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TNF α and SOCS3 regulate IRS-1 to increase retinal endothelial cell apoptosis

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Abstract

Rates of diabetes are reaching epidemic levels. The key problem in both type 1 and type 2 diabetes is dysfunctional insulin signaling, either due to lack of production or due to impaired insulin sensitivity. A key feature of diabetic retinopathy in animal models is degenerate capillary formation. The goal of this present study was to investigate a potential mechanism for retinal endothelial cell apoptosis in response to hyperglycemia. The hypothesis was that hyperglycemia-induced TNF α leads to retinal endothelial cell apoptosis through inhibition of insulin signaling. To test the hypothesis, primary human retinal endothelial cells were grown in normal glucose (5 mM) or high glucose (25 mM) and treated with exogenous TNF α , TNF α siRNA or suppressor of cytokine signaling 3 (SOCS3) siRNA. Cell lysates were processed for Western blotting and ELISA analyses to verify TNF α and SOCS3 knockdown, as well as key pro- and anti-apoptotic factors, IRS-1, and Akt. Data indicate that high glucose culturing conditions significantly increase TNF α and SOCS3 protein levels. Knockdown of TNF α and SOCS3 significantly increases anti-apoptotic proteins, while decreasing pro-apoptotic proteins. Knockdown of TNF α leads to decreased phosphorylation of IRS-1^{Ser307}, which would promote normal insulin signaling. Knockdown of SOCS3 increased total IRS-1 levels, as well as decreased IR^{Tyr960}, both of which would inhibit retinal endothelial cell apoptosis through increased insulin signaling. Taken together, our findings suggest that increased TNF α inhibits insulin signaling in 2 ways: 1) increased phosphorylation of IRS-1^{Ser307}, 2) increased SOCS3 levels to decrease total IRS-1 and increase IR^{Tyr960}, both of which block normal insulin signal transduction. Resolution of the hyperglycemia-induced TNF α levels in retinal endothelial cells may prevent apoptosis through disinhibition of insulin receptor signaling.

Keywords

Retinal endothelial cells; Apoptosis; Insulin resistance; TNF α ; Suppressors of cytokine signaling; Diabetes

1. Introduction

In the United States, 8.3% of the population (25.8 million people) are living with diabetes, with another 7 million undiagnosed people and 79 million people living with pre-diabetes (ADA website). While there are 2 types of diabetes, 90% of people have type 2 diabetes, non-insulin dependent diabetes. Unfortunately, both type 1 and type 2 diabetes produce the same severe complications (heart disease, kidney disease, vision loss, and amputation), costing the United States over 174 million dollars in 2007 (JDRF website). The end-organ damage from either form of diabetes results from either an inability to produce insulin (type 1) or the inability to properly process insulin in the tissues, termed insulin resistance (type 2). In addition to its ability to regulate circulating glucose levels, insulin also serves a number of other important functions, for example regulating growth, inhibiting cell death, and controlling muscle function through glucagon production. Insulin acts as an anti-apoptotic factor through activation of insulin receptor signaling. Phosphorylation of the insulin receptor leads to activation of the insulin receptor substrate complexes (IRS-1, IRS-2, IRS-3, IRS-4), and phosphorylation of Akt. Akt decreases the cleavage of caspase 3 to block apoptosis [1,2].

One of the major complications of diabetes in the retina is death of cells in the ganglion cell layer [3-5]. Since the ganglion cells are responsible for transducing vision to the central nervous system, it is clear that hyperglycemia decreases electrical activity of the retina in animal models of diabetes [5,6]. Additionally, degenerate capillaries and pericyte ghosts are key to the hypoxia noted in diabetic retinopathy in animal models [7-9]. Work has indicated that insulin treatment in whole retinal samples in vivo or in retinal explants will increase Akt activity, primarily through IRS-2 actions [10,11]. We have recently found that β -2-adrenergic receptors regulate insulin receptor signaling through IRS-1 actions to inhibit apoptosis in retinal Müller cells (Walker et al., in press, Molecular Vision). This followed our previous findings that β -adrenergic receptors can significantly decrease TNF α levels, which are increased in hyperglycemic conditions in retinal Müller cells [12,13]. We found similar results in retinal endothelial cells [14,15]. Taken together, these findings suggest that dysfunctional insulin signaling may be involved in a number of the complications of diabetic retinopathy.

Insulin signaling, as well as apoptosis, can be regulated by a large number of divergent pathways. There is clear data that the cellular markers of diabetic retinopathy in animals can be prevented through the use of Enbrel, a TNF α receptor inhibitor or in TNF α knockout animals [16,17]. In many other tissues, TNF α (and other cytokines) is linked with insulin resistance, common to type 2 diabetes [18,19]. Furthermore, there is strong data from adipocytes that TNF α can inhibit insulin receptor signaling through IRS-1^{Ser307} phosphorylation [20,21]. It is unclear if such a pathway exists in retinal endothelial cells, as well as the potential signaling cascades involved linking TNF α to altered retinal endothelial cell apoptosis.

In addition to TNF α , the suppressors of cytokine signaling (SOCS) proteins have been suggested to be involved in insulin resistance through disruption of IRS-1 [22,23]. In obese

mice, work has shown that SOCS1 and SOCS3 respond to increased TNF α levels [23]. The mammalian SOCS family contains 8 members, which include the cytokine-induced Src homology 2 (SH2) containing protein (CIS) and SOCS1-7 [24]. SOCS proteins respond to a variety of cytokines, hormones, and some growth factors [24,25]. Additionally, factors that activate the janus kinase (JAK) pathway also will increase SOCS1 and SOCS3 levels [24]. SOCS1 and SOCS3 are involved in the reparative abilities of ciliary neurotrophic factor (CNTF) for retinal ganglion cells following optic nerve injury [25,26]. In streptozotocin-induced type 1 diabetic rats, increased SOCS protein levels and decreased Akt phosphorylation occurred concurrently in retinal cells, suggesting that these proteins may be involved in the diabetic retina [27]. SOCS proteins in other cell types can have two different actions on the insulin-IRS-1 complex. Increased SOCS3 levels inhibited insulin receptor phosphorylation and coupling to IRS-1, such that insulin receptor tyrosine phosphorylation is reduced in rat hepatoma cells [28]. The other mechanism by which SOCS proteins can promote cell death is through IRS-1 degradation [29]. TNF α can increase SOCS1 and SOCS3 levels, which promotes proteosomal degradation of IRS-1 (and IRS-2) in HEK293 cells [29]. Work in adipocytes (3T3-L1) cells demonstrated that SOCS3 is involved in insulin resistance [30]. In contrast, work in 3T3-L1 adipocytes showed that TNF α -induced increases in SOCS3 levels were not sufficient to induce IRS-1 degradation [31]; however in this study, the authors find that TNF α stimulation caused IRS-1 degradation at a high level, with only minimal increases in SOCS3 mRNA and protein levels [31].

In the present study, we hypothesized that hyperglycemia-induced TNF α leads to increased retinal endothelial cell apoptosis through SOCS3 activation. To address this hypothesis, we used TNF α siRNA and SOCS3 siRNA for investigations of retinal endothelial cell apoptosis in both normal (5 mM) and high (25 mM) glucose. Work also included investigations of IRS-1 and insulin receptor signaling proteins.

2. Materials and methods

2.1. Cell culture

Human retinal endothelial cells were acquired from Cell Systems Corp (Kirkland, WA). Cells were grown in M131 medium supplemented with microvascular growth supplement at either 5 mM glucose (NG) or 25 mM glucose (HG). Cells were grown to 80–90% confluence before serum starvation for 18–24 hours in the appropriate glucose conditions. Some dishes were collected and used as non-treated controls (NT).

Once cells were serum starved, some dishes of cells in each glucose condition were treated with 10 ng/ml recombinant TNF α (R&D Systems, Minneapolis, MN) for 30 minutes. In additional experiments, cells in normal glucose and high glucose were transfected with scrambled siRNA, 20 nM TNF α siRNA (Dharmacon, Lafayette, CO) or 20 nM SOCS3 siRNA (Dharmacon, Lafayette, CO) using lipofectamine RNAimax (Invitrogen) transfection reagent for 24 hours. Prior to use for experiments, cell lysates from siRNA-treated cells were processed to verify successful knockdown using a TNF α ELISA (Fisher) or SOCS3 Western blotting.

2.2. Western blotting

After the appropriate treatment, cells were collected into lysis buffer and processed for Western blotting as we have done in the past [32,33]. Briefly, following cell lysis, protein concentrations are obtained using the Bradford assay. Western blotting was completed; membranes were exposed to primary antibodies to SOCS3 (1:500, Cell Signaling, Danvers, MA), total and phosphorylated Akt (Ser 473, 1:500, Cell Signaling, Danvers, MA), cytochrome c (1:500, Cell Signaling, Danvers, MA), Bax (1:500, Cell Signaling, Danvers, MA), Bcl-xL (1:500, Cell Signaling, Danvers, MA), total and phosphorylated IRS-1 (Ser307, 1:500, Cell Signaling, Danvers, MA), total and phosphorylated insulin receptor (Tyr960, 1:500, Cell Applications, San Diego, CA) overnight at 4 °C. Secondary antibodies conjugated to horseradish peroxidase utilized were anti-mouse (1:5000) or anti-rabbit (1:5000; Promega, Madison, WI). Antibody-antigen interactions were visualized using enhanced chemiluminescence (ECL Reagent; Amersham Biosciences, Little Chalfort, England). Densito-metric analysis was carried out using Kodak Image Station 4000MM. Data are expressed as mean densitometry in arbitrary units (A.U.) and in the case of phosphorylated proteins, data are expressed as a ratio of phosphorylated protein levels to total protein levels in arbitrary units. Using Prism software, mean densitometry numbers or the ratio of mean densitometry of phosphorylated protein to total protein was used to compare data from untreated cells (NT) to treated cells with $P < 0.05$ being accepted as significant.

2.3. ELISA analyses

A TNF α ELISA (Fisher, Pittsburgh, PA) and a caspase 3 ELISA (Cell Signaling, Danvers, MA) were used according to the manufacturer's instructions, except that equal protein loading was used for the cleaved caspase 3 to allow for analyses using the optical density (O.D.) values.

2.4. Statistics

Data from Western blot band intensity or protein levels from ELISA analyses were compared using a Kruskal–Wallis non-parametric test, followed by Dunn's testing. Comparisons were made to normal glucose untreated for all samples and between siRNA-treated or TNF α -treated to untreated samples.

3. Results

3.1. TNF α increases SOCS3 protein levels, while decreasing total IRS-1 levels

Based upon our hypothesis, increased TNF α levels observed in response to high glucose should increase SOCS3 levels, such that either TNF α or high glucose treatment leads to the increased SOCS3 protein levels observed in Fig. 1A ($*P < 0.05$ vs. NG not-treated), which is eliminated when cells are treated with TNF α siRNA (Fig. 1C, $\#P < 0.05$ vs. HG NT). SOCS3 has been reported to lead to increased phosphorylation of insulin receptor on tyrosine 960, which eliminates insulin receptor/IRS-1 binding in hepatoma cells [28]; therefore one would expect increased SOCS3 levels and increased phosphorylation of insulin receptor on tyrosine 960 compared to untreated high glucose treated retinal endothelial cells. This

response should be eliminated when TNF α siRNA is applied to REC cultured in high glucose, suggesting this is a TNF α -mediated event. Indeed, treatment with TNF α increased IR^{Tyr960} phosphorylation, which was reduced when TNF α is knocked down (Fig. 1D, # P <0.05 vs. HG scsiRNA).

3.2. TNF α increases IRS-1^{Ser307} in REC

While increased SOCS3/IR^{Tyr960} is one pathway by which TNF α may induce insulin resistance and REC apoptosis [28], TNF α can also increase serine 307 phosphorylation on IRS-1 to insulin receptor signaling, eliminating Akt activation [20]. Data in the REC treated with high glucose and TNF α show increased IRS-1^{Ser307} (Fig. 2, * P <0.05 vs. NG NT), while cells treated with TNF α siRNA have significantly reduced IRS-1^{Ser307} levels (Fig. 2, # P <0.05 vs. HG NT and HG TNF α).

3.3. Knockdown of TNF α decreases cell death proteins and increases cell survival proteins in cells cultured in high glucose

Since both increased IRS-1^{Ser307} or increased SOCS3/IR^{Tyr960} levels can induce insulin resistance, leading to cell death, we measured cell death proteins (caspase 3, cytochrome c, Bax) and cell survival proteins (Akt, Bcl-xL) in REC treated in normal and high glucose alone and following TNF α siRNA application. Analyses of all key cell death/survival proteins suggest that knockdown of TNF α can eliminate the effects of hyperglycemia on REC (Fig. 3).

3.4. Knockdown of SOCS3 produces the same responses in cell death/cell survival proteins as knockdown of TNF α

In order to dissect out the pathway utilized by TNF α , we investigated the same cell survival/cell death proteins we assessed following TNF α siRNA application. We found that SOCS3 siRNA applied to REC cultured in high glucose led to increased cell survival proteins (Akt, Bcl-xL) and decreased cell death markers (cytochrome C, Bax, caspase 3) compared to REC treated with high glucose only (Fig. 4).

3.5. SOCS3 regulates total IRS-1 protein levels and insulin receptor phosphorylation on tyrosine 960

Since hyperglycemic culturing conditions increased TNF α to increase SOCS3, we used SOCS3 siRNA to determine whether SOCS3 is directly involved in the changes in total IRS-1 (leading to degradation) or insulin receptor phosphorylation on tyrosine 960 (inhibiting insulin receptor/IRS-1 binding). Data indicate that knockdown of SOCS3 leads to a significant increase in total IRS-1 levels (Fig. 5, # P <0.05 vs. HG scsiRNA treated REC), suggesting that SOCS3 directly regulates IRS-1 levels in REC cultured in high glucose. This is further supported by data in REC cultured in high glucose treated with SOCS3 siRNA and probed for IR^{Tyr960} (inhibitory site to IR/IRS-1 binding). In high glucose conditions, IR^{Tyr960} is increased, which would decrease insulin receptor/IRS-1 binding (Fig. 6 * P <0.05 vs. NG) but it is inhibited after SOCS3 siRNA application (Fig. 6, # P <0.05 vs. HG only), suggesting that SOCS3 does inhibit IR/IRS-1 binding activities.

4. Discussion

4.1. TNF α in insulin resistance in type 2 diabetes

Diabetic retinopathy is the leading cause of blindness in working age adults. While a plethora of potential factors are involved in the retinal damage, ultimately, the disease results from dysfunctional insulin production or insulin processing. Others have demonstrated that the retina has insulin receptors [34] and that diabetes significantly decreases normal insulin receptor actions [10,35]. Using in vivo and ex vivo retinal explants, Reiter et al. found that altered IRS-2 to Akt signaling was key to insulin signaling in the retina [10]. Work in retinal Müller cells in vitro demonstrated that altered phosphorylation of IRS-1^{Ser307} may be linked to Müller cell death during hyperglycemia (Walker et al., in press). In the present findings, high glucose induced increased IRS-1^{Ser307} phosphorylation in human retinal endothelial cells, which was eliminated through knockdown of TNF α . While a number of pathways are likely involved in the pathogenesis of insulin resistance, TNF α appears to be a key player. Work in multiple systems has suggested that obesity and many of the markers of type 2 diabetes are associated with increased TNF α levels [19,36]. Key work on the effects of TNF α in insulin resistance came from work on adipocytes [37], which revealed that TNF α preferentially phosphorylates IRS-1^{Ser307}, which is inhibitory to insulin receptor signaling [20,38]. Work in obese mice with characteristics of type 2 diabetes and a null mutation for TNF α show improved insulin sensitivity [39], again suggesting that TNF α is a factor in insulin resistance. Interestingly, many of the drugs currently on the market to treat type 2 diabetes have a component involving a reduction in TNF α levels [40,41].

4.2. SOCS3 in insulin resistance

While TNF α can directly inhibit insulin signaling through its phosphorylation of IRS-1^{Ser307}, it also can activate the SOCS proteins [30], which can also block insulin signaling. In our work, we show that high glucose leads to increased SOCS3 levels in retinal endothelial cells. Activation of SOCS3 can lead to insulin resistance in 2 separate ways: increased insulin receptor phosphorylation on tyrosine 960 [42] or through IRS-1 degradation by proteasomes [29]. In these experiments with knockdown of SOCS3, we found that SOCS3 likely inhibits insulin signaling in both ways in retinal endothelial cells, as we found decreased IR^{Tyr960} phosphorylation and increased total IRS-1 levels following SOCS3 siRNA application. Taken together, these data suggest that hyperglycemia-induced TNF α increases SOCS3 levels and actions in human retinal endothelial cells, which likely contributes to increased insulin resistance and endothelial cell death.

4.3. Retinal endothelial cell apoptosis

Insulin resistance induces a number of problems within the target organs, including cardiovascular diseases [36], general inflammation [43], periodontal disease [36], as well as altered glucose tolerance [44]. Key to a number of these problems is cell death. Work in the endothelial cell specific insulin receptor knockout mice revealed that dysfunctional insulin signaling produced altered vasoactive mediators and vascular tone [45]. We have previously found that hyperglycemia induces retinal endothelial cell death [46]. Additional experiments revealed that hyperglycemia reduced insulin receptor phosphorylation in these cells [47] or

in whole retinal lysates from diabetic animals [6]. These early findings led us to question whether the altered TNF α response in retinal endothelial cells during hyperglycemia played a role in the observed apoptosis. Since work in adipocytes suggested that TNF α can inhibit insulin signaling through phosphorylation of IRS-1^{Ser307}, we pursued these studies to investigate whether hyperglycemia-induced TNF α levels produced increased retinal endothelial cell death through altered insulin signal transduction. This is associated with altered phosphorylation of IRS-1, such that insulin receptor signaling is inhibited with increased levels of proapoptotic proteins in human retinal endothelial cells. Since we observe similar responses of pro- and anti-apoptotic proteins following TNF α or SOCS3 siRNA application, the data suggest that both TNF α and SOCS3 pathways are involved in retinal endothelial cell apoptosis. Since there are targeted drugs for TNF α receptor actions, which are effective against degenerate capillary formation in the diabetic retina [16], future work will investigate whether blockade of hyperglycemia-induced TNF α is effective in eliminating insulin resistance or whether co-inhibition of TNF α and SOCS3 is required to prevent retinal endothelial cell apoptosis.

4.4. Other interpretations

While we did find that TNF α and SOCS3 are involved in insulin resistance in retinal endothelial cells and apoptosis, TNF α was induced by hyperglycemia or given exogenously. For a physiologically relevant study, a co-culture of retinal Müller cells and retinal endothelial cells would be preferable, since Müller cells likely produce the TNF α present in the retina and retinal Müller cells do possess insulin receptors [13]. Additionally, we focused this study on the role of TNF α and SOCS3. Any number of cytokines can activate the SOCS proteins, potentially leading to insulin resistance. Future work on physiological insulin resistance will focus on in vivo studies of the type 2 diabetic rat retina, as well as other key cell types in the retina.

5. Conclusions

This study found that retinal endothelial cell death was increased in response to hyperglycemia likely due to increased TNF α and SOCS3 proteins. SOCS3 inhibits insulin receptor signaling through both phosphorylation of the insulin receptor on tyrosine 960, as well as degradation of IRS-1 by the proteasome pathway. TNF α induces SOCS3 protein, as well as inhibits insulin signaling through phosphorylation of IRS-1^{Ser307}. Taken together, these studies provide a potential mechanism by which both TNF α and SOCS3 induce insulin resistance and endothelial cell apoptosis in the diabetic retina.

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Fig. 1.

Panel A. Bar graph and representative blots for SOCS3 in retinal endothelial cells cultured in normal glucose (NG) or high glucose (HG) and treated with nothing (NT) or treated with TNF α (TNF α). Panel B. Bar graph of TNF α levels to demonstrate successful knockdown of TNF α levels with TNF α siRNA in both normal and high glucose. Panel C. SOCS3 levels in REC cultured in normal glucose or high glucose and treated with TNF α or TNF α siRNA. Panel D. Total and phosphorylated insulin receptor (Tyr 960) in REC cultured in normal glucose (NG) or high glucose (HG) and treated with TNF α or TNF α siRNA. For all panels, * P <0.05 vs. NG NT, # P <0.05 vs. HG NT or scsiRNA. N=3–4 independent assays for each panel.

Fig. 2.

Bar graph and representative blot of total and phosphorylated IRS-1 on serine 307 in REC cultured in normal glucose (NG) or high glucose (HG) and treated with nothing (NT), TNF α (TNF α) or TNF α siRNA. * P <0.05 vs. NG NT, # P <0.05 vs. HG NT. N =4 independent dishes.

Fig. 3.

Bar graphs and representative blots for cytochrome C, Bax, Bcl-xL and Akt, as well as ELISA data for cleaved caspase 3 for cells cultured in normal glucose (NG) or high glucose (HG) and treated with scrambled siRNA (scsiRNA) or TNF α siRNA. Top panels are apoptotic factors, while Bcl-xL and Akt are anti-apoptotic proteins. * $P < 0.05$ vs. NG scsiRNA, # $P < 0.05$ vs. HG scsiRNA. $N = 3$.

Fig. 4.

Bar graphs and representative blots for cytochrome C, Bax, Bcl-xL and Akt, as well as ELISA data for cleaved caspase 3 for cells cultured in normal glucose (NG) or high glucose (HG) and treated with scrambled siRNA (scsiRNA) or SOCS3 siRNA. Top panels are apoptotic factors, while Bcl-xL and Akt are anti-apoptotic proteins. * $P < 0.05$ vs. NG scsiRNA, # $P < 0.05$ vs. HG scsiRNA. $N = 3$.

Fig. 5.

Panel A. Representative blot and bar graph to show successful SOCS3 knockdown using SOCS3 siRNA. Panel B. Bar graph and blot for total IRS-1 levels in REC cultured in normal glucose (NG) and high glucose (HG) and treated with scrambled siRNA (scsiRNA) or SOCS3 siRNA. * $P < 0.05$ vs. NG scsiRNA, # $P < 0.05$ vs. HG scsiRNA. N=3.

Fig. 6.

Bar graph and blot for phosphorylated insulin receptor on tyrosine 960 and total insulin receptor in REC cultured in normal glucose (NG) and high glucose (HG) and treated with scrambled siRNA (scsiRNA) or SOCS3 siRNA. * $P < 0.05$ vs. NG scsiRNA, # $P < 0.05$ vs. HG scsiRNA. $N = 3$.