MINIREVIEW

Tumor Necrosis Factor-α–Induced Insulin Resistance in Adipocytes (44471)

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Abstract. **Recent studies examining the link between insulin resistance and the development of obesity and noninsulin-dependent diabetes mellitus are consistent with the involvement of tumor necrosis factor–α (TNF-α) as a central mediator. In insulin resistant obese mouse models, neutralization of TNF-α in circulation has been demonstrated to restore insulin-mediated glucose uptake. Adipose tissue has been shown to be a site for synthesis of TNF-α, with the degree of adiposity directly correlated with the level of synthesis. Studies conducted on obese human patients have demonstrated a correlation between levels of TNF-α, the extent of obesity, as well as the level of hyperinsulinemia observed. Mechanistic studies in cell culture have suggested that TNF-α functions to render cells insulin resistant through regulation of the synthesis of the insulin responsive glucose transporter as well as through interference with insulin signaling. This review will address these issues and additionally introduce the reader to the molecular aspects of TNF-α, its receptors as well as TNF-α–initiated signaling cascades, that are necessary to understand the function of this cytokine in the regulation of adipose tissue metabolism.** [P.S.E.B.M. 2000, Vol 223]

Our laboratory has been interested in the regulation of glucose transporter gene expression in adipocytes. Although adipocytes express two transporters, GLUT1 (a ubiquitous, basal homeostatic transporter) and GLUT4 (the insulin-responsive glucose transporter) (1), the focus of our work and this review is on the regulation of GLUT4. Why the concern with the regulation of GLUT4 in adipocytes? The case is frequently made that expression of GLUT4 in muscle is both quantitatively and qualitatively more important than that in adipose tissue, particularly when speaking of the relative contribution to glucose homeostasis and insulin resistance. However, in a transgenic mouse model, work by Shepherd *et al.* (2) demonstrated that the number of GLUT4 transporters is rate-limiting for glucose transport in adipose tissue and that increasing glucose transport into fat can enhance whole-body glucose tolerance. The enhanced expression of GLUT4 in fat was suggested to alter nutrient partitioning to increase adipose mass, and potentially set into motion events that foster the replication of immature adipocytes, thus providing a demonstration that the adipocyte may play a very active role in the development of obesity and diabetes.

This view is reinforced by the description of the ability of adipose tissue to secrete TNF-α as well as being the major site of synthesis and secretion of the *ob* gene product, leptin. Recent reviews have highlighted the endocrine function of the adipose cell and suggested that, "the adipocyte is at the center of a key regulatory system for maintenance of energy stores, and henceforth obesity must be viewed as a disorder both of and by the adipocyte" (3, 4). Most importantly noninsulin-dependent diabetes mellitus, which is usually accompanied by hyperinsulinemia and peripheral insulin resistance, also leads to reduced levels of GLUT4 message and protein in adipose tissue (5, 6), consistent with an attenuation of synthesis. Thus, these arguments provide a compelling rationale for the investigation of the insulin resistance at the level of adipose tissue and the involvement of TNF-α in the generation of that resistance at the levels of both signaling and GLUT4 gene expression.

Our review will focus on this latter function and detail

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the ability of TNF-α to activate signal transduction pathways in the adipocyte that may either interfere with insulin-initiated signal transduction or alter transcription of the insulin responsive glucose transporter.

TNF-α

In 1985, Old *et al.* (7) identified a protein from the serum of rabbits that had been treated with endotoxin, and that protein was responsible for the hemorrhagic necrosis of some transplantable tumors. The protein was named tumor necrosis factor (TNF-α). At the same time, another group led by Anthony Cerami identified a circulating protein proposed to be responsible for the development of cachexia, which they named cachetin (8). Upon sequence comparison, TNF-α and cachetin were demonstrated to be identical (9). Since these initial discoveries, TNF-α has been realized to be more than a "tumor necrosing factor" with the demonstration of many target cells and tissues, its involvement in immune and inflammatory responses, its synthesis and secretion by adipose tissue, as well as its function as a mediator of insulin resistance in diabetes mellitus (10–18).

TNF-α is encoded by a signal-copy gene, which gives rise to a protein of 26 kDa (19). The protein is synthesized and processed as a membrane-associated protein that after proteolytic cleavage is released into circulation as a homotrimer of ≈ 51kDa (20). The membrane-bound form of TNF-α is active; however, based on data using metalloprotease inhibitors, it is the cleaved circulating form that is responsible for the mortality associated with endotoxic shock (21, 22).

TNF-α Receptors

The TNF-α receptors belong to a superfamily that has at least 12 members, including the Nerve Growth Factor and CD95 (APO1/Fas) receptors (23). Two TNF receptors have been identified and are referred to as TNFR1 (or p55 in rodents, p60 in humans) (24, 25) and TNFR2 (p75 in rodents, p80 in humans) (26, 27). Although most cells express both receptors, the ratio may vary (28). The receptors are homologous only in their extracellular domains to the extent of 30% (29) with the lack of homology between the intracellular domains suggesting that the receptors initiate different signaling pathways and have different biological functions (30–32). TNFR1, similar to CD95 (APO-1/Fas) receptor, has an 80-amino acid sequence, as part of the intracellular region, referred to as the "death domain." This region has been demonstrated to be responsible for transmission of the TNF-α–induced cytotoxic signal (33–36).

Most of the biological activities of TNF-α including apoptosis, antiviral activity, and activation of transcription factor NF-kB have been proposed to be mediated by TNFR1 (37). Occupation of TNFR2 by a ligand does initiate signaling mechanisms and under certain conditions this includes apoptosis and activation of NF-κB (38). In addition, signaling through TNFR2 has been shown to be critically involved in lipopolysaccharide-induced leukostasis and downregulation of TNFR1-dependent neutrophil influx in a lung inflammation model (39).

Tartaglia *et al.* (40) have suggested that TNFR2 also functions in ligand passing to TNFR1. This hypothesis is based on the lower affinity and faster rate of dissociation of TNF-α with respect to TNFR2 ($K_{d\ TNFR2}$ = 100 p*M* and $t_{1/2}$ for dissociation = 10 min; $K_{d\ TNFR1}$ = 500 p*M* and $t_{1/2}$ for dissociation = 3 hr). The theory suggests that at low concentrations of TNF-α, TNFR2 would preferentially bind the cytokine and through its rapid rate of dissociation, the concentration of TNF-α would increase locally, making it available for binding to TNFR1.

TNF-α–Induced Signaling Cascade

Occupation of the receptor by ligand results in homotrimerization of the receptor, which initiates signal transduction. Signaling by TNFR1 appears to depend on the presence of the cytosolic region known as the death domain (DD) (33, 34). The DD has no enzymatic activity but serves as a docking region for the assembly of a protein complex that will lead to activation of specific cellular enzymatic activities. Briefly, when TNF-α binds to TNFR1 the following events take place (summarized in Fig. 1): TRADD (TNF receptor associated death domain protein) is recruited and binds to DD as an adapter that in turn recruits downstream signal transducers, FADD (Fas-associated death domain protein), and TRAF2 (TNF receptor associated factor-2) (41). FADD is thought to interact directly through its N-terminal domain with the apoptotic protease, FLICE (FADD-like interleukin 1β converting enzyme, also known as MACH), initiating cell death. TRAF2 signals the activation of two separate pathways and appears to be essential for mediation of survival. In the first pathway, TRAF2 recruits NIK (NF-κB inducing kinase) (42). Prior to activation, NF-κB is maintained inactive in the cytosol in a complex with the inhibitory subunit IκB. The recruitment of NIK to the receptor complex results in the phosphorylation of IκB, the phosphorylated protein dissociates from the complex, and the NF-κB subunit translocates to the nucleus where it can transactivate the appropriate target genes. The second pathway, which is independent of NIK, results in the activation of the JNKs or SAPKs (Jun N-terminal kinase or stress activated protein kinase) (43). These kinases function to activate a number of transcription factors, including ATF-2, c-Jun, and Elk-1 (43, 44).

Finally, RIP (receptor interacting protein), a serine/threonine kinase, is recruited by TRAF2 and has been suggested, based on studies with dominant negative mutants, to also play a role in the activation of NF-κB (45).

The proteins TRADD and FADD are also thought to mediate TNF-α activation of the *acidic* sphingomyelinase (SMase), leading to the generation of ceramide and apoptosis (45–50). TNFR1 has been shown to activate the *neutral* SMase through FAN (factor associated with neutral SMase), a protein that binds to the cytosolic domain of

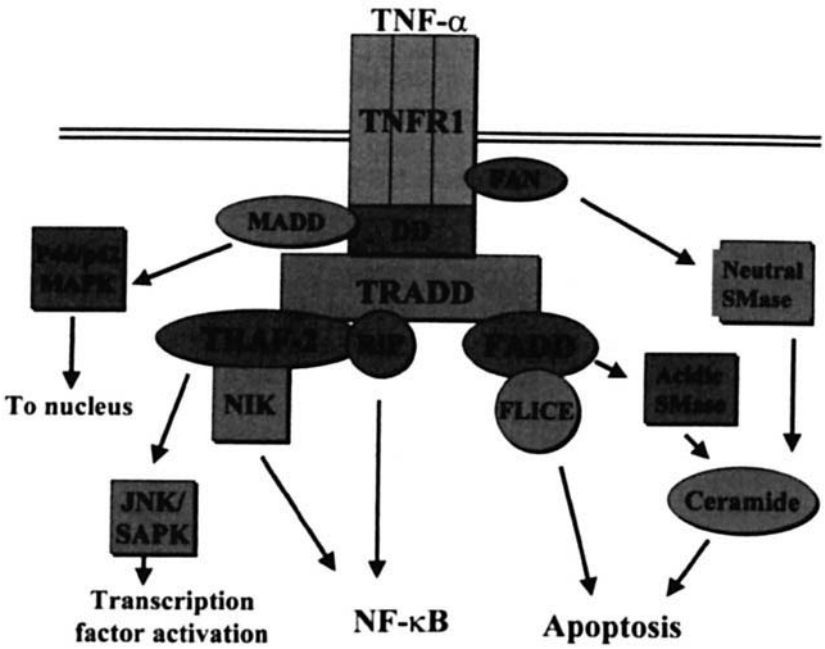

Figure 1. TNF-α–initiated signaling cascades. TNF-α binds to receptor TNFR1 and initiates signal transduction by recruiting proteins that associate with specific domains. Briefly, when the receptor is occupied by ligand the cytosolic region referred to as the Death Domain (DD), recruits TRADD (TNF receptor associated death domain protein). This adapter recruits FADD (Fas-associated death domain protein), and TRAF2 (TNF receptor associated factor-2). FADD interacts with FLICE (FADD-like interleukin 1β converting enzyme to initiate cell death. TRAF2 recruits NIK (NF-κB inducing kinase) as well as independently activating JNKs (c-Jun N-terminal kinases) and SAPKs (stress-activated protein kinases). RIP (receptor interacting protein) is recruited by TRAF-2 and leads to activation of nuclear factor-κB (NF-κB). TRADD and FADD have also been implicated in activation of the acidic SMase (sphingomyelinase), whereas FAN (factor associated with neutral SMase) activates the neutral SMase. MADD (mitogen-activated protein [MAP] kinase death domain protein) binds to the DD and activates the p44/42 MAP kinase.

TNFR1 at a region 9 amino acids distal to and distinct from the DD.

Additionally, recent studies have shown that occupation of TNFR1 can result in the association of MADD (mitogen-activated protein kinase death domain protein) with the DD. MADD was cloned as a DD-binding protein that activates p44/p42 MAP kinase (or ERKs 1 and 2) in the nonapoptotic pathway (51).

Numerous studies support a model in which TNF-α occupation of TNFR1 activates two contradictory signal transduction pathways: one leads to apoptosis, the other through activation of NF-κB, protects against it. The fate of the cell appears to depend on which pathway predominates.

On a final note, for its participation in signaling, TNFR2 does not interact with TRADD but binds directly to TRAF2, which can result in activation of both NF-κB and the JNK/SAPK pathways.

TNF-α in Adipose Tissue

In 1982, Kawakami *et al.* (52) described an endotoxin-induced macrophage secretory substance that inhibited the synthesis of adipose tissue lipoprotein lipase (LPL). In addition, the substance appeared generally to suppress anabolic storage processes while stimulating catabolic processes (53). It was this observation, inhibition of anabolism while stimulating catabolism, that led the investigators to name the substance "cachectin" suggesting that the regulatory events initiated by the substance may play a role in the cachexia observed in chronic diseases. The substance was demonstrated to be a protein isolated and found to be identical to TNF-α (9). While predominantly synthesized and secreted by phagocytic cells of the immune system, TNF-α has been shown to be made in the adipocytes (and to a minor degree in both skeletal and cardiac muscle) of both mice and humans (54). In the adipose tissue of rodents with genetic obesity and insulin resistance, increased levels of both RNA (5–10-fold) and protein (2-fold) were described, supporting a link between obesity, diabetes, and TNF-α. In 1993 a direct link between TNF-α and obesity-linked insulin resistance was established when neutralization of TNF-α in several rodent models of obesity and insulin resistance was shown to improve insulin sensitivity and insulin receptor signaling (9, 10). Most interestingly, a direct correlation was established between the degree of adiposity, increased TNF-α production and insulin resistance (15).

Mechanisms of TNF-α–Mediated Insulin Resistance

Effects on Signal Transduction. TNF-α has been suggested to regulate insulin responsiveness by interfering with insulin signaling as well as through alteration of glucose transporter gene expression. The insulin signaling pathway is initiated by the binding of insulin to the insulin receptor (IR), which activates the endogenous receptor tyrosine kinase and results in autophosphorylation of tyrosine residues on the β-subunit of the IR and phosphorylation of tyrosine residues within insulin receptor substrate-1 (IRS-1) (55). Once phosphorylated, IRS-1 serves as a docking molecule for signaling molecules containing an SH2 (Src homology domain 2). IRS-1 contains multiple potential Ser/Thr phosphorylation sites (14, 56, 57) and phosphorylation of IRS1 on serine and threonine residues interferes with the subsequent insulin-stimulated tyrosine phosphorylation of IRS-1 by the IR (57–59). Spiegelman and colleagues (14) have presented data indicating that exposure of adipocytes to TNF-α results in the increased serine phosphorylation of IRS-1 resulting in the inhibition of insulin-induced tyrosine phosphorylation of IRS-1 (14). The IRS-1 mediated inhibition of IR tyrosine kinase activity could occur by direct or indirect interactions between the IR and IRS-1 (60). Serine-

phosphorylated IRS-1 might associate with the IR in a manner that blocks the autophosphorylation reaction. On the other hand, serine-phosphorylated IRS-1 might act indirectly on the IR through an association with an inhibitor that acts on the IR in a stoichiometric or catalytic fashion (61). Regardless of the alternative, IRS1 is the critical intermediary in this model (Fig. 2). TNF-α–induced serine/threonine phosphorylation of IRS-1 may occur through TNF activation of a variety of kinases. Potential candidates known to be activated by TNF-α include protein kinases C and A, β-casein kinase, and p44/42 and p38 MAP kinases (55, 60–63). Protein kinase C in particular has been associated with phosphorylation of the IR leading to an inhibition of insulin-induced tyrosine phosphorylation and insulin action in intact cells (64).

Effects on Synthesis of GLUT4. Clearly, the reduced levels of GLUT4 message and protein in the adipose tissue of patients with noninsulin-dependent diabetes mellitus or obesity (5, 6) are consistent with an attenuation of synthesis. To investigate the potential for TNF-α–induced regulation at the level of *GLUT4* gene expression, the 3T3-L1 adipocytes were exposed to TNF-α. Treatment of the cells with concentrations of TNF-α as low as 0.2 n*M* resulted in repression of *GLUT4* gene transcription and decreased GLUT4 mRNA stability (17, 18). The effect of TNF-α on GLUT4 transcription was rapid, detectable (10%–15% suppression) within 60 min and maximal (80%–90% suppression) within 4 hr. The regulation was not dependent on new protein synthesis and appeared to be controlled by a kinase/phosphatase system (17). It is important to note that the effects on transcription could be detected within an hour whereas the loss of protein occurred over several days. Moreover, the loss of GLUT4 content was paralleled by the loss of C/EBP-α mRNA and protein (17).

An in-depth analysis of the effect of TNF-α on synthesis relative to an effect on insulin signal transduction revealed that prolonged exposure of the 3T3-L1 adipocytes to TNF-α resulted in a substantial reduction in the cellular content of IRS-1 and GLUT4 mRNA and protein as well as a lesser reduction in the amount of insulin receptor (18). Nevertheless, the remaining proteins appear to be biochemically indistinguishable from those in untreated adipocytes. Both the IR and IRS-1 were phosphorylated on tyrosine to the same extent in response to acute insulin stimulation following exposure to TNF-α. Furthermore, the ability of the insulin receptor to phosphorylate exogenous substrate in the test tube was also normal following its isolation from TNF-α–treated cells. These results were confirmed by the reduced but obvious level of insulin-dependent glucose transport and GLUT4 translocation observed in TNF-α–treated adipocytes. The authors concluded that the observed insulin resistance resulted from decreased content of the requisite proteins involved in insulin action, consistent with an effect on synthesis (18).

The question now became, how was the effect on synthesis mediated? Kaestner (65) had demonstrated the presence of a C/EBP (CCAAT enhancer binding protein) site at position –273 in the GLUT4 promoter and suggested that at least in part, control of *GLUT4* gene transcription resided with the C/EBP family (α-, β-, and δ-isoforms) of transcription factors interacting at this site. With this consideration, it was proposed that loss of C/EBP-α played a role in the TNF-induced decreased transcription of the *GLUT4* gene. Because of the coordinate loss of both GLUT4 and C/EBP-α, it was not clear that C/EBP-α mediated a direct effect on the transcription of GLUT4, and we hypothesized that transcriptional suppression could be mediated by manipulation of the specific C/EBP isoforms occupying the promoter element. To address this question, the ability of TNF-α to alter the occupation of the C/EBP site in the GLUT4 promoter was examined using electrophoretic mobility shift assays (66). In the fully differentiated adipocytes, the data suggested that the C/EBP site is a ligand for predominantly αα homodimers. However, after exposure to TNF-α a shift

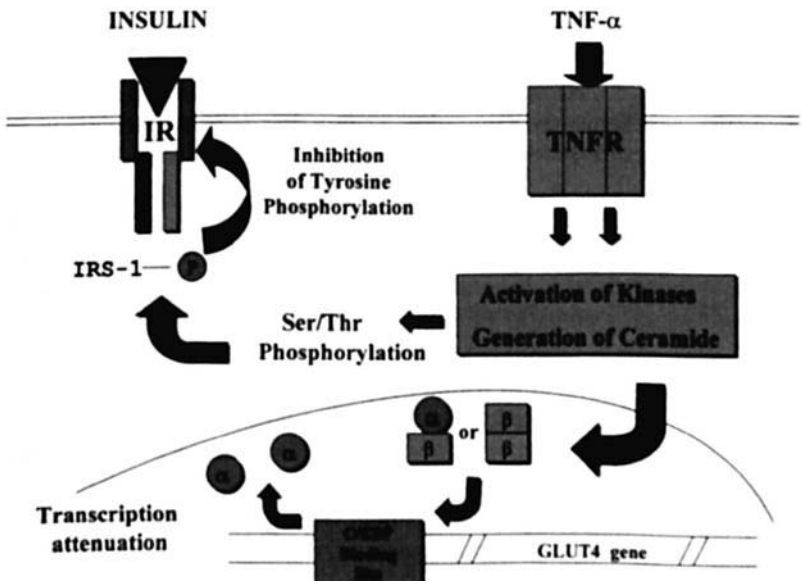

Figure 2. TNF-α induced insulin resistance. TNF-α has been suggested to induce insulin resistance in adipocytes by two mechanisms. With no specific order intended, there is an activation of kinases and generation of ceramide. Ceramide has been suggested as the mediator of the effects on transcription of the *GLUT4* gene, whereas at the C/EBP (CCAAT enhancer binding protein) site in the GLUT4 promoter, homodimers of C/EBP-α are replaced with heterodimers of the α- and β-isoforms or homodimers of C/EBP-β. The alternative translation (truncated) product of the C/EBP-β mRNA, which is a transcriptional repressor, may play a role in the attenuation of transcription. Additionally, *via* TNF-induced Ser/Thr phosphorylation of IRS-1 (insulin receptor substrate 1), insulin signaling is inhibited.

in occupancy of the site occurred, and the ligands were now α/β heterodimers and β/β homodimers. In terms of the analysis, it is important to realize that the α- and β-isoforms of C/EBP possess alternative translation-initiation codons that result in the formation of truncated forms of the two proteins. In the case of the β-isoform, a truncated protein of 18 kDa (relative to the 30-kDa full-length protein known as p30 C/EBPβ or liver-enriched activating protein, LAP) lacking the transactivation domain is produced. The truncated protein known as LIP (p19 C/EBPβ or liver-inhibitory protein) can form homodimers or heterodimerize with other family members and as it lacks the transactivation domain can attenuate the transcriptional activation properties of the other isoforms (67, 68). The data suggest that LIP is present in the dimers binding to the C/EBP site, which suggests that attenuation of transcription may occur through dimer formation (homo- and hetero-) with the truncated form of C/EBP-β.

As both C/EBPα and β (LIP and LAP) are present in the cell at the same time, the question arises as to what regulates specific dimer formation. The data are consistent with partner selection in dimer formation being controlled by selective translocation of the β-isoform (LAP and LIP) from the cytosol to the nucleus after exposure of the cells to TNF (summarized in Fig. 2).

Although the data provide a testable hypothesis, questions still arise as to what signals initiate: i) Dissociation of the αα-homodimers from the C/EBP site; ii) Translocation of C/EBPβ from the cytosol to the nucleus; and iii) Formation of the appropriate homo- and hetero dimers necessary to attenuate transcription.

Signaling Processes that Mediate the Effect of TNF-α on GLUT4 Synthesis

As described above, signal transmission is initiated through the recruitment and direct association of specific proteins to the cytosolic domain of the TNF receptors (69) and is continued through cytosolic kinases with the eventual activation of specific enzymes and transcription factors. In attempting to define the specific TNF-α–initiated pathway responsible for control of *GLUT4* gene transcription, the cytokine was demonstrated to activate an adipocyte sphingomyelinase with generation of ceramide (70). Use of cell-permeable analogs of ceramide mimicked the TNF-α effect and resulted in a marked reduction in the transcription of the *GLUT4* gene (70), suggesting that it might function as a mediator of TNF-α action. In the same study, consistent with previous reports by Lozano *et al.* (71) and Muller *et al.* (72), protein kinase C-ζ was activated by exposure of the cells to either TNF-α or ceramide analogs. The activation was transient on time frame consistent with its role in a regulatory event (70). Thus, the data describe a potential pathway from TNF-α occupation of its receptor to activation of a sphingomyelinase, generation of ceramide, and attenuation of *GLUT4* gene transcription. Potentially, ceramide may use protein kinase C-ζ as a mediator of transcriptional regulation.

TNF-α Induction of Oxidant Stress

In recent studies the possibility that oxidative stress and TNF-α may regulate adipocyte GLUT4 expression and function has been addressed. 3T3-L1 adipocytes exposed to either prolonged oxidative stress or TNF-α displayed impaired insulin responsiveness. This was associated with both reduced GLUT4 protein and mRNA content, decreased mRNA stability (17, 73, 74), as well as impaired insulin-induced GLUT4 translocation (18, 74). The impairment in GLUT4 translocation following oxidative stress was associated with disruption of the normal insulin-stimulated cellular distribution of IRS-1 and phosphatidyl inositol (PI) 3-kinase to the low density microsomal (LDM), and impairment of insulin-stimulated protein kinase B activation (75). The investigators concluded that striking similarities existed between the responses to both conditions, and that the data supported the possibility of a common mechanism mediating the effects of responses to TNF-α and oxidative stress. Consistent with this notion, TNF-α caused a rapid reduction in reduced glutathionine (GSH) levels, with an increase in the level of GSSG, GSSG/GSH ratio, as well as the protein mixed disulfide content (76, 77). The reduction in GSH levels has been suggested to mediate the TNF-α–induced activation of the sphingomyelinase and the resulting generation of ceramide (78). Thus, these data would suggest that the generation of ceramide and subsequent regulation of *GLUT4* gene transcription may result from a primary event where the adipocyte addresses an oxidative stress through the GSH – GSSG equilibrium.

Other Considerations

The data summarized address the ability of TNF-α to control expression of *GLUT4* gene transcription. While not yet complete, the available data have contributed to our understanding of major components of the signal transduction pathway. However, as detailed earlier, when the adipocytes are exposed to TNF-α, in addition to the effect on transcription of the *GLUT4* gene, there is a marked destabilization of the GLUT4 mRNA. The half-life decreases from 9 hr to ≈ 4.5 hr (17, 18). Using *in vitro* RNA gel shift assays, protein(s) have been demonstrated to bind to the GLUT4 3′untranslated region coincident with the alteration of stability (79). However, mechanism of these events has yet to be addressed.

The downregulation of both GLUT4 transcription and mRNA stability rapidly renders the cell unable to produce GLUT4 protein. Investigations into the TNF-α–initiated signals that control mRNA stability are consistent with a *lack* of involvement of both ceramide and the p44/42 MAP kinase (77). Thus, much work remains to understand the regulation.

Perspective

The data support a critical role for TNF-α in the regulation of glucose utilization and adipose cell metabolism and provide the basis for the development of targeted intervention in insulin-resistant patients. The description of the synthesis of TNF-α by adipose tissue, the cloning and characterization of the TNF receptors, the identification of the TNF-α–initiated signaling mechanisms, as well as genes that are regulated by TNF-α provide primary targets for disruption of TNF-α action. Previous studies have shown that passive immunization against TNF-α has been effective in protecting animals from the lethal effect of endotoxin (80) and in three animal models of insulin resistance and obesity, neutralization of TNF-α resulted in a return to glycemic control (9, 10). However, in the one human study reported to date, the antibody-mediated neutralization of TNF-α had no effect on insulin sensitivity in obese noninsulin-dependent diabetes mellitus (NIDDM) subjects (81). In this study, TNF-α levels in all subjects receiving the antibody decreased significantly, in most cases falling below the limits of detection of the assay (81). With the consideration that other factors such as elevated free fatty acids may have been involved in the observed insulin resistance in these patients, the utility of adsorption/neutralization of TNF-α as a therapeutic strategy for the amelioration of insulin resistance merits more investigation. In addition, it is believed that as knowledge of the various target genes and signaling intermediates of TNF-α grows other strategies will develop directed toward the specific steps in the pathway(s).

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