reverts back to pre-hibernation. These changes in metabolism throughout the hibernation cycle

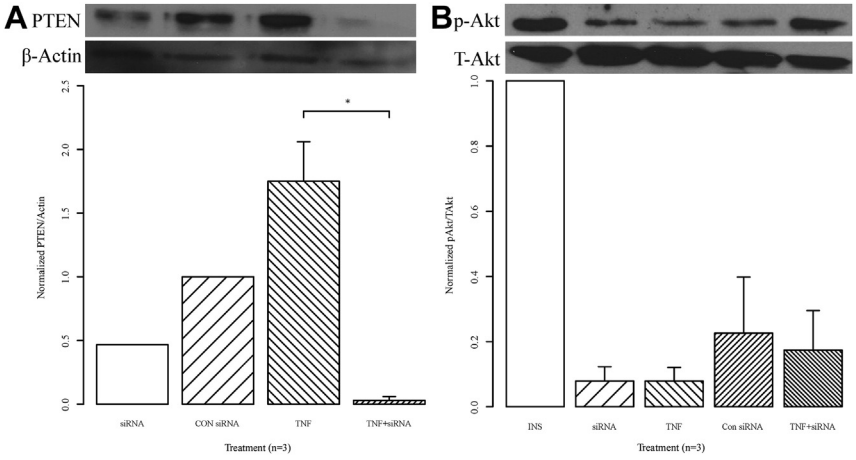

Fig. 3. siRNA knockdown of PTEN significantly prevented TNFα-induced increase in PTEN without affecting p-Akt levels. A: Representative western blot above with bar graph quantifying the PTEN and β-Actin antibody band densities calculated as percentages of control. Data are expressed as mean ± SD (n = 3). B: Representative western blot above with bar graph quantifying the p-Akt and T-Akt antibody band densities calculated as percentages of control. Data are expressed as mean ± SD (n = 3). Insulin was added as a positive control.

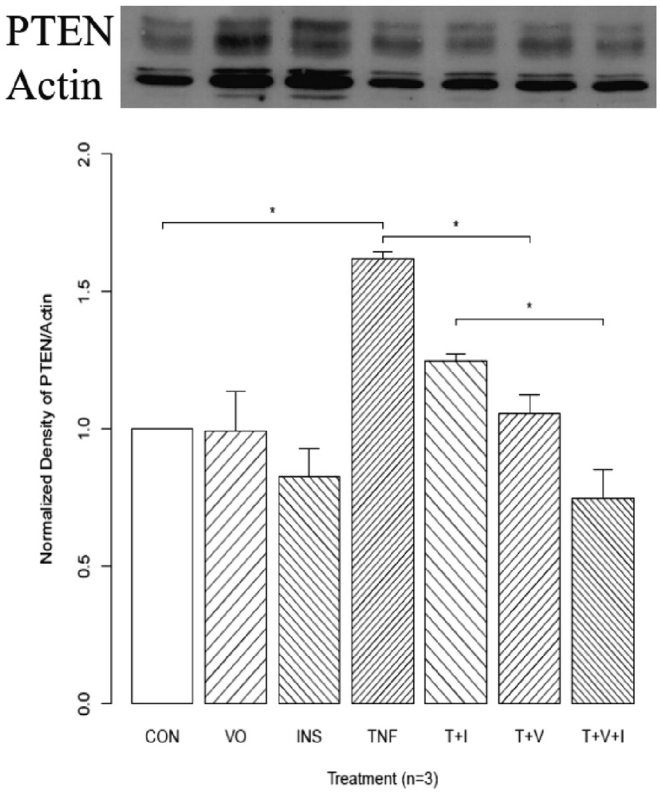

Fig. 4. VO-OHpic significantly prevented TNFα-induced increase in PTEN with and without insulin treatment. With the representative blot above and the quantified band bar plot below, PTEN levels are compared with β-Actin loading control normalized to control. Data are expressed as mean ± SD (n = 3).

were then shown to be mediated by the PTEN/p-Akt switch. It seems very reasonable that this switch is mediated through a cascade of hormones, which might be targeted by selective drug therapy. Both of these studies can be linked with the TNFα-induced IR tissue culture model presented here.

TNFα has previously been found to cause insulin resistance (IR) in many cells and is thought to be a key player in obesity-associated insulin resistance in humans [6]. Many mechanisms have previously been proposed for TNFα-induced IR, and our lab has recently found PTEN gene expression to be increased in TNFα treated H-411E rat hepatoma cells in tissue culture [14]. Using the same H-411E cell line as before, we have now shown that TNFα causes a significant increase in PTEN mRNA and protein. The increase in PTEN protein might sensitize TNFα's ability to induce apoptosis through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). These findings identify yet another part of the critical mechanism whereby TNFα induces apoptosis. Thus, PTEN has been identified as a central player in both insulin signaling/resistance and apoptosis. PTEN is then a key therapeutic target for IR in obesity-associated DM2 or metabolic syndrome. It is likely that drugs inhibiting PTEN in insulin-sensitive tissue potentially represent a new approach which would likely be additive in effect to other existing therapies.

Conflict of interest

The authors declare no conflict of interest.

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Contribution statement

SS conceived the study and secured its support. DB drafted the article. SS, GM, and DB designed the study. GM, DB, JC, and SC obtained the data. SS, GM, DB, JC, and SC interpreted the data. All authors reviewed the article and approved the final draft.

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