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Tumor necrosis factor (TNF) interferes with insulin signaling through the p55 TNF receptor death domain

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Abstract

Tumor necrosis factor (TNF) contributes to insulin resistance by binding to the 55 kDa TNF receptor (TNF-R55), resulting in serine phosphorylation of proteins such as insulin receptor (IR) substrate (IRS)-1, followed by reduced tyrosine phosphorylation of IRS-1 through the IR and, thereby, diminished IR signal transduction. Through independent receptor domains, TNF-R55 activates a neutral (N-SMase) and an acid sphingomyelinase (A-SMase), that both generate the sphingolipid ceramide. Multiple candidate kinases have been identified that serine-phosphorylate IRS-1 in response to TNF or ceramide. However, due to the fact that the receptor domain of TNF-R55 mediating inhibition of the IR has not been mapped, it is currently unknown whether TNF exerts these effects with participation of N-SMase or A-SMase. Here, we identify the death domain of TNF-R55 as responsible for the inhibitory effects of TNF on tyrosine phosphorylation of IRS-1, implicating ceramide generated by A-SMase as a downstream mediator of inhibition of IR signaling.

Keywords: Tumor necrosis factor; Sphingomyelinase; Ceramide; Insulin; Diabetes; A-SMase; N-SMase; IRS-1

The insulin signaling system has a pivotal function in physiological processes such as cellular growth, survival, reproduction, and carbohydrate and fat metabolism [1]. After ligand stimulation of the insulin receptor (IR) tyrosine kinase, a number of endogenous substrates, including insulin receptor substrates (IRS)-1-4 and the adapter protein Shc, are phosphorylated on tyrosine residues. These proteins then serve as docking sites for downstream effector molecules such as phosphoinositide 3-kinase, and in consequence, activate signaling kinases including the Ser/Thr kinase Akt. Akt and its downstream signals are crucial for the metabolic actions of insulin such as GLUT4 translocation and glucose transport, glycogen synthesis through activation of glycogen synthesis kinase-3, and mammalian target of rapamycin activation and protein synthesis. The mitogenic actions of insulin are mediated by binding of Grb2 to tyrosine-phosphorylated Shc and IRS-1/2, leading to the activation of the mitogen activated protein (MAP) kinase pathway. Other signaling pathways triggered by insulin may also contribute to these responses [2].

Insulin resistance is a pathological state in which target cells fail to respond to ordinary levels of circulating insulin. Insulin resistance results from a consequence of both genetic and environmental factors, and represents an important prerequisite in the development of type 2 diabetes mellitus [3,4]. Insulin resistance alone does not cause diabetes as long as pancreatic β-cells produce sufficient insulin to compensate for the reduced sensitivity. Nevertheless, type 2 diabetes mellitus eventually develops, most likely because hyperinsulinemia itself

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exacerbates the pre-existing resistance until β-cells fail to compensate [5]. Obesity is a characteristic feature of many patients suffering from type 2 diabetes and since the inflammatory cytokine tumor necrosis factor (TNF) is highly expressed in adipose tissues of obese animals and humans, TNF has been proposed as one of the risk factors of insulin resistance in obesity [6,7]. In support of this hypothesis, obese mice deficient for TNF or TNF receptors do not develop insulin resistance [8,9].

At the molecular level, it has been shown that TNF can interfere with insulin signaling in multiple ways. In addition to transcriptional attenuation of insulin signaling [10], TNF can interfere with insulin signaling by inducing serine phosphorylation of IRS proteins such as IRS-1 [2]. Upon serine phosphorylation, IRS-1 displays a reduced ability to interact with the IR, to be phosphorylated by the IR on tyrosine residues, and to subsequently transduce signals of the IR [11,12]. Alternatively, serine phosphorylation may switch IRS-1 into an inhibitor of the tyrosine kinase function of the IR [13]. Aside from functional impairment, serine phosphorylation can also result in proteasome-mediated degradation of IRS-1 [14].

Several kinases have been implicated as candidates for serine phosphorylation of IRS-1 [2]. c-Jun aminoterminal kinase (JNK) has been identified as a potential kinase mediating phosphorylation of IRS-1 at Ser^{302} and Ser^{307} [15,16]. JNK is a kinase that can be activated by TNF through generation of the lipid mediator ceramide [17]. In addition, ceramide is able to activate PKCζ, an isoform of atypical PKC that likewise phosphorylates IRS-1 at serine residues [18,19]. Other studies suggest the involvement of p42/p44 MAP kinases and p38 MAP kinase in IRS-1 serine phosphorylation [20,21]. Inhibitor κB kinase (IKK) activation by TNF has similarly been implicated [22], potentially explaining why IKK inhibition with salicylates or targeted disruption of *ikk*-β reverses obesity-induced insulin resistance [23]. Further studies suggest that Akt, mammalian target of rapamycin, and glycogen synthesis kinase-3 are additional kinases that mediate serine phosphorylation of IRS-1 [24–26].

TNF-mediated inhibition of IR signaling can be mimicked by treatment of cells with exogenous sphingomyelinase (SMase) or cell-permeable ceramide analogs [12,27]. Ceramide and fatty acids have additionally been described as inducers of insulin resistance in skeletal muscle [28–30]. Moreover, inhibition of ceramide generation reverses TNF-induced insulin resistance [31]. Therefore, SMase activation by TNF followed by intracellular accumulation of ceramide may be responsible for the activation of serine kinases leading to inhibition of IR signaling [27,32]. TNF transduces its signals through two distinct cell surface receptors of 55 kDa (TNF-R55) and 75 kDa (TNF-R75) molecular weight. For inhibition of insulin signaling, activation of TNF-R55 is sufficient [27]. TNF-R55 rapidly activates two distinct forms of SMases, an endosomal-lysosomal acid (A-) SMase and a membrane-bound neutral (N-) SMase. The generation of ceramide in the lysosomal compartment by A-SMase has been linked to the activation of JNK [17,33], PKCζ [34], and apoptosis [17,35,36] whereas activation of N-SMase leads to an accumulation of ceramide at the plasma membrane of a cell. This pool of membrane-bound ceramide is responsible for the activation of the protein kinase Raf-1 by TNF, most likely by stimulating CAPK/KSR which in turn phosphorylates Raf-1 [37]. Both types of SMases are independently activated by distinct cytoplasmic domains of TNF-R55 [38]. A region corresponding to the previously identified death domain [39] is responsible for A-SMase activation and also for mediating the cytotoxic effects of TNF, activation of nuclear factor-κB, JNK, p38, and p42/44 MAP kinase [40–42]. A second, independent region N-terminal of the death domain, designated N-SMase activation domain (NSD; [43]), recruits the WD-repeat protein factor associated with N-SMase activation (FAN) which in turn activates N-SMase [44].

At present, it remains unclear whether TNF-R55 participates in inhibition of IR signaling by activation of the A-SMase- or the N-SMase pathway. In this study, we have identified the death domain of TNF-R as the responsible domain that mediates the attenuation of IR signaling. In consequence, our data implicate A-SMase, but not N-SMase, as the enzyme that is most likely responsible for relaying the inhibitory signals of TNF to the IR.

Materials and methods

Reagents. Highly purified human recombinant TNF was provided by G. Adolf (Bender Research Institute, Vienna, Austria). Insulin from porcine pancreas and anisomycin was obtained from Sigma. The rabbit polyclonal antibodies specific for the IR or IRS-1 and the mouse monoclonal anti-phosphotyrosine antibodies PY99 and PY20 were purchased from Santa Cruz. The expression construct pRK5.hIR, coding for the full-length human IR, was kindly provided by C. Wallasch and A. Ullrich (Max-Planck-Institute for Biochemistry, Martinsried, Germany).

Mice. Cryoconserved sperm (kindly provided by K. Pfeffer, University of Düsseldorf, Germany and T. Plitz, Serono International S. A., Geneva, Switzerland) was utilized to generate C57Bl/6 TNF-$R55^{-/-}$ TgΔ30 mice by in vitro fertilization (Transgenics Laboratory, ZMBH Heidelberg, Germany). The animals were interbred under standard laboratory conditions and their genotype was verified as described [45].

Cell culture. Embryonal fibroblasts (EF) from wild-type (WT) TNF-$R55^{-/-}$TgΔ30 and TNF-$R55^{-/-}$TgΔcyt mice [45] have been described [42]. EF were immortalized by stable transfection with the SV40 large T antigen expression vector pMSSVLT [46]. Transfectants of the murine pre-B cell line 70Z/3 stably expressing the intact human TNF-R55 have been described elsewhere [42]. EF and 70Z/3 transfectants were maintained in a mixture of Click's/RPMI 1640 (50/50%v/v) supplemented with 10%v/v FCS, 10 mM glutamine, and 50 μg/ml each of streptomycin and penicillin. 293 (human embryonic kidney)

cells were originally obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in high glucose DMEM supplemented as above. Primary WT myoblasts from an expanded culture designated i28 have been described previously [47,48] and were cultured on collagen type I-coated petri dishes in Ham's F-10 medium supplemented with 20%v/v FCS and 50 μg/ml each of streptomycin and penicillin. Primary TNF-R55$^{-/-}$TgΔ30 myoblasts were established from the hind-leg muscles of TNF-R55$^{-/-}$TgΔ30 mice [45] identically as described for primary WT i28 myoblasts [47,48]. Initial expansion and enrichment in myoblasts was achieved by repeated preplating and growth in minimum essential medium with D-valine containing 20%v/v FCS. When the expanded culture was highly enriched, TNF-R55$^{-/-}$TgΔ30 myoblasts were further propagated in Ham's F-10 medium supplemented with 20%v/v FCS and 2.5 ng/ml bFGF. For differentiation into myotubes, the medium was changed to low-glucose (1 g/L) DMEM supplemented with 10%v/v horse serum for 2 days. All cells were grown in a humidified incubator containing 5%w/v CO_2.

Transfections, immunoprecipitations, and immunoblots. For EF, 2×10^6 cells per timepoint were seeded in 10 cm tissue culture dishes, allowed to adhere, and serum starved (Click's/RPMI 1640 + 0.1%w/v BSA) for 24 h. After stimulation, the cells were washed twice with cold PBS and lysed in TNE buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% v/v NP40, and 2 mM EDTA) containing 10 μg/ml pepstatin/aprotinin/leupeptin, 1 mM sodium orthovanadate, and 5 mM NaF. After centrifugation at 10,000*g* and 4 °C for 15 min, the protein concentration of the supernatants was measured using a BCA assay (Pierce). Immunoprecipitation was performed overnight on ice using 2 μg anti-IR or anti-IRS-1 antibody followed by collection of the immunocomplexes by a 1 h incubation with γ-bind–Sepharose (Amersham Biosciences) and subsequent washing of the immunocomplexes for four times in cold lysis buffer. The immunoprecipitated proteins were separated on 7.5%w/v gels by SDS–PAGE. After electrophoretic transfer to nitrocellulose (Whatman-Biometra), reactive proteins were detected using antisera specific for phosphotyrosine and the ECL detection kit (Amersham Biosciences). Experiments with 70Z/3 transfectants were basically carried out as above, except for the use of 1×10^7 suspension cells per timepoint, the addition of 10 mM sodium pyrophosphate, and the substitution of pepstatin/aprotinin/leupeptin for Complete protease inhibitor mixture (Roche) in the lysis buffer as recommended by the manufacturer. Transient transfection of 293 cells was performed by the calcium phosphate precipitation method using 5 μg pRK5.hIR in combination with 5 μg pEF.FAN$_{1–917}$, pEF.FAN$_{703–917}$ (pEF.ATG containing cDNAs coding for FAN$_{1–917}$ or FAN$_{703–917}$ [44]), pRK5.FADD$_{DN}$ or pRK5.TRAF2$_{DN}$ [49] or the expression vectors pEF.ATG [44] or pRK5 [49] without insert. Twenty-four hours after transfection, cells were serum starved (DMEM + 0.1%w/v BSA) for another 24 h and either stimulated while still attached to petri dishes or after detachment and transfer into Eppendorf caps. Cells were lysed and expression of the IR or tyrosine phosphorylation of the IR and of IRS-1 was detected by Western blot using anti-IR antibody or the phosphotyrosine-specific antibody PY20 as described above. For analysis of myoblasts, 5×10^6 cells per timepoint were seeded in 10 cm tissue culture dishes and differentiated into myotubes for 2 days as described above. Prior to stimulation, differentiated cells were serum starved (DMEM + 0.1% w/v BSA) for 2 h. After stimulation, the cells were washed twice with cold PBS and scraped into 750 μl cold phosphorylation buffer (50 mM Hepes, pH 7.8, 2.5 mM EDTA, 1% v/v Triton X-100, 150 mM saccharose, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, and Complete protease inhibitor mixture (Roche)). After 60 min on ice, the cells were homogenized by repeated passing through a 27-gauge needle. Immunoprecipitation and Western blot were performed as above using 1.5 μg anti-IRS-1 antibody. Reactive proteins were detected using antibodies specific for phosphotyrosine (PY99) or IRS-1. Tyrosine phosphorylation of IRS-1 was quantified after scanning of the autoradiographs using the software package PCBAS (Raytest). For all Western blot experiments, equal loading was either verified by reblotting the membranes previously used for detection of tyrosine phosphorylation with antibodies for IR or IRS-1 or by parallel immunoprecipitation/Western blot using IR or IRS-1 antibodies (not shown).

Results

To characterize the TNF-R55 domain mediating interference with insulin signaling, we initially analyzed EF from mice deficient for TNF-R55 but expressing a transgene of the murine TNF-R55 that either lacks the C-terminal 30 amino acids (TNF-R55$^{-/-}$TgΔ30) or the entire cytoplasmic domain (except for the five membrane-proximal amino acids, TNF-R55$^{-/-}$TgΔcyt, [45]). In mice carrying the TNF-R55$^{-/-}$TgΔ30 transgene, the deletion eliminates signaling through the death domain while the NSD remains unaffected. In the TNF-R55$^{-/-}$TgΔcyt deletion, both domains are lacking [42,45]. As shown in Fig. 1A, treatment with insulin transiently increased tyrosine phosphorylation of the IR in TNF-R55$^{-/-}$TgΔcyt, TNF-R55$^{-/-}$TgΔ30, and in control EF from WT mice. However, pretreatment with TNF did not induce a decrease of IR tyrosine phosphorylation in either cell line. Since a selective reduction of IRS-1 but not IR tyrosine phosphorylation has been described after treatment of rat Fao hepatoma cells with bacterial SMase [50], we additionally analyzed the effects of TNF on IRS-1 in WT EF. Again, no inhibition of

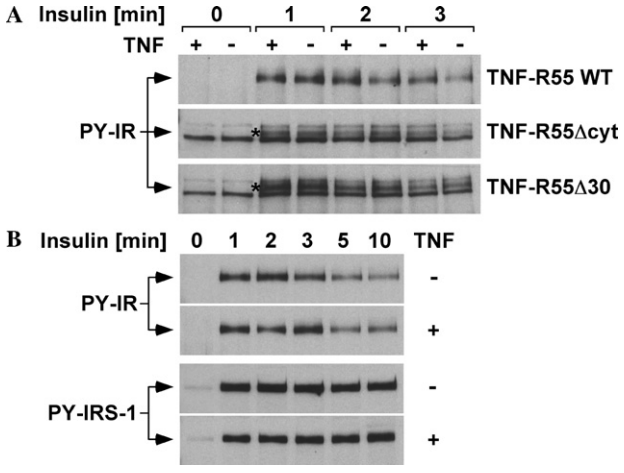

Fig. 1. TNF does not inhibit insulin signaling in murine EF. (A) Murine EF from TNF-R55 WT, TNF-R55$^{-/-}$TgΔcyt and TNF-R55$^{-/-}$TgΔ30 mice were serum starved for 24 h. Thirty minutes prior to stimulation with 100 nM insulin for the indicated times, 100 ng/ml hTNF was added (+) or not (−). The IR was immunoprecipitated from the cell lysates and phosphorylation on tyrosine residues (PY-IR) was detected by Western blot analysis using PY99 antibody. For TNF-R55$^{-/-}$TgΔcyt and TNF-R55$^{-/-}$TgΔ30 EF, the position of the tyrosine phosphorylated IR is additionally marked by an asterisk. (B) parallel analysis of murine EF from TNF-R55 WT mice for tyrosine phosphorylation of both immunoprecipitated IRS-1 (PY-IRS-1) and IR (PY-IR).

insulin-induced tyrosine phosphorylation by TNF was detectable, either for IR or for IRS-1 (Fig. 1B). The analysis of EF deficient for FAN (resulting in defective activation of N-SMase by TNF, [51]) or FADD (defective activation of A-SMase, [52]) yielded identical results, as did the use of primary instead of immortalized EF, transient overexpression of the IR in WT EF or an increase in preincubation time with TNF (data not shown). In summary, these observations suggest that in EF (which do not represent a natural target of insulin), the signaling pathways of TNF-R55 and the IR may not cross-talk, explaining the inability of TNF to inhibit insulin signaling.

In a separate approach, we employed the murine pre-B cell line 70Z/3 that does not express endogenous TNF-R55 but is capable of displaying TNF-specific responses after transfection of the human TNF-R55 [53]. For this cell line, a set of stable transfectants exists that stably express the intact human TR55 or various deletion mutants thereof [38,42,43]. These deletions either inactivate the death domain but leave the NSD intact or affect both domains, prevent signaling through both the A-SMase and the N-SMase pathways [43]. When we analyzed 70Z/3 cells stably expressing the intact TNF-R55, we however did not observe a reduction of insulin-induced IRS-1 tyrosine phosphorylation by TNF (Fig. 2). In contrast, treatment with anisomycin used as a positive control [16] clearly inhibited IRS-1 tyrosine phosphorylation. Independent experiments in which we extended the time of preincubation with TNF for up to 24 h likewise did not show a reduction of insulin-induced IRS-1 tyrosine phosphorylation (data not shown), indicating that, similar to EF, in 70Z/3 pre-B cells the signaling pathways of TNF and insulin likewise do not interact, preventing mapping of the TNF-R55-inhibitory domain in this cell system.

As a third alternative, we employed the human embryonal kidney cell line 293. These cells have been previously utilized to analyze IR signal transduction after transient or stable transfection of individual components of the signaling pathways [54–57]. To obtain further insight into the impact of TNF on insulin signaling, we therefore examined 293 cells after transient transfection of the IR in combination with constructs coding for proteins that are involved in signaling pathways initiated by either the NSD or the death domain of TNF-R55. As exemplified in Fig. 3A for the IR, 293 cells strongly overexpressed transfected constructs for up to 48 h. Tyrosine phosphorylation of the IR and of IRS-1 was substantially reduced in cells pretreated with TNF when compared to cells treated with insulin alone (Fig. 3B). However, this response was neither altered by additional transfection of FAN (enhancing signals through the NSD, [44]) or dominant negative constructs of FAN (reducing NSD-mediated signals of TNF, [44]), nor by transfection of dominant negative constructs of FADD or TRAF2 which impair death domain-mediated signals ([49], Fig. 3B). In subsequent analyses, we found that the observed reduction in IR tyrosine phosphorylation after preincubation with TNF (Fig. 3C, upper panel) was not specific for TNF but rather a general effect caused by detaching the cells and preincubating them in suspension before the addition of insulin. When the cells were left adherent to petri dishes during preincubation/stimulation, no reduction of tyrosine phosphorylation by TNF was detectable anymore (Fig. 3C, lower panel). Likewise, when cells were preincubated in suspension in the presence of either TNF or medium without TNF, insulin-induced tyrosine phosphorylation of the IR was equally reduced in both instances (Fig. 3D). In summary, these data suggest that caution should be exerted when analyzing TNF interference with insulin signaling in 293 cells or when interpreting data obtained from this system [58].

Since the above cell systems do not represent natural targets for insulin, we decided to conduct further investigations in cells that represent physiological targets of insulin. In addition to adipocytes and hepatocytes, skeletal muscle cells have been previously utilized for the analysis of the effects of TNF on insulin signaling. Skeletal muscle is responsible for 75% of the glucose disposal of the body and appears to play a major role in insulin-regulated glucose homeostasis [59]. Primary murine myoblasts from an expanded culture derived from WT mice (designated i28) differentiate in vitro into multinucleated myotubes with markers characteristic of skeletal muscle [47,48]. These myotubes are responsive to both TNF and insulin, and represent a suitable model for studying the cross-talk between TNF and insulin signalling cascades [30,60]. As shown in Fig. 4A, in myotubes from WT i28 primary myoblasts, tyrosine phosphorylation of IRS-1 induced by insulin was substantially inhibited by preincubation with TNF when compared to myotubes treated with insulin only, confirming that TNF-R55 is expressed in these cells as a signaling competent molecule that actually links to the signaling pathways of the IR. Subsequently, primary myoblasts obtained from TNF-R55$^{-/-}$TgΔ30 mice expressing a

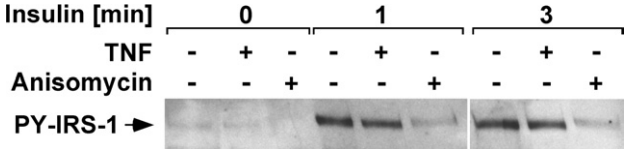

Fig. 2. Inhibition of IR tyrosine phosphorylation in murine 70Z/3 pre B-cells. 70Z/3 cells stably transfected with the human TNF-R55 were pretreated with either 100 ng/ml hTNF for 2 h or with 5 μg/ml anisomycin for 30 min (+) or not (−). Subsequently, cells were stimulated with 100 nM insulin for the indicated times. IRS-1 was immunoprecipitated from the cell lysates and tyrosine phosphorylation (PY-IR) was detected by Western blot analysis using PY99 antibody.

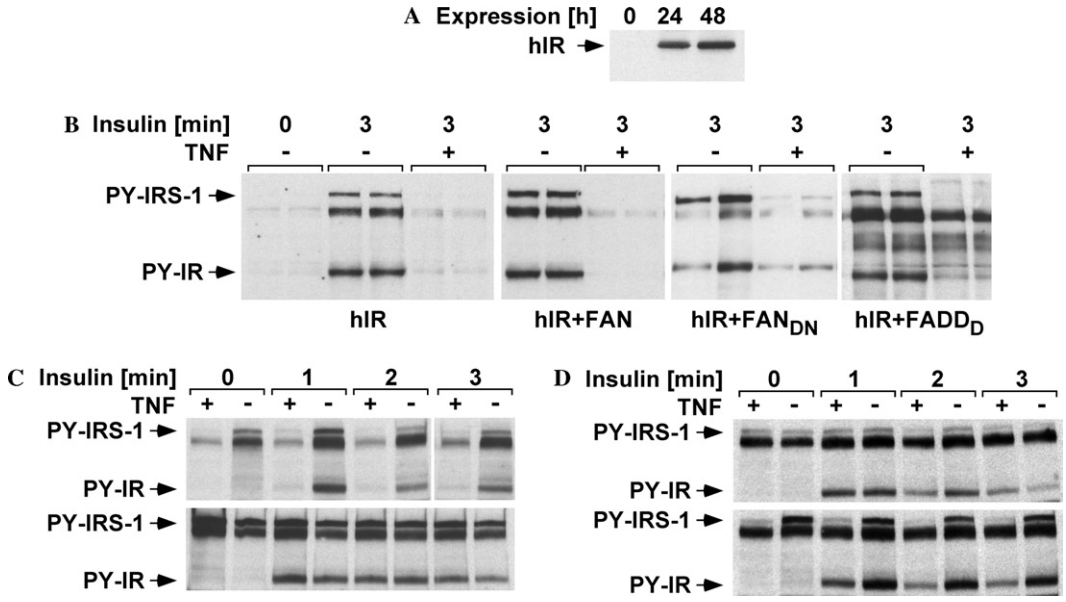

Fig. 3. Inhibition of IR tyrosine phosphorylation in 293 cells depends on stimulation conditions, not on the presence of TNF. (A) 293 cells were transiently transfected with an expression construct for the human IR. IR protein levels at the indicated times were monitored by Western blot analysis. (B) 293 cells transiently transfected with the human IR and expression constructs for FAN and dominant negative (DN) forms of FAN, FADD or TRAF2 were serum starved for 24 h after transfection for another 24 h and left unstimulated (0) or treated with 100 nM insulin for 3 min without (−) or with prior incubation with 100 ng/ml hTNF for 30 min (+) after detachment. Cell lysates were loaded in duplicate for control. Control transfection with the human IR in combination with expression vectors carrying no insert yielded identical results (not shown). (C) Upper panel, 293 cells transiently transfected with the human IR were serum starved as in B, detached, and preincubated in suspension in the presence of 100 ng/ml hTNF for 30 min (+) before 100 nM insulin was added for the indicated times. Alternatively, cells were detached, and immediately stimulated with 100 nM insulin (−). Lower panel, cells were stimulated identically but left attached to petri dishes during stimulation. (D) Upper panel, 293 cells transiently transfected with the human IR were serum starved as in B, detached and preincubated in suspension for 10 min either with the addition of 100 ng/ml hTNF (+) or with the addition of an equal volume of medium containing no TNF (−) before being stimulated with 100 nM insulin for the indicated times. Lower panel, to illustrate that IR tyrosine phosphorylation shown in the upper panel is indeed reduced, lysates shown under "+" in the upper panel were separated again next to lysates from cells that had been detached and immediately been stimulated with 100 nM insulin (−). Proteins reactive to phosphotyrosine (antibody PY20) corresponding in size to the IR (PY-IR) and to IRS-1 (PY-IRS-1) are indicated.

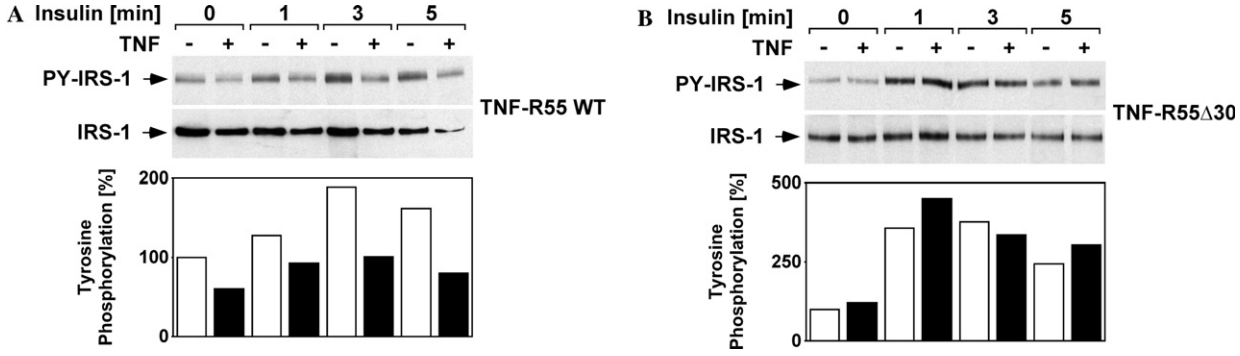

Fig. 4. Inhibition of insulin-mediated IRS-1 tyrosine phosphorylation by TNF depends on a functional death domain of TNF-R55. (A) Upper panel, myotubes from i28 WT primary myoblasts were serum starved for 2 h in the presence (+) or absence (−) of 50 ng/ml hTNF. Subsequently, the cells were stimulated with 200 nM insulin for the indicated times. IRS-1 was immunoprecipitated from the cell lysates and the amount of IRS-1 phosphorylated on tyrosine (PY-IRS-1) or the total amount of IRS-1 protein (IRS-1) was detected by Western blot analysis. Lower panel, tyrosine phosphorylation of IRS-1 protein was quantified by densitometric analysis and is shown relative to cells neither treated with TNF nor with insulin. (B) Parallel analysis using myotubes from TNF-R55$^{-/-}$TgΔ30 cells. One out of several experiments with similar results is shown ($n = 3$).

TNF-R55 that carries a nonfunctional death domain but an intact NSD (see above) were differentiated into myotubes and analyzed for changes in insulin-induced tyrosine phosphorylation in response to TNF. In contrast to i28 WT primary myotubes, insulin-stimulated TNF-R55$^{-/-}$TgΔ30 primary myotubes did not show a reduction in tyrosine phosphorylation of IRS-1 when pretreated with TNF (Fig. 4B), suggesting that the death domain of TNF-R55 is responsible for the suppression of insulin signaling.

Discussion

In this study, we wanted to clarify which domain of TNF-R55 is responsible for inhibition of insulin-induced tyrosine phosphorylation of the IR and IRS-1, and therefore ultimately for insulin resistance caused by TNF. Our initial results obtained in several cell systems such as embryonal fibroblasts, 70Z/3 pre-B cells or 293 human embryonal kidney cells that are principally well established for the study of TNF signal transduction (e.g., [38,42,43,49]), nevertheless demonstrate that in these cell systems, TNF does not affect insulin-induced tyrosine phosphorylation. This is most likely due to the fact that cells of fibroblastoid origin, pre-B cells such as 70Z/3 or kidney-derived cells such as 293 do not belong to the natural targets of insulin. Although these cells have been extensively used for the analysis of TNF signaling and although they clearly respond to insulin with an increased tyrosine phosphorylation of the IR and of IRS-1, obviously, one or more components required for relaying the suppressive signals of TNF are either lacking or inhibited in these cells. However, the ability of TNF to interfere with insulin signaling can be restored, e.g., when EF are differentiated into adipocytes that represent physiological targets of insulin [61], pointing out the necessity to utilize cells that naturally respond to insulin when studying the role of TNF in insulin resistance.

In contrast to the above cell systems, differentiated myotubes as a model for skeletal muscle do represent natural targets of insulin. Our results obtained in primary myotubes point to the death domain of TNF-R55 as being responsible for the inhibitory action of TNF on insulin signaling. This result suggests that TNF-R55 participates in inhibition of IR signaling by activation of the A-SMase-pathway which depends on a functional death domain. This assumption fits very well into a model that has linked the generation of ceramide in the lysosomal compartment by A-SMase to the activation of JNKs [17]. JNKs have been implicated in the phosphorylation of IRS-1 at $Ser^{302/307}$ [15,16], thereby interfering with insulin action in cultured cells. Moreover, JNKs apparently play a central role in obesity and insulin resistance in vivo [62]. JNK-inhibitory peptides have already been successfully used to improve insulin resistance and glucose tolerance in mice, representing a promising novel approach for the treatment of diabetes [63].

In addition to JNKs, p42/p44 MAP kinases and p38 MAP kinase can similarly mediate IRS-1 serine phosphorylation in response to TNF [20,21]. We have previously demonstrated that p42/p44 MAP kinases are also activated through the death domain of TNF-R55 [42], as is p38 MAP kinase [41]. The activation of IKK by TNF may be another mechanism to induce interference with insulin signaling [22]. de Alvaro et al. [64] have recently shown that IKK activation by TNF occurs in a p38 MAP kinase-dependent manner, once more implicating the death domain in TNF-induced insulin resistance. Finally, PKCζ, an isoform of atypical PKC that is also

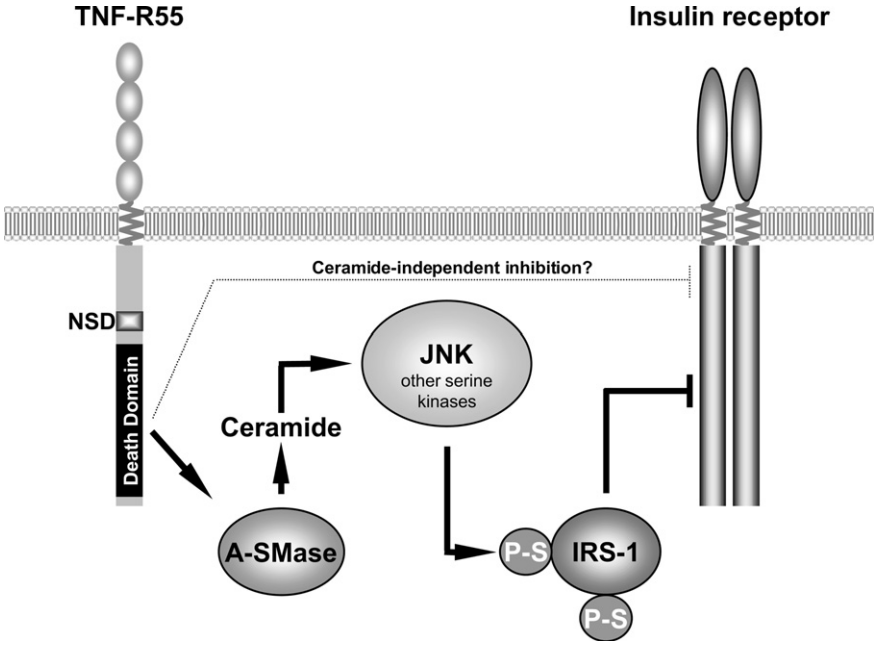

Fig. 5. Simplified model for interference of TNF-R55 with insulin signaling. The activated TNF-R55 enhances the intrinsic activity of A-SMase through its death domain, resulting in accumulation of ceramide. Ceramide in turn stimulates the kinase function of JNKs (and eventually other serine kinases), leading to increased phosphorylation of IRS-1 on serine residues. Serine phosphorylated IRS-1 is now able to inhibit signals from the IR. Independently, TNF-R55 may initiate other, ceramide-independent inhibitory signals through its death domain.

capable of phosphorylating IRS-1 at serine residues [18,19], can be activated by ceramide generated by the A-SMase pathway [65]. However, PKCζ is most likely involved in serine phosphorylation of IRS-1 in response to insulin rather than to TNF [18,19].

Paz et al. [12] have previously speculated that TNF-mediated insulin resistance may involve activation of kinases in response to ceramide generated by FAN and N-SMase. Based upon our results, a role of N-SMase, FAN or the NSD in the inhibition of insulin signaling by TNF is unlikely. Rather, we suggest a model in which binding of TNF to TNF-R55 leads to the death domain-dependent activation of A-SMase and, in consequence, to the activation of JNKs (and eventually other serine kinases) by A-SMase-generated ceramide, resulting in increased serine phosphorylation of IRS-1 and finally, attenuation of insulin signaling (Fig. 5). At present, we cannot rule out the possibility that the death domain additionally initiates ceramide-independent signals that interfere with insulin signaling. In addition, ceramide generated through the A-SMase pathway may inhibit insulin signaling through mechanisms distinct from activation of serine kinases. For example, Stratford et al. [66] have described that ceramide interferes with insulin stimulation of Akt by blocking translocation of Akt as well as by stimulating the dephosphorylation of Akt. Therefore, the targeting of death domain-mediated signals or inhibition of A-SMase-dependent ceramide metabolism may provide novel options in the prevention or treatment of insulin resistance and type II diabetes mellitus.

Acknowledgments

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