Thioredoxin Regulates Adipogenesis through Thioredoxin-interacting Protein (Txnip) Protein Stability

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Txnip (thioredoxin-interacting protein) is a critical mediator of metabolism and adipogenesis in vivo. The mechanisms of action of Txnip are believed to operate at least in part by inhibiting the redox signaling functions of thioredoxin. We tested here whether Txnip suppressed adipogenesis by inhibiting thioredoxin and discovered a reversal of roles; Txnip inhibits adipogenesis directly, and thioredoxin binding regulates Txnip by enhancing Txnip protein stability. Unlike Txnip, a Txnip mutant that cannot bind thioredoxin (C247S) did not prevent adipocyte differentiation, but was degraded more quickly by proteasomal targeting. Finding that endogenous Txnip protein is also rapidly degraded at the onset of adipogenesis suggested that Txnip degradation is required for adipocyte differentiation. Thioredoxin overexpression stabilized Txnip protein levels to inhibit adipogenesis, and adipogenic stimuli such as insulin promoted Txnip-thioredoxin dissociation to the more labile free Txnip state. As an α-arrestin protein, Txnip has two C-terminal tail PPXY motifs that mediate E3 ubiquitin ligase binding and Txnip protein stability. Mutating the PPXY motifs prevented Txnip degradation, even when thioredoxin binding was lost, and restored the ability of C247S Txnip to inhibit adipogenesis. These studies present a novel reconsideration of Txnip-thioredoxin signaling by showing that thioredoxin regulates the intrinsic function of Txnip as an inhibitor of adipogenesis through protein stabilization.

Txnip, or thioredoxin-interacting protein, critically regulates metabolism and the cell redox state, and it binds thioredoxin (TXN) and inhibits TXN disulfide reductase activity (1–3). The diverse physiologic and developmental roles of Txnip include regulation of glucose and lipid homeostasis, glycolytic-aerobic metabolic switching, nutrient sensing, and adipogenesis (4–10). Evidence is also mounting for the role of Txnip in diabetes pathogenesis. Glucose and insulin reciprocally stimulate and suppress Txnip expression, and its expression correlates with insulin resistance in humans. Txnip is directly linked to inflammasome activation and inhibits insulin-stimulated glucose uptake into fat and muscle (8, 11). Furthermore, glucotoxicity and β-cell apoptosis are spared in mouse Txnip deletion models, and Txnip-null mice are protected from dietary and genetically induced peripheral insulin resistance despite having increased adiposity (5, 7, 12).

The mechanism(s) of action of Txnip in these diverse events is unclear. Attention has mainly focused on the well defined capacity of Txnip to inhibit TXN; however, in vivo models consistently fail to show altered TXN activity or directly correlate TXN function to changes in cell signaling (9, 13, 14). Although in vitro studies suggest that Txnip competitively displaces TXN from other signaling molecules, Txnip may promote TXN dissociation by altering cellular redox through undefined mechanisms. Supporting this, a single Txnip cysteine-to-serine mutation (C247S) abolishes TXN binding and catalytic inhibition yet still suppresses glucose uptake and glycolytic metabolism like Txnip (15, 16). Unlike Txnip, however, C247S Txnip fails to stimulate hepatic gluconeogenesis (13), implying that some of the functions of Txnip are TXN-dependent, whereas others are TXN-independent.

Txnip is one of six structurally related mammalian arrestin domain-containing proteins (ArrDC), described sometimes as “α-arrestins” to distinguish them from the structurally related visual/β-arrestins (17). Txnip is the only α-arrestin that interacts with and inhibits TXN. Little is known about the other mammalian α-arrestins. One member, ArrDC4, also suppresses glucose uptake and glycolysis (15), whereas ArrDC3 has been shown to regulate β2 adrenergic receptor ubiquitination as an E3 ubiquitin ligase adaptor (18). Insights into α-arrestin functions may be derived from well described β-arrestin biology. β-Arrestins are diverse multifunctional scaffolds that desensitize G-protein-coupled receptor signaling while regulating downstream signaling (19, 20). β2-arrestin was also recently shown to play a role in insulin receptor signaling and the development of insulin resistance in mice (21). α-Arrestins and β-arrestins differ most in their C-terminal tails; β-arrestins contain clathrin association motifs, whereas α-arrestins contain PPXY motifs that bind ubiquitin ligases (22). Txnip has two C-terminal tail PPXY motifs that bind the E3 ligase Itch, which mediates Txnip protein stability (23). A relationship between TXN and Itch binding could thus represent a potential mechanism whereby the redox-sensitive interaction between Txnip and TXN might modulate the stability or intrinsic signaling properties of Txnip as a ubiquitin ligase adaptor.
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To clarify what aspects of Txnip functions are TXN-dependent or -independent, we examined the role of Txnip in adipocyte development, hypothesizing that Txnip affects adipogenesis through inhibition of TXN function. Surprisingly, we learned that TXN affects intrinsic Txnip functions by mediating Txnip protein stability. We show here that Txnip protein degradation, as promoted by TXN dissociation, is a key step in the progression of adipogenesis and that impairing Txnip pro teaseomal targeting blocks adipogenesis even when TXN binding is prevented. These data reveal the intrinsic redox-independent functional property of Txnip as an inhibitor of adipocyte development and reveal a novel mechanism by which the TXN redox state regulates adipogenesis.

EXPERIMENTAL PROCEDURES

Reagents and Plasmid Construction—Unless indicated, all reagents were obtained from Sigma-Aldrich except cell culture reagents, which were obtained from Invitrogen. Anti-Txnip antibodies were from MBL International (JY2), anti-human thioredoxin1 was from BD Pharmingen (TRX 1–104), and anti-mouse TXN1 was from Cell Signaling. Untagged human Txnip and Txnip C247S lentiviral vectors were as described previously (13). Full-length Txnip and C247S species were sub cloned, absent the initiating methionine, into the pCDH-CMV-MCS-EF1-GreenPuro backbone (System Biosciences) modified to express an N-terminal FLAG epitope (DYKDDDDK). Human Txnip PPXY-to-AAXA and human thioredoxin C32S and C35S substitution mutations were made by the “Round the Horn” site-directed mutagenesis technique. 5’-abutting sense and antisense primers were designed with mutated residues and C35S substitution mutations were made by the “Round the Horn” site-directed mutagenesis technique.

Cell Culture and Adipocyte Differentiation—HEK 293TN cells were obtained from System Biosciences (Mountain View, CA). 3T3-L1 cells were obtained from ATCC (Manassas VA) and were differentiated by standard DMI induction, as described (5). Transient transfection was achieved with PureFection (System Biosciences). Lentiviral pseudovirus production and cell transduction were as described (5). Stable cDNA-expressing cell lines were obtained 96 h after viral transduction by antibiotic selection with 5 μg/ml puromycin and maintained thereafter in medium containing 2 μg/ml puromycin.

Detection of Txnip-TXN Complexes—Cells were fast acid-precipitated in 0.1g/ml trichloroacetic acid in PBS and reacted with 25 mM N-ethylmaleimide, and labeled proteins were resolved by non-reducing SDS-PAGE as described (15).

Statistical Analysis—Data are reported as means ± S.E. Two sample comparisons were made by two-sided Student’s t tests.

Additional Methods—More detailed methods are presented in the supplemental data.

RESULTS AND DISCUSSION

Txnip Exhibits Both TXN-dependent and TXN-independent Functions in Adipocytes—3T3-L1 adipocytes were used to define the mechanisms of action of Txnip as Txnip regulates multiple aspects of adipocyte biology including respiration, lipogenesis, insulin action, and adipocyte differentiation (5, 8).

We used the Txnip single mutant (C247S), which neither binds TXN nor inhibits the antioxidant functions of TXN, to test the TXN-independent effects of Txnip in adipocytes (16). As we had shown that C247S suppresses glucose uptake in the same way as Txnip (15), we first asked whether TXN binding affected insulin sensitivity during adipocyte glucose uptake. 2-Deoxyglucose (2DG) transport was measured with increasing doses of insulin in differentiated 3T3-L1 adipocytes transduced with Txnip and C247S lentivirus. C247S Txnip was equivalent to Txnip in suppressing basal 2DG uptake and insulin-stimulated 2DG transport over three logs of insulin relative to a GFP control lentivirus (Fig. 1A, *p < 0.05 relative to GFP).

To clarify what aspects of Txnip functions are TXN-dependent or -independent, we examined the role of Txnip in adipocyte development, hypothesizing that Txnip affects adipogenesis through inhibition of TXN function. Surprisingly, we learned that TXN affects intrinsic Txnip functions by mediating Txnip protein stability. We show here that Txnip protein degradation, as promoted by TXN dissociation, is a key step in the progression of adipogenesis and that impairing Txnip pro teaseomal targeting blocks adipogenesis even when TXN binding is prevented. These data reveal the intrinsic redox-independent functional property of Txnip as an inhibitor of adipocyte development and reveal a novel mechanism by which the TXN redox state regulates adipogenesis.
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This indicated that the loss of TXN binding did not alter the effect of Tnip on glucose transport nor insulin responsiveness. Another α-arrestin that neither binds nor inhibits TXN, ArrDC4, also suppressed insulin-stimulated glucose uptake in the same way as Tnip and C247S Tnip (Fig. 1B). Thus, suppression of insulin-stimulated glucose uptake is a conserved function of at least some arrestin domain-containing proteins that is independent of TXN inhibition.

We then tested whether the known inhibition of adipogenesis by Tnip was TXN-dependent. Overexpressing Tnip in preadipocytes prevents adipocyte differentiation, whereas both Tnip gene silencing and gene deletion enhance adipogenesis (5). Tnip, C247S Tnip, and ArrDC4 were stably introduced into 3T3-L1 cells, and preadipocytes were differentiated by standard DMI induction (8). Surprisingly, although both Tnip and ArrDC4 overexpression suppressed adipogenesis, C247S Tnip had no effect relative to GFP-expressing control cells (Fig. 1C). Varying the adipogenic stimulant components to alter potency and pathway selectivity did not reveal any distinction between C247S and the GFP control (Fig. 1C). We considered two possible explanations. 1) Some aspects of adipocyte biology require TXN inhibition by Tnip, such as adipogenesis, whereas others are TXN-independent, such as glucose transport; or 2) C247S Tnip protein is less stable than Tnip under certain conditions, such as hormonal stimulation of adipogenesis.

Tnip Is Rapidly Degraded at the Onset of Adipogenesis by Hormone Induction—To address the hypothesis that Tnip degradation plays a role in adipogenesis, we first examined endogenous Tnip protein levels in 3T3-L1 preadipocytes after DMI induction. Tnip protein dramatically disappeared within minutes after DMI stimulation (Fig. 2A); the rapidity suggested Tnip protein degradation. As Tnip is subject to robust transcriptional regulation, we also examined RNA levels at the same time points. Unlike Tnip protein, Tnip mRNA levels increased 2-fold over the first hour after DMI (1.7 ± 0.3-fold when normalized to TATA binding protein transcript levels, p < 0.05, n = 4), supporting stimulated protein turnover.

We next looked at Tnip protein levels following DMI treatment of 3T3-L1 cells overexpressing Tnip versus C247S Tnip. Tnip protein was more resistant to DMI-induced turnover in the Tnip-overexpressing cells relative to C247S (Fig. 2B) despite higher transgene mRNA expression in the C247S Tnip 3T3-L1 cells (2.0 ± 0.5-fold when normalized to TATA binding protein transcript levels, p < 0.05, n = 3). As enhanced C247S Tnip degradation relative to Tnip could explain these observations, we hypothesized that TXN may play a critical role in Tnip protein stability and that one function of the adipogenic stimuli is to promote Tnip turnover to facilitate adipocyte differentiation.

TXN Inhibits Tnip Proteasomal Degradation—To determine whether TXN binding stabilized Tnip, plasmids carrying Tnip and C247S were transfected into HEK 293TN cells, and protein levels were followed after inhibition of protein synthesis (Fig. 3A). C247S Tnip levels declined more precipitously than Tnip: Tnip protein was nearly twice that of C247S Tnip at 4 h after treatment with cycloheximide, when normalized to uninhibited levels (Tnip 37 ± 0.3% versus 21 ± 0.7% for C247S Tnip, p < 0.05, n = 5). Proteasomal inhibition prevented both Tnip and C247S Tnip disappearance after cycloheximide treatment, indicating that proteasomal targeting and degradation was responsible for C247S Tnip protein turnover (Fig. 3B), as has been shown for Tnip (23, 24).

We then tested whether co-expression of TXN would prevent Tnip but not C247S Tnip degradation. Uniform amounts of Tnip or C247S Tnip were transfected into 293TN cells with increasing amounts of TXN or catalytically inactive C32S and C35S TXN. Cys42 and Cys45 are TXN vicinal active site cysteines that participate in target protein disulfide reduction by intermolecular disulfide complex formation and disulfide exchange (25). Tnip protein levels were potently increased in parallel with increased TXN expression, whereas the TXN active site mutants had no significant effect on Tnip levels (Fig. 3C). Furthermore, neither TXN nor C32S and C35S TXN altered C247S Tnip protein levels, supporting the concept that Tnip-TXN complex formation with catalytically active TXN promotes Tnip protein stability.

TXN Stabilizes Tnip Protein in 3T3-L1s and Impairs Adipogenesis—We next hypothesized that overexpressing TXN in 3T3-L1 cells would stabilize Tnip protein as it had in 293TN, and in turn, inhibit adipocyte differentiation. TXN and GFP control-expressing 3T3-L1 lines were generated by lentiviral transduction and antibiotic selection. TXN overexpression attenuated the rapid degradation of Tnip following DMI induction relative to GFP control 3T3-L1 (Fig. 4A). As with 293TN results, the catalytically inactive C35S TXN mutant did not impede DMI-stimulated Tnip degradation like TXN (Fig. 4B).

Resolving TXN and GFP control 3T3-L1 lysates under non-reducing conditions revealed a new supershifted ~110-kDa species immunoreactive to both TXN and Tnip (Fig. 4C). TXN overexpression blunted the disappearance of the supershifted complex relative to GFP controls after DMI induction. The apparent size of this Tnip-TXN species, twice that predicted for Tnip (~45 kDa) + TXN (~12 kDa), suggested that a higher-order dimerized Tnip-TXN complex was formed. β-Arrestin dimerization has been described and proposed to serve as an inactivating “storage form” or a mechanism contrib-
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We then tested whether stable TXN overexpression would also suppress adipogenesis. As predicted, 3T3-L1 overexpressing TXN accumulated markedly less lipid than GFP control cells (Fig. 4D). Consistently, catalytically inactive TXN species failed to suppress adipogenesis (Fig. 4D). These data support the concept that complex formation with a catalytically active TXN promotes Txnip protein stability, which may play a central role in 3T3-L1 adipogenic progression.

Adipogenic Stimulants Decrease the Stability of the Txnip-TXN Complex—Intact Txnip-TXN complexes can be trapped using a free sulfhydryl alkylation method, allowing comparison of the relative abundance of free Txnip to TXN-complexed Txnip (15). Using this approach, we tested whether complexed Txnip was more stable than free Txnip and how adipogenic stimulants influence complexed and free Txnip stability.

Transfected cells were then co-incubated with cycloheximide and the proadipogenic stimulants insulin and IBMX, an inhibitor of phosphodiesterase activity that increases cellular cyclic adenosine monophosphate (cAMP) levels. cAMP is also known to stimulate Txnip degradation through proteasomal targeting (8, 24). IBMX and insulin decreased both complexed and free Txnip levels when compared with vehicle-treated controls (Fig. 5B and supplemental Fig. 1B). Forskolin, an activator of adenylate cyclase, and the cAMP analog 8-Br-cAMP also decreased complexed and free Txnip when compared with untreated controls, similar to IBMX (Fig. 5B). This suggested that insulin and cAMP decreased the stability of both com-
plexed and free Txnip. Complexed and free Txnip levels were examined over a broader time course in the presence of IBMX and were found to decline at the same rate (Fig. 5C). Thus, 3T3-L1 adipogenic stimulants enhanced Txnip degradation by altering the stability of the Txnip-TXN complex. This also suggests a second mechanism by which these agents might promote adipogenesis, enhancing Txnip degradation to promote adipogenesis. However, this model did not allow us to determine whether cAMP and insulin promoted Txnip degradation through complex dissociation, enhanced free Txnip degradation, proteasomal targeting of the intact Txnip-TXN complex, or all of the above.

The PPXY E3 Ubiquitin Ligase Association Motifs of Txnip Control Txnip Degradation in the Absence of TXN Binding—As the previous studies could not distinguish between complex dissociation versus complex degradation, we generated degradation-resistant Txnip and C247S Txnip species to test whether insulin and cAMP promoted complex dissociation. α-Arrestins contain two C-terminal PPXY motifs that bind WW motifs notably present in the Nedd4-like family of E3 ubiquitin ligases (26). The PPXY motifs of Txnip were recently shown to be critical for binding the E3 ligase Itch, which in turn regulates Txnip ubiquitination and degradation (23). We mutated each PPXY motif to AAXA separately or in combination for both Txnip and C247S Txnip, generating eight different Txnip or C247S Txnip species (Fig. 6A). Protein stability was tested for each after treatment with cycloheximide. Neither individual AAXA mutation significantly improved Txnip or C247S Txnip protein stability (Fig. 6B, AAXA1 or AAXA2); however, the double AAXA mutation markedly enhanced both Txnip and C247S protein stability (Fig. 6B, AAXA1,2). Complex trapping by NEM alkylation confirmed that C247SAA1,2 did not restore binding with TXN (supplemental Fig. 1C), indicating that TXN binding was not essential to ensure Txnip protein stability. It also suggested a mechanism by which the TXN-Txnip complex might prevent Txnip proteasomal targeting, either by interfering with the association of Txnip with the E3 ligase Itch or by preventing the effective targeting by Itch of Txnip to the proteasome. In a basal state, β-arrestin C-terminal tails are tethered to the arrestin N-domain, obscuring C-terminal protein-protein interaction motifs. β-Arrestin activation promotes conformational changes that release the C terminus, which increases accessibility to binding partners (27). As an α-arrestin, the C terminus of Txnip may be similarly tethered, preventing E3 ubiquitin ligase association, and TXN binding to a Cys residue in the arrestin C-domain could inhibit conformational changes to unmask the PPXY motifs; however, as the structure of Txnip is not yet solved and the β-arrestin residues that tether the C-tail are not conserved in α-arrestins (17), this is speculative.

We then used TxnipAA1,2 to test whether insulin and IBMX promoted Txnip dissociation from TXN. In this context,
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FIGURE 7. Txnip inhibits adipocyte differentiation independent of TXN. A, FLAG epitope-tagged Txnip and C247S double AAXA mutants were stably expressed in 3T3-L1 by lentiviral transduction. Transgene protein levels were assayed by anti-FLAG staining following DMI induction for the indicated time points. B, adipocyte formation for 3T3-L1 stably expressing Txnip mutant transgenes. Lipids were stained with Oil Red O at day 10 after DMI induction.

Txnip-TXN dissociation would increase the ratio of free TxnipAA1,2 to TXN-complexed TxnipAA1,2, conversely, a primary effect that enhanced proteasomal targeting would not affect the ratio. Consistent with complex dissociation, insulin treatment stimulated the gradual decline of the TxnipAA1,2–TXN complex with a reciprocal rise in the formation of the free TxnipAA1,2 species, whereas the total amount of free TxnipAA1,2 + complexed TxnipAA1,2 was unchanged (Fig. 6C). A similar dose-response effect was seen for insulin (Fig. 6D), and IBMX also produced the same insulin-mediated effect of shifting the complex to the free TxnipAA1,2 species relative to the vehicle control-treated cells. Thus, adipogenic stimulants contribute to Txnip degradation by promoting Txnip-TXN dissociation.

Impairing Txnip Ubiquitin Ligase Binding Inhibits Adipogenesis in the Absence of TXN Binding—The non-degradable Txnip species also allowed us to directly challenge the role of Txnip degradation in adipocyte development and whether TXN binding is essential to this function. Introducing the non-degradable C247SAA1,2 species into 3T3-L1 should have no effect on differentiation if TXN binding and inhibition are critical Txnip functions. Conversely, if the primary role of TXN in adipocyte development is to promote Txnip stability, then we expected that this Txnip species, which neither binds TXN nor undergoes proteasomal targeting, would effectively suppress adipogenesis. Lentiviral TxnipAA1,2 and C247SAA1,2 were stably expressed in 3T3-L1s with N-terminal FLAG epitope tags to distinguish them from endogenous Txnip. No appreciable DMI-induced degradation was seen for either TxnipAA1,2 or C247SAA1,2 species, consistent with the effects seen in 293TN cells (Fig. 7A). This confirmed that adipogenic stimulants promoted Txnip degradation by augmenting proteasomal targeting.

We then tested whether the double AAXA mutations prevented adipocyte development in 3T3-L1 cells and whether TXN inhibition was important. As seen previously, Txnip-expressing cells suppressed adipocyte formation, whereas C247S Txnip cells showed no discernable inhibition of adipocyte formation (Fig. 7B). TxnipAA1,2-expressing 3T3-L1 cells were more potent in suppressing adipocyte development than Txnip. This likely reflected that TxnipAA1,2 was not susceptible to proteasomal degradation following complex dissociation, whereas the Txnip transgene was; hence, TxnipAA1,2 adipogenic inhibition was enhanced. Most significantly, whereas C247S Txnip had no effect on inhibiting adipogenesis, C247SAA1,2 potently suppressed adipocyte formation. This indicated that TXN inhibition is not the central mechanism by which Txnip and TXN inhibit adipogenesis. Instead, these results support the alternate hypothesis that Txnip intrinsically regulates adipocyte development by suppressing the progression of differentiation. Thus, TXN contributes to the adipogenic signaling properties of Txnip through stabilization of Txnip protein.

This work provides new insights into adipocyte development through a novel reevaluation of how the redox state may regulate Txnip metabolic signaling. This represents a shift away from the thioredoxin inhibition function of Txnip and highlights a new role for TXN–mediated Txnip protein stabilization. These conclusions have broader implications for the members of the arrestin domain-containing protein family, which share several regulatory functions including promotion of aerobic metabolism, modulation of insulin-responsive glucose uptake, and inhibition of adipocyte development. The unique association of Txnip with TXN through a mixed disulfide bond that is sensitive to the redox state of the cell provides a means to modulate its arrestin-like signaling in response to cellular stresses and changes in cellular metabolic state through complex dissociation and Txnip degradation. As several studies have shown that adipogenesis is influenced by anti- or pro-oxidants (28–30), it will be interesting to see whether changes in the Txnip-TXN interaction and Txnip stability underlie oxidative stress effects on differentiation or on other known Txnip metabolic properties such as the development of insulin resistance.

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