TNF inhibits insulin induced STAT5 activation in differentiated mouse muscle cells pmi28

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Abstract Tumor necrosis factor (TNF) plays a central role in the state of insulin resistance leading to type II diabetes. We here describe the crosstalk of TNF with insulin signaling cascades in the mouse muscle cell line pmi28. TNF downregulated insulin induced insulin receptor kinase activity and insulin induced activation of the transcription factor STAT5. Our results provide evidence that the inhibitory crosstalk between TNF and insulin in skeletal muscle cells comprises an interference with the expression of STAT5 regulated genes which may play an important role in the manifestation and/or progression of insulin resistance in muscle cells.

Key words: Insulin resistance; Tumor necrosis factor; Skeletal muscle; STAT5

1. Introduction

Insulin plays a key role for the regulation of metabolism in many mammalian cells, and many components of the insulin signaling network have been identified (for review see White and Kahn [1]). The major intracellular substrates of the insulin receptor tyrosine kinase (IRβ) are insulin receptor substrates IRS-1 and IRS-2, which act as an interface between stimulated receptors and signaling proteins with SRC homology 2 (SH2) domains, and upon phosphorylation bind a variety of signaling molecules including PI-3 kinase, Syp, Nck, Grb2 and Fyn. In addition to IRS, the SH2/SH3 domain containing adapter, Shc, has been shown to directly interact with the IR. An additional physiological substrate of the insulin receptor, STAT5 (signal transducer and activator of transcription), has recently been described which, unlike the cytokine mediated activation of STATs, appears to be independent of JAK [2]. Thus, STAT5 is likely to participate in the regulation of the transcriptional program of insulin responsive tissues. In support of this, STAT5A−/− and STAT5B−/− knockout mice have been examined and each mutant shows a highly specific phenotype with female lactation defects in STAT5A−/− mice and male growth retardation as well as development of obesity in STAT5B−/− animals [3,4].

Insulin resistance is defined as the reduced ability of cells or tissues to respond to physiological levels of insulin and is a characteristic condition of early stage non-insulin dependent diabetes mellitus (NIDDM) [5]. Obesity is the strongest risk factor for NIDDM. Insulin resistance has also been described in a number of disease conditions including cancer, sepsis, endotoxemia, trauma and alcoholism [6]. These conditions are known to cause altered levels of cytokine expression. In particular, TNF has been recognized as an important mediator associated with the above disease states including obesity: the expression of TNF is increased in adipose tissue and skeletal muscle cells of obese, diabetic patients [7,8] and TNF alters protein and lipid metabolism in adipose tissue [9].

Of relevance, knockout mice devoid of either the TNF-α gene or the two TNFR genes develop insulin resistance neither in a diet induced nor in a genetically determined (*ob/ob*) obesity model [10], pointing to the essential contribution of TNF in the acquisition of this disease state. As to the molecular mechanisms of TNF action, it is apparent from several studies with affected tissues [10,11] and from in vitro cell culture models [12] that IR kinase activity and downstream signaling events are affected. So far, in vitro models of insulin resistance are based on cell lines of liver or fat tissue origin and have focused on the crosstalk of TNF with insulin at the level of IRS-1 and 2 [13]. From these studies, the time course of TNF effects on IR signaling and the mechanisms of action appears to differ depending on the cell types analyzed [12,14,15]. In particular, the relevance of serine phosphorylation of IRS-1 and/or IRS-2 versus downregulation of protein levels of these molecules remains unresolved at present. Other potential targets in the diverse signal pathways emanating from the IR and their role in development of insulin resistance have not been studied so far.

We here describe a new cellular model, the murine muscle cell line pmi28, that retains in vitro differentiation potential and expresses markers characteristic of differentiated muscle such as sarcomeric myosin [16–18]. This cell line, upon differentiation, is both TNF and insulin responsive and represents a suitable model for studying the crosstalk between TNF and insulin signaling cascades. Evidence is presented that this interaction occurs not only by reduction of IR phosphorylation but also at the transcriptional level by inhibition of STAT5 activation.

2. Materials and methods

2.1. Cell culture

Myoblasts from a primary culture designated pmi28 were used [17,18]. The culture was established from the hind-leg muscles of a

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Abbreviations: AP, alkaline phosphatase; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; IRβ, insulin receptor β chain; IRS-1, insulin receptor substrate-1; JAK, janus kinase; NF-κB, nuclear factor κB; NIDDM, non-insulin dependent diabetes mellitus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PI-3 kinase, phosphatidylinositol-3 kinase; PMSF, phenylmethylsulfonyl fluoride; PY, phosphotyrosine; SH, SRC homology; STAT, signal transducer activator of transcription; TNF, tumor necrosis factor

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7 day old male BALB/c mouse. Initial expansion and enrichment in myoblasts was achieved by repeated replating and growth in minimum essential medium with D-valine (Gibco BRL) containing 20% fetal bovine serum (FBS). When the expanded culture was highly enriched (100% myoblasts as evaluated by immunofluorescent staining for desmin), the cells were further propagated in F10 medium. Pmi28 myoblasts expanded over 2–6 passages have been implanted in vivo and shown, among several other expanded primary cultures, to integrate into and improve muscle contractile strength in syngeneic animals [17]. Pmi28 cells have been propagated in vitro over 40 passages without loss of myogenic properties or proliferative capacity [17]. In the present experiments, the mouse myoblast cell line pmi28 was cultured in nutrient mixture Ham's F10 (Biochrom) supplemented with 20% FCS (PAA). For differentiation pmi28 cells were grown to confluence in tissue culture plates for primary cell culture (Sarstedt) and medium was changed into low glucose (1 g/l) Dulbecco's MEM (Biochrom) supplemented with 10% horse serum for 2 days (Gibco BRL), resulting in the formation of syncytia. Prior to the experiments, differentiated pmi28 cells were serum starved and as indicated preincubated with TNF (50 ng/ml) for 2 h and stimulated with insulin (200 nM) for 10 min or for the indicated times.

2.2. Cytokines, antibodies and reagents

Recombinant huTNF was a kind gift of BASF and insulin from bovine pancreas was from Sigma. Monoclonal antibodies against sarcomeric myosin (MF-20) were a kind gift of Prof. Dr. Anna Starzinski-Powitz (University of Frankfurt/M, Germany). Antibodies against insulin receptor β (C-19), STAT5 (G-2) and phosphotyrosine (PY99) were from Santa Cruz. All inhibitors of proteases and phosphatases were from Biomol. Secondary AP linked antibodies, goat anti-mouse IgG and IgM (H+L) and goat anti-rabbit IgG (H+L), were from Dianova. NF-κB oligonucleotides (5′-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3′) were synthesized by MWG and STAT5 oligonucleotides (5′-AGATTTCTAGGAATTCAATCC-3′) and mutant oligonucleotides (CTAGG→AGTTT substitution in the STAT5 binding motif) were from Santa Cruz.

2.3. Immunofluorescence

Cells were washed and fixed with 3.5% paraformaldehyde for 10 min, rinsed three times with PBS and once with 0.15 M glycine in PBS. The cells were permeabilized with 0.1% SDS for 1 min, blocked with blocking buffer (5% goat serum and 0.05% Tween in PBS) for 30 min and washed three times with PBS. After incubation with MF-20 primary antibody (1:10) in blocking buffer for 2 h, the slides were rinsed three times with PBS and then incubated with secondary CY3 linked antibody in blocking buffer for 1.5 h. Rinsing with PBS (four or five times) was followed by mounting with Mowiol.

2.4. Immunoprecipitation

After stimulation the cells were washed twice with PBS (4°C) and scraped in 750 μl ice-cold phosphorylation buffer (50 mM HEPES pH 7.8, 2.5 mM EDTA, 1% Triton X-100, 150 mM saccharose, 100 mM NaF, 10 mM NaPP, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 100 nM okadaic acid, 100 nM calyculin A, 1 mM *p*-nitrophenyl phosphate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) or in WCL (0.5% NP-40, 50 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM NaF, 1 mM NaPP, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 100 nM okadaic acid, 100 nM calyculin A, 1 mM *p*-nitrophenyl phosphate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). After sonification and cell lysis, the

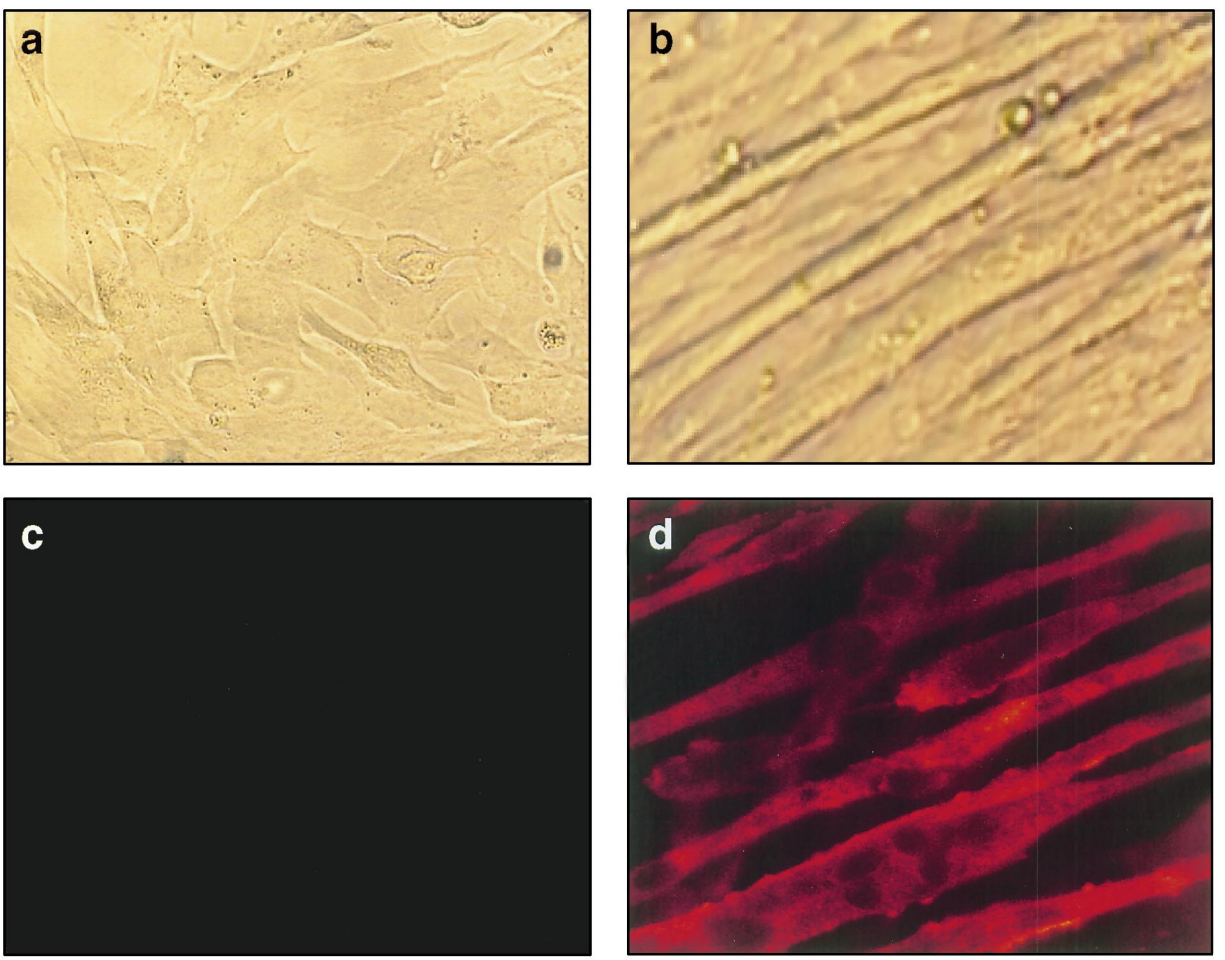

Fig. 1. Differentiation of pmi28 primary myoblasts into muscle syncytia. Pmi28 myoblasts were grown to confluence and differentiation medium was added for 2 days. Immunofluorescence staining against myosin (MF-20) was performed as described in Section 2. a: Phase contrast of undifferentiated cells. b: MF-20 immunofluorescence staining of undifferentiated cells. c: Phase contrast of differentiated cells. d: MF-20 immunofluorescence staining of differentiated cells.

lysates were centrifuged (10 000 rpm, 15 min, 4°C) and immunoprecipitation was performed as described [19].

2.5. Kinase assay

The cells were lysed as described above. After immunoprecipitation of IRβ, the protein A pellets were washed thrice with buffer C [19] and resuspended in 30 μl of kinase buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$). After addition of 4 μCi [γ-^{32}P]ATP (Amersham) in 10 μl kinase buffer, the samples were incubated for 15 min at 37°C. The reaction was stopped with equal amounts of 2× reducing Laemmli buffer and the samples were separated on 7.5% SDS-PAGE and blotted onto nitrocellulose. Analysis of the kinase assays was performed with a phosphorimager (Molecular Dynamics).

2.6. Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were ^{32}P-labeled with polynucleotide kinase and EMSA was performed as described [19].

3. Results

3.1. Differentiation of pmi28 cells

Myoblasts from an expanded primary culture of a male BALB/c mouse skeletal muscle described in [17,18] were differentiated according to Section 2. After 2 days of differentiation the cells exhibited the typical phenotype of polynucleated (syncytia) muscle fibers. In order to determine the number of differentiated cells, expression of a specific muscle differentiation marker, sarcomeric myosin [16], was analyzed by immunofluorescence microscopy with antibody MF-20 (Fig. 1). In all experiments described below, at least 90% of the cells expressed this differentiation marker.

3.2. Differentiated pmi28 cells express functional insulin and TNF signal pathways

To verify the function of the IR signaling cascade and to establish optimum experimental conditions for studies of

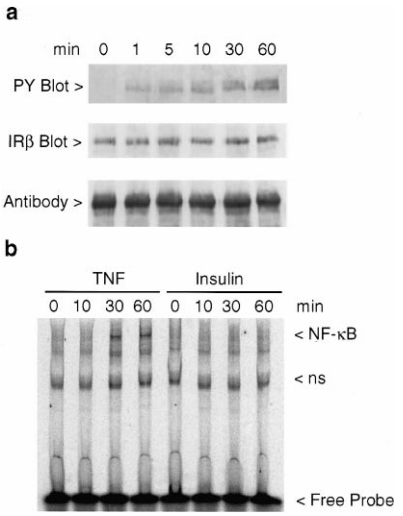

Fig. 2. Kinetics of insulin induced tyrosine phosphorylation of IRβ in differentiated pmi28 cells. Differentiated pmi28 cells were serum starved for 2 h and stimulated with insulin (200 nM). a: Cells were lysed in phosphorylation buffer, immunoprecipitated, samples separated on SDS-PAGE, blotted to nitrocellulose and stained with PY or IRβ antibodies. All experiments were performed three times with similar results. b: TNF induced signaling evaluated by NF-κB translocation in differentiated pmi28 cells. Differentiated pmi28 cells were serum starved for 2 h and stimulated with TNF (50 ng/ml) or insulin (500 nM) for 10, 30 or 60 min. Nuclear extracts were prepared and incubated with NF-κB oligonucleotide. The gel was dried and imaged on a phosphorimager.

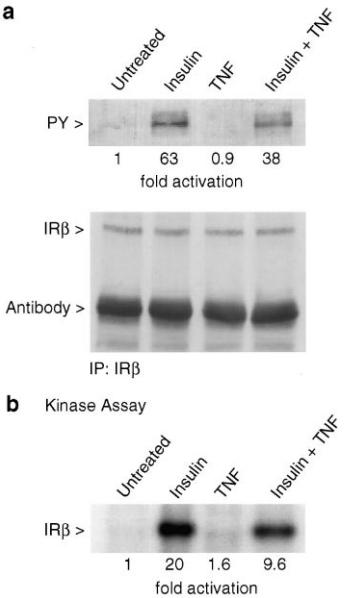

Fig. 3. Influence of TNF and insulin on tyrosine phosphorylation of IRβ in pmi28 muscle cells. Cells were serum starved and preincubated with TNF (50 ng/ml). After stimulation with insulin (500 nM) for 10 min the cells were lysed in phosphorylation buffer. IRβ immunoprecipitates were run on SDS-PAGE and blotted to nitrocellulose. a: The blot was stained with PY or IRβ antibodies. b: Kinase assay with immunoprecipitated IRβ receptor (insert) as described in Section 2 and densitometric evaluation of the kinase assay. Experiments were performed three times with similar results.

TNF-insulin crosstalk, first the kinetics of IRβ tyrosine phosphorylation was analyzed by Western blot with a phosphotyrosine specific antibody. Upon insulin treatment of differentiated pmi28 cells, IRβ was tyrosine phosphorylated approximately 60% of maximum within 1 min of stimulation, indicating that this activation is an extremely rapid process, reaching its peak after 10 min and remaining at a high activation level for the time period analyzed (60 min, Fig. 2a).

TNF mediated inhibition of insulin signal pathways in adipocytes and hepatocytes has been associated with TNFR1 signaling [15,20]. To investigate whether differentiated pmi28 cells respond to TNF treatment, cells were treated with human TNF, which selectively binds to and activates murine TNFR1, but not TNFR2 [21], and activation of NF-κB as a typical TNF response was measured by EMSA. Nuclear translocation of NF-κB was observed with a typical maximum after 30 min of TNF treatment, indicating intact TNFR1 signaling in differentiated pmi28 cells; as expected, insulin did not induce NF-κB in these cells (Fig. 2b). Accordingly, in differentiated pmi28 cells, both insulin and TNF signal pathways are operative.

3.3. Inhibition of insulin induced tyrosine phosphorylation of IRβ by TNF

To examine the potential crosstalk of TNF with insulin signal pathways in pmi28, cells pretreated with TNF were subsequently stimulated with insulin for 10 min, a time point of maximum tyrosine phosphorylation of IRβ in cells not pretreated with TNF (Fig. 2). Immunoprecipitates from cell extracts were blotted and probed with phosphotyrosine specific antibodies. In addition, kinase activity of IR was tested in an in vitro immunocomplex kinase assay. Compared to un-

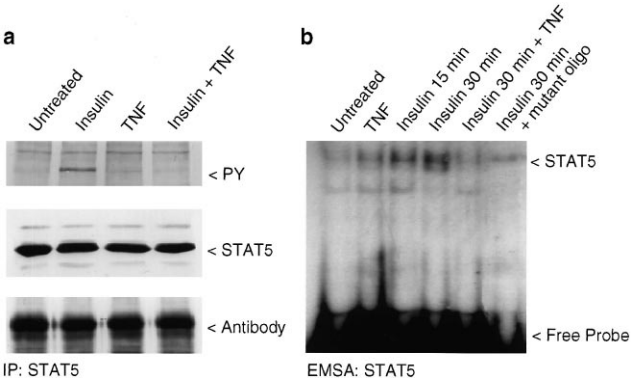

Fig. 4. Inhibition of insulin induced activation of STAT5 by TNF in differentiated pmi28 cells. a: Tyrosine phosphorylation of STAT5. Cells were serum starved and preincubated with TNF (50 ng/ml) for 2 h. After stimulation with insulin (200 nM) for 30 min the cells were lysed with WCL buffer. STAT5 was immunoprecipitated, separated on SDS-PAGE and transferred to nitrocellulose membrane. The blot was stained with PY or STAT5 antibodies. b: EMSA of STAT5. Differentiated pmi28 cells were pretreated with TNF for 2 h and/or stimulated with insulin (200 nM) for 15 or 30 min. Nuclear extracts were prepared and incubated with STAT5 oligonucleotide or mutant oligonucleotide deficient in STAT5 binding. The gel was dried and imaged on a phosphorimager. Experiments were performed three times with similar results.

treated control cells, TNF pretreatment for 2 h resulted in an approximately 40% and 50% decrease of insulin stimulated tyrosine kinase activity of IR, as revealed by Western blotting (Fig. 3a) and in vitro autophosphorylation activity (Fig. 3b). Neither the amount of expressed IR nor its electrophoretic mobility was changed during a TNF pretreatment period of up to 2 h (Fig. 3a). Accordingly, the differentiated mouse muscle cell line pmi28 exerts a similar TNF interference with IR activity as shown previously for mouse adipocytes and the human hepatocarcinoma line HepG2 [20,21].

3.4. Crosstalk between insulin and TNF receptor cascades at the level of STAT5

Recently STAT5B has been described as a novel physiological substrate of the insulin receptor and a role in the physiological regulation of insulin responses has been proposed [2]. STAT5 is expressed in differentiated pmi28 cells. Immunoprecipitates from nuclear extracts were assayed for tyrosine phosphorylation by Western blotting. As shown in Fig. 4a, STAT5 was tyrosine phosphorylated upon insulin stimulation of pmi28. This IR action was totally inhibited in cells pretreated with TNF. To assess a functional activation of STAT5, its specific DNA binding activity was investigated. Nuclear extracts were subjected to non-denaturing gel mobility shift assays with ^{32}P-labeled double stranded oligonucleotide probes comprising the STAT5 binding sequence of the β-casein gene promoter [22]. Band shift analyses of pmi28 nuclear extracts verified that insulin stimulates nuclear translocation and STAT5 DNA binding activity, reaching maximum after 30 min (Fig. 4b). This activation was completely blocked when cells were preincubated with TNF (Fig. 4b).

4. Discussion

The two major achievements described here are (i) the establishment of an in vitro model to study molecular mechanisms of insulin resistance in cells derived from the physiologically most relevant of the affected tissues, the skeletal muscle, and (ii) the demonstration that, aside from the known downregulation of the IR induced signal pathways, the IR mediated activation of STAT5 is abolished by TNF. Our data thus open up new perspectives as to the underlying mechanisms of insulin resistance and point, for the first time, to a TNF-insulin crosstalk that becomes manifested at the transcriptional level with potentially persistent inhibition of STAT5 regulated genes.

The tissue and cell specific patterns of signal pathways make it necessary to establish suitable cellular models for each of the affected tissues. The interference occurs at several independent levels of intracellular signal cascades. For example in different adipocyte models, controversial data exist as to whether TNF induced insulin resistance is predominantly accomplished by affecting the functional state or rather the protein expression levels of IRS-1 and IRS-1 regulated molecules [12,14,23].

The murine myoblast cell line studied here, pmi28, differs from other, previously described cell lines of similar tissue origin by retention of its in vitro differentiation potential into polynucleated syncytia. Upon differentiation, these cells show, with respect to IR kinase activity, a response pattern that resembles data obtained with biopsies of muscle tissue from mouse NIDDM models [10], indicating that differentiated pmi28 are suitable for analyses of molecular mechanisms of TNF-insulin crosstalk.

The principal role of TNF in insulin resistance is stressed from data recently obtained with obese TNF or TNFR1/2 knockout mice [10], both of which do not acquire insulin resistance and show, compared to obese wild type mice, a fully restored IR signal capacity in adipocytes, liver and muscle. Interestingly, in this in vivo model, obese, insulin resistant animals presented significantly reduced protein levels neither of IR nor of the glucose transporter GLUT4, supporting the view that TNF does not primarily promote insulin resistance by downregulation of protein levels of signal or effector proteins controlling glucose uptake [10]. Thus, at present, the common denominator of the different models of insulin resistance is the quantitatively and/or qualitatively reduced IR kinase activity and insufficient substrate phosphorylation as the crucial initiating event. This opens up the possibility that TNF induced insulin resistance is, at least in part, due to interference with IR induced signal cascades distinct from IRS-1/2 and funnelling downstream to unknown targets. We propose that one candidate, the transcriptional activator STAT5, could be an important operator in this scenario as an immediate substrate of IR kinase.

The physiologic function of STAT5 is not yet fully elucidated [24], however, STAT5 is capable of acting in concert with other transcription factors, in a bimodal way, for example, with the glucocorticoid receptor. Activated STAT5 antagonizes glucocorticoid action at the glucocorticoid responsive elements and glucocorticoids synergize with STAT5 at specific STAT5 response elements [25,26], providing a link to glucocorticoid modulation of glucose homeostasis [27]. The potential role of STAT5 in acquisition of NIDDM is supported by very recent observations made in STAT5B knockout mice, which, besides other defects, develop obesity [4]. It will be interesting to see whether or not these animals are prone to NIDDM. The relevance of STAT5 and other members of this family of transcription factors is further underscored by the

interesting finding, that in diabetic (*db/db*) mice expressing a defective leptin receptor, defective STAT3, 5 and 6 signaling was found, suggesting an important role of these transcription factors in mediating the anti-obesity effects of leptin [28]. Similar to TNF, leptin has also been implicated in the crosstalk with insulin signaling cascades [29].

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References

[1] White, M.F. and Kahn, C.R. (1994) J. Biol. Chem. 269, 1–4.
[2] Chen, J., Sadowski, H.B., Kohanski, R.A. and Wang, L.-H. (1997) Proc. Natl. Acad. Sci. USA 94, 2295–2300.
[3] Liu, X., Robinson, G.W., Wagner, K.-U., Garrett, L., Wynshaw-Boris, A. and Henninghausen, L. (1997) Genes Dev. 11, 179–186.
[4] Udy, G.B., Towers, R.P., Snell, R.G., Wilkins, R.J., Park, S.-H., Ram, P.A., Waxman, D.J. and Davey, H.W. (1997) Proc. Natl. Acad. Sci. USA 94, 7239–7244.
[5] Kahn, B.B. (1998) Cell 92, 593–596.
[6] Hotamisligil, G.S. and Spiegelman, B.M. (1994) Diabetes 43, 1271–1278.
[7] Hotamisligil, G.S., Shargil, N.S. and Spiegelman, B.M. (1993) Science 259, 87–90.
[8] Saghizadeh, M., Ong, J.M., Garvey, W.T., Henry, R.R. and Kern, A.A. (1996) J. Clin. Invest. 97, 1111–1116.
[9] Hauner, H., Petruschke, T., Russ, T., Röhrig, K. and Eckel, J. (1995) Diabetologia 38, 764–771.
[10] Uysal, K.T., Wiesbrock, S.M., Marino, M.W. and Hotamisligil, G.S. (1997) Nature 389, 610–614.
[11] Saad, M.J., Araki, E., Miralpeix, M., Rothenberg, P.L., White, M.F. and Kahn, C.R. (1992) J. Clin. Invest. 90, 1839–1849.
[12] Feinstein, R., Kanety, H., Papa, M.Z., Lunenfeld, B. and Karasik, A. (1993) J. Biol. Chem. 268, 26055–26058.
[13] Paz, K., Hemi, R., LeRoith, D.L., Karasik, A., Elhanany, E., Kanety, H. and Zick, Y. (1997) J. Biol. Chem. 272, 29911–29918.
[14] Stephens, J.M., Lee, J. and Pilch, P.F. (1997) J. Biol. Chem. 272, 971–976.
[15] Peraldi, P., Hotamisligil, G.S., Buurman, W.A., White, M.F. and Spiegelman, B.M. (1996) J. Biol. Chem. 271, 13018–13022.
[16] Bader, D., Masaki, T. and Fischman, D.A. (1982) J. Cell. Biol. 95, 763–770.
[17] Irintchev, A., Langer, M., Zweyer, M., Theisen, R. and Wernig, A. (1997) J. Physiol. 500, 755–758.
[18] Irintchev, A., Langer, M., Zweyer, M. and Wernig, A. (1997) Basic Appl. Myol. 7, 161–166.
[19] Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D. and Pfizenmaier, K. (1995) EMBO J. 14, 1961–1969.
[20] Kantety, H., Feinstein, R., Papa, M.Z., Hemi, R. and Karasik, A. (1995) J. Biol. Chem. 270, 23780–23784.
[21] Hotamisligil, G.S., Peraldi, P., Budavari, A., Ellis, R., White, M.F. and Spiegelman, B.M. (1996) Science 271, 665–668.
[22] Groner, B. and Gouillieux, F. (1995) Curr. Opin. Genet. Dev. 5, 587–594.
[23] Brüning, J.C., Winnay, J., Bonner-Weir, S., Taylor, S.I., Accli, D. and Kahn, R.C. (1997) Cell 88, 561–572.
[24] Darnell Jr., J.E. (1997) Science 277, 1630–1635.
[25] Stöcklin, E., Wissler, M., Gouilleux, F. and Groner, B. (1996) Nature 383, 726–728.
[26] Stöcklin, E., Wissler, M., Morrigl, R. and Groner, B. (1997) Mol. Cell. Biol. 17, 6708–6716.
[27] Ewart, H.S., Somwar, R. and Klip, A. (1998) FEBS Lett. 425, 179–183.
[28] Ghilardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M.H. and Skoda, R.C. (1996) Proc. Natl. Acad. Sci. USA 93, 6231–6235.
[29] Cohen, B., Novick, D. and Rubinstein, M. (1996) Science 274, 1185–1190.