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Impaired regulation of the TNF-α converting enzyme/tissue inhibitor of metalloproteinase 3 proteolytic system in skeletal muscle of obese type 2 diabetic patients: a new mechanism of insulin resistance in humans

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Abstract

Aims/hypothesis—TNF-α levels are increased in obesity and type 2 diabetes. The regulation of TNF-α converting enzyme (TACE) and its inhibitor, tissue inhibitor of metalloproteinase 3 (TIMP3), in human type 2 diabetes is unknown.

Methods—We examined TACE/TIMP3 regulation: (1) in lean and obese normal glucose tolerant (NGT) individuals and in type 2 diabetes patients; (2) following 6 h of lipid/saline infusion in NGT individuals; and (3) in cultured human myotubes from lean NGT individuals incubated with palmitate. Insulin sensitivity was assessed by a euglycaemic clamp and TACE/TIMP3 was evaluated by confocal microscopy, RT-PCR, western blotting and an in vitro activity assay. Circulating TNF-α, TNF-α-receptor 1 (TNFR1), TNF-α-receptor 2 (TNFR2), IL-6 receptor (IL-6R), vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) levels were evaluated.

Results—TIMP3 levels were reduced and TACE enzymatic activity was increased in type 2 diabetes skeletal muscle. *TACE* expression, and TACE, TNF-α, TNFR1 and IL-6R levels were increased in type 2 diabetes, and positively correlated with insulin resistance. A 6 h lipid infusion into NGT individuals decreased insulin-stimulated glucose metabolism by 25% with increased TACE, decreased expression of the gene encoding TIMP3 and increased IL-6R release. Palmitate induced a dramatic reduction of TIMP3 and increased the TACE/TIMP3 ratio in cultured myotubes.

Conclusions/interpretation—TACE activity was increased in skeletal muscle of obese type 2 diabetes patients and in lipid-induced insulin resistance. We propose that dysregulation of membrane proteolysis by TACE/TIMP3 of TNF-α and IL-6R is an important factor for the development of

skeletal muscle insulin resistance in obese type 2 diabetes patients by a novel autocrine/paracrine mechanism.

Keywords

Human type 2 diabetes mellitus; Insulin resistance; TACE; TIMP3

Introduction

Insulin resistance is the hallmark of obesity and type 2 diabetes and is a major risk factor for cardiovascular disease [1]. Obesity and type 2 diabetes are characterised by a low-grade inflammation state that may also contribute to the increased risk of cardiovascular disease in these patients [2,3]. TNF-α is a pro-inflammatory cytokine that was first linked to insulin resistance by Hotamisligil et al. [4–7] in the *ob/ob* mouse, a model of obesity and type 2 diabetes [8,9]. Although the role played in insulin resistance by TNF-α is well established in animals, its contribution to the development of impaired insulin action in humans is controversial [4, 10,11]. While patients with type 2 diabetes and obesity have increased plasma levels of inflammatory cytokines, e.g. TNF-α and IL-6 [12–14], the infusion of TNF-α-neutralising antibody does not significantly improve insulin sensitivity in obese type 2 diabetes patients [15].

Pro-TNF-α is expressed as a 26 kDa membrane-bound protein and processed into a 17 kDa soluble form, TNF-α, that is released from the cell surface by the action of a disintegrin and metalloproteinase (ADAM-17) also called TNF-α converting enzyme (TACE) [16,17]. Tissue inhibitor of matrix metalloproteinase 3 (TIMP3) controls TNF-α levels in vivo [18]. Among the four TIMPs, only TIMP3 binds to the extracellular matrix and contains an amino acid sequence (PFG) required for TACE inhibition [19]. It has been previously shown that *Timp3* acts as a modifier gene whereby its deficiency contributes to the onset of diabetes in insulin receptor (IR) heterozygous mice, supported by the development of glucose intolerance and hyperinsulinaemia in *Insr* and *Timp3* double-heterozygous mice [20]. Short-term in vivo inhibition of TACE results in a marked reduction of hyperglycaemia and vascular inflammation in this model of insulin resistance and type 2 diabetes. While *Tace* (also known as *Adam17*) heterozygous mice are partially protected from diabetes and obesity induced by high-fat diet, *Tace* homozygous mice show a lean hypermetabolic phenotype [20–22].

Here, we have explored the role of TACE and TIMP3 in mediating insulin resistance in non-diabetic obese and type 2 diabetes individuals, and have tested the effects of 6 h lipid infusion, a treatment known to induce insulin resistance, on the regulation of TACE/TIMP3 in normal individuals.

Methods

Participants

Fourteen lean and 14 obese individuals with normal oral (75 g) glucose tolerance and 14 type 2 diabetes patients were studied [23]. Their clinical and laboratory characteristics are shown in the Table 1. BMI was <25 kg/m^2 in all lean participants. There was no evidence of major organ disease (except diabetes) based upon history, physical examination, clinical chemistry, urinalysis and ECG. Weight was stable for at least 3 months prior to the study and, other than oral glucose-lowering drugs (metformin or glibenclamide [known as glyburide in the USA and Canada]), no participant was taking medication. Fasting blood samples were taken from an antecubital vein to determine HbA_{1c}. Insulin sensitivity was assessed with a euglycaemic–hyperinsulinaemic (80 mU m^{-2} min^{-1}) clamp [24] or by the homeostatic model assessment of insulin resistance index (HOMA-IR) [25]. In each participant, following a 10–12 h overnight

fast, a percutaneous biopsy was obtained from the vastus lateralis muscle under local anaesthesia using a Bergström needle.

In a separate group, 12 normal glucose tolerant (NGT) individuals (Electronic supplementary material [ESM] Table 1) were infused in random order with either lipid (Lyposin III (Hospira, Inc., Lake Forest, IL, USA), 20% (wt/vol.) triacylglycerol emulsion) or 0.9% (wt/vol.) saline solution at a rate of 60 ml/h for 6 h. Percutaneous vastus lateralis muscle biopsies were obtained from these individuals before and after the 6 h infusion. Finally, after the second biopsy was taken, a 2 h euglycaemic insulin (80 mU m^{-2} min^{-1}) clamp was performed on all participants (ESM Fig. 1). Each participant gave written informed consent and all studies were previously approved by the University of Texas Health Science Center Institutional Review Board.

Confocal microscopy

Confocal microscopy experiments were carried out as previously described [26,27] and as detailed in the ESM Methods.

Subcellular fractionation

Subcellular fractionation was carried out with human skeletal muscle by differential centrifugation as described [28,29] with minor modifications, as detailed in ESM Methods (Fig. 1a).

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was extracted from human skeletal muscle, as detailed in ESM Methods. RNA expression of *TACE*, *TIMP3*, *IR* (also known as *INSR*), *IRS-1* (also known as *IRS1*) and *Akt* (also known as *Akt1*) (gene assays on demand Hs00234224_m1, HS00165949_m1, Hs00961557_m1, Hs00178563 and Hs99999145_m1, respectively) was measured by TaqMan real-time RT-PCR using an ABI PRISM 7900Ht System (Applied Biosystems, Foster City, CA, USA) and normalised to 18S rRNA selected as an endogenous control. Each reaction was carried out in duplicate. Efficiency of each probe was determined by serial dilutions of RNA skeletal muscle standard (Ambion, Austin, TX, USA) and analysis performed by the $2^{-\Delta\Delta C_t}$ method.

ELISA

A sandwich ELISA method was performed using anti-mouse antibodies conjugated to horseradish peroxidase, for human soluble TNF-α receptor 1 (sTNFR1), human soluble TNF-α receptor 2 (sTNFR2), human soluble IL-6 receptor (sIL-6R), human soluble vascular cell adhesion molecule-1 (sVCAM), and human soluble intercellular adhesion molecule-1 (sICAM) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The minimum detectable concentration was 0.77 pg/ml for sTNFR1, 6.5 pg/ml for sIL-6R, 0.6 pg/ml for sTNFR2, 0.35 ng/ml for sICAM and 0.6 ng/ml for sVCAM. The intra-assay and inter-assay CV values were 4.4% and 6.1% for sTNFR1, 4.5% and 5.1% for sIL-6R, 3.5% and 4.0% for sTNFR2, 4.8% and 10.1% for sICAM, and 3.1% and 7.0% for sVCAM, respectively. Sera were diluted before assay.

Western blotting

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane with 5% (wt/vol.) non-fat dried milk (Bio-Rad, Hercules, CA, USA) in TRIS-buffered saline (20 mmol/l TRIS [pH 7.4], 150 mmol/l NaCl and 0.02% (vol./vol.) Tween-20) and probed with primary antibodies to TACE and TIMP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and to pro-TNF-α, TNF-α, β-actin, alpha subunit of IR (IRα), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), AKT, phospho-AKT (Ser 473), 5′-AMP-activated protein kinase

(AMPK), phospho-AMPK and phospho-acetyl-CoA carboxylase (all antibodies were from Cell Signaling Technology, Danvers, MA, USA) followed by secondary antibodies as appropriate, and visualised by enhanced chemiluminescence, as described [30].

TACE activity assay

TACE activity was determined by the cleavage of the 12 residue peptide that spans residues Ala-76 to Val-87 in pro-TNF-α (ADAM-17 substrate IV) as previously described [31] and detailed in ESM Methods.

Generation of primary myotubes

Primary skeletal muscle cells were grown from satellite cells obtained from muscle tissue as previously described [32,33]. All donors were lean and were NGT. Human myotubes were treated with fatty acid-free BSA or 200 and 400 μmol/l palmitate for 4 h. Palmitate was mixed with acid-free BSA at a 5:1 palmitate/BSA ratio. After treatment, cells were lysed in lysis buffer (20 mmol/l TRIS, pH 7.5, 5 mmol/l EDTA, 10 mmol/l Na_3 PO_4, 100 mmol/l NaF, 2 mmol/l Na_3VO_4, 1% (vol./vol.) NP-40, 10 μmol/l leupeptin, 3 mmol/l benzamidine, 10 μg/ml aprotinin and 1 mmol/l phenylmethylsulfonyl fluoride). TIMP3, TACE, IR and β-actin protein content were measured by western blotting and densitometric analysis using a Bio-Rad GS800 (Bio-Rad, Hercules, CA, USA) densitometer as previously described [31,32].

Adenovirus infection

Adenoviruses expressing green fluorescent protein (GFP) only or GFP and TACE (Vector Biolabs, Philadelphia, PA, USA) were used to infect myotubes as described in ESM Methods.

Statistical analysis

Comparison between groups and association between variables were performed using Student's *t* test, one-way ANOVA, two-way ANOVA and Pearson's correlation coefficient (*r*) where appropriate (SPSS v.13 for Windows; SPSS, Chicago, IL, USA). Data are expressed as means ± SEM. $p<0.05$ was considered statistically significant.

Results

TNF-α, TACE, TIMP3 and IR distribution in human skeletal muscle

Fluorescence immunostaining and quantification by confocal microscopy were used to assess the abundance and subcellular localisation of TACE, TIMP3, TNF-α and IR in human skeletal muscle. TACE and IR distribution displayed a punctate staining pattern throughout the tissue but with a predominant localisation on the surface sarcolemma (ESM Figs 2a and 3a for TACE; ESM Figs 2c and 4b for IR), whereas TIMP3 showed a diffuse and reticular localisation pattern (ESM Fig. 2b). TNF-α was localised as a thin layer on the plasma membrane surface (ESM Figs 3b and 4a). Secondary antibodies alone did not show any specific staining, indicating the specificity of the pattern observed (data not shown). Merged images showed regions with co-localisation of TNF-α, TACE, TIMP3 and IR on the sarcolemmal surface (ESM Figs 2d, 3c, and 4c). To confirm the co-localisation pattern observed, we carried out quantitative analysis of co-localised staining in the images. Application of this method resulted in positive values for the product of differences of the mean (PDM) and Pearson's coefficient (Rr) in each pair analysed (IR–TACE 0.42; TACE–TIMP3 0.74; IR–TIMP3 0.56; TACE–TNF-α 0.38; and IR–TNF-α 0.63), with intensity correlation analysis (ICA) exhibiting right-skewed plots indicative of partial co-localisation. Channel co-localisation indicated that TACE signal resides in a subset of the IR and TIMP3 signal (ESM Fig. 2h, j), IR is a subset of the TIMP3 and TNF-α signal (ESM Figs 2m and 4g), and TNF-α is a subset of TACE and IR signal (ESM Figs 3g and 4f). The overlapping patterns of abundance of IR, TACE, TNF-α and TIMP3 in human

skeletal muscle suggest the possibility that TNF-α binds TNF-α receptor (TNFR) 1 or 2 and this may result in phosphorylation of serine/threonine kinases (JNK, IKKalpha/beta and ERK 1/2), which in turn negatively regulates insulin signalling at the skeletal muscle plasma membrane and this could directly inhibit IR signalling and, thus, glucose disposal by the muscle, in a paracrine fashion.

TACE and TIMP3 intracellular distribution in human skeletal muscle

To define TACE and TIMP3 subcellular localisation, we performed human skeletal muscle subcellular fractionation (Fig. 1a) and immunoblot analysis in each of three fractions (internal membrane, cytosol and plasma membrane), and in total homogenate. TIMP3 was present in all three subcellular compartments although enriched in plasma membrane (Fig. 1c), while TACE was found to be highly enriched in plasma membrane (Fig. 1b). In order to define the relative enrichment obtained during the subcellular fractionation procedure, we performed western blotting experiments with anti-GAPDH antibodies as well as with antibodies to IRα. GAPDH, a cytosolic protein, was virtually absent in the plasma membrane fraction (ESM Fig. 5a), while IRα was enriched more than twofold in the plasma membrane fraction (ESM Fig. 5b). Furthermore, Coomassie staining of two separate subcellular fractionation experiments demonstrated that the protein band compositions of the three subcellular fractions that we examined, i.e. plasma membranes, internal membranes and cytosol, were highly reproducible and markedly different from each other as well as from the total homogenate (ESM Fig. 5c).

TACE, TIMP3, pro-TNF-α and TNF-α expression in human skeletal muscle from NGT individuals and type 2 diabetes patients

We analysed expression of *TACE* in human skeletal muscle by quantitative RT-PCR amplification of RNA from lean NGT and obese NGT individuals and obese type 2 diabetes patients (Table 1). A significant ($p<0.01$) increase in *TACE* expression in type 2 diabetes patients (2.59±0.65) compared with lean NGT individuals (1.00±0.26) was observed (Fig. 2a). A modest but statistically insignificant increase in *TACE* expression was also observed in obese NGT individuals (1.52±0.31). *TACE* RNA expression was positively correlated with insulin resistance, measured as HOMA-IR ($r=0.549$, $p<0.001$; data not shown) and was inversely correlated with insulin-stimulated glucose disposal rate (*M*) during the euglycaemic insulin clamp, $r=-0.369$, $p<0.05$ (data not shown). *TACE* RNA expression also correlated with BMI ($r=0.411$, $p=0.02$), suggesting a positive association with obesity and an inverse relationship with insulin sensitivity. We next determined if the increase in *TACE* RNA expression was paralleled by a rise in protein levels; we performed western blots from skeletal muscle homogenates from lean NGT and obese NGT individuals and obese type 2 diabetes patients. Densitometric analysis of the western blots showed no significant increase in TACE levels between NGT and type 2 diabetes individuals (Fig. 2b). Because of the inhibitory action of TIMP3 over TACE and to examine further the regulation of TACE production in type 2 diabetes, we quantified the abundance of TIMP3 in type 2 diabetic, lean NGT and obese NGT individuals. We found a 70% reduction in TIMP3 levels in skeletal muscle of type 2 diabetes compared with lean NGT participants ($p<0.01$) (Fig. 2c) by western blotting; obese NGT individuals showed a 40% of decrease in TIMP3 levels vs lean NGT individuals. We then quantified the abundance of pro-TNF-α as well as TNF-α in the skeletal muscle of lean, obese and type 2 diabetic participants. We observed a significant increase in the levels of pro-TNF-α as well as TNF-α in the skeletal muscle of type 2 diabetic patients (Fig. 2d, e). β-Actin levels were similar in the three study groups (Fig. 2f).

In vitro assay of TACE activity in skeletal muscle in lean and obese participants and type 2 diabetes patients

To investigate whether decreased TIMP3 levels resulted in increased enzymatic TACE activity in skeletal muscle homogenates, we used a fluorogenic peptide substrate assay. Figure 3a, b demonstrates a linear increase in human skeletal muscle protein and TACE activity. In skeletal muscle from type 2 diabetes patients TACE activity was increased by 33% compared with NGT individuals ($p<0.05$); no difference was observed between lean NGT and obese NGT individuals (Fig. 3c, d). Interestingly, TACE activity correlated positively with NEFA concentration ($r=0.460$, $r^2=0.211$, $p<0.05$) (Fig. 3e) and correlated negatively with *M* value (insulin sensitivity) ($r=-0.471$, $r^2=0.222$, $p<0.05$) (Fig. 3f).

Determination of soluble circulating TACE substrates

In addition to shedding pro-TNF-α as TNF-α, TACE converts several other membrane-bound proteins to their soluble forms. To evaluate the effect of increased TACE levels on its substrates, TNF-α, circulating TNFR1, TNFR2, IL-6R and cell-surface adhesion molecule (intercellular adhesion molecule-1 [sICAM] and vascular cell adhesion molecule-1 [sVCAM]) levels were measured in serum using an ELISA. TNF-α levels were significantly increased in type 2 diabetic patients compared with obese NGT as well as lean NGT individuals (Table 1). sTNFR1 was significantly increased in type 2 diabetes vs NGT lean ($p<0.05$) and NGT obese individuals ($p<0.05$) (Fig. 3g). sTNFR1 levels were inversely and significantly correlated with insulin sensitivity, measured as insulin-stimulated glucose disposal rate (*M*) during a euglycaemic insulin clamp ($r=-0.519$, $p<0.01$) (Table 2). sIL-6R was significantly higher in diabetic patients vs lean NGT individuals ($p<0.01$) (Fig. 3h). sIL-6R levels were inversely and significantly correlated with insulin sensitivity ($r=-0.441$, $p<0.02$, Table 2). Both increased sTNFR1 and sIL-6R levels significantly correlated with elevated HbA_{1c} levels and BMI (Table 2). No differences in TNFR2, sICAM and sVCAM serum concentrations were observed between lean, obese and diabetic groups, and there were no significant correlations with BMI, HbA_{1c} and *M* values (Table 2 and data not shown). In multivariate analysis (Table 3), insulin sensitivity was inversely correlated with TNFR1 and 2 and IL-6R. Altogether, these pro-inflammatory cytokines explained 46% ($r^2=0.457$, $p=0.02$) of variance in the insulin-mediated rate of glucose uptake. Likewise, when a multivariate was performed with HbA_{1c} as the dependent variable, these inflammatory mediators could explain 72% ($r^2=0.719$, $p<0.0005$) variability in HbA_{1c} concentration. Also, in a multivariate analysis (ESM Table 2), plasma TNF-α was associated with fasting plasma glucose, HbA_{1c}, and TNFR1 and 2, and inversely associated with insulin sensitivity. Furthermore, sICAM made an independent contribution to plasma TNF-α concentrations. Altogether these variables explained 67% ($p=0.02$) of variance in plasma TNF-α concentrations.

Effect of lipid infusion in TACE/TIMP3 expression

Obesity and type 2 diabetes are characterised by increased circulating levels of NEFAs. Lipid infusion acutely raises the plasma and intracellular fatty acid levels, induces insulin resistance and stimulates inflammation in muscle [34–36]. To examine the effect of lipid infusion on TACE and TIMP3 activities, we performed a 6 h lipid or saline infusion in 12 lean NGT individuals. Lipid infusion increased the plasma NEFA concentration from 618±80 to 1,863 ±423 μmol/l and markedly decreased insulin-stimulated total body glucose disposal by 20% ($p<0.001$) (Fig. 4a). After 6 h of lipid vs saline infusion, *TACE* expression increased fourfold (Fig. 4b), and *TIMP3* expression decreased threefold (Fig. 4c) in skeletal muscle biopsies, while *IR* expression showed a decrease, although not significant, following lipid infusion (Fig. 4d). Both *IRS-1* and *Akt* demonstrated a marked 80% decrease in RNA levels (Fig. 4e, f). In addition, after 2 h of lipid infusion, plasma sIL-6R concentration increased 1.3-fold ($p<0.05$) and remained elevated during the insulin clamp (80 mU m^{-2} min^{-1}) (Fig. 4g). After 6 h of lipid

vs saline infusion, protein levels of IRS-1, AKT, TIMP3 and TACE were unaffected (ESM Fig. 6), suggesting that lipotoxicity may act chronically increasing *TACE* expression but also increasing its activity.

Effect of palmitate in TACE/TIMP3 expression in human myotubes

To examine further the direct effect of lipotoxicity on TACE activity we incubated human myoblasts from lean NGT individuals with increasing concentrations of palmitate, a saturated fatty acid. In an in vitro set, palmitate produced a modest but statistically significant 12–18% decrease in TACE protein levels (Fig. 5a) and a dramatic decrease in TIMP3 levels by ~40% at 200 μmol/l and ~70% at 400 μmol/l over a 4 h time period (Fig. 5b). Therefore, the TACE/TIMP3 stoichiometry was increased from 1.5- to 2.5-fold after palmitate treatment, suggesting that also in this in vitro system the TACE/TIMP3 dyad is overactive, as observed in vivo, both in type 2 diabetic patients and after lipid infusion (Fig. 5c). β-Actin level was unchanged by palmitate treatment (Fig. 5d). Finally, to look for metabolic effects of TACE activation we infected primary human myotubes with adenovirus encoding *TACE* or *GFP* for control (Fig. 6a). We observed that increased TACE determined a significant increase in pro-TNF-α and TNF-α shedding, mimicking the in vivo situation (Fig. 6b–d). Increased TNF-α shedding was associated with a significant reduction in insulin-induced phosphorylation of AKT (Fig. 6e, f). By contrast, we did not observe an effect of increased TNF-α shedding on phosphorylation of AMPK and its substrate ACC (Fig. 6g–i). β-Actin level was similar in both the groups (Fig. 6j).

Discussion

In humans, skeletal muscle accounts for 60–70% of glucose uptake in the postprandial period and for 80–90% of glucose disposal during i.v. glucose/insulin infusion [37,38]. Therefore, identification of factors that impair muscle glucose uptake in insulin-resistant states, such as obesity and type 2 diabetes, is of great clinical importance. Among the various cytokines synthesised by skeletal muscle under basal conditions and in response to inflammation, TNF-α is of particular relevance because it impedes glucose uptake in human skeletal muscle by inhibiting insulin signal transduction, while concomitantly causing endothelial dysfunction [12,39–42]. A number of studies, but not all, have demonstrated increased circulating levels of TNF-α and increased mRNA expression in adipose tissue and skeletal muscle of obese and insulin-resistant humans [13,39–44]. A large body of evidence documents that adipose tissue is an active endocrine organ that secretes multiple proteins to modulate insulin sensitivity in muscle/liver and intracellular pathways involved in inflammation and atherosclerosis [45]. An emerging concept is that the differential localisation and release of TNF-α in individual tissues could be a critical determinant of insulin resistance.

In animal models, it is generally accepted that circulating TNF-α levels may not reflect autocrine or paracrine action of the cytokine [10]. In adipose tissue, membrane-associated TNF-α acts locally to inhibit adipogenesis through TNFR1 [46]. In mice, this could, in part, be explained by the low levels of *Tace* expression in adipose tissue compared with skeletal muscle (M. Federici, unpublished data). In rats, both membrane-associated and soluble TNF-α are present in adipose tissue, while in skeletal muscle soluble TNF-α is predominant [10]. Consistent with this, antibody neutralisation of circulating TNF-α increases glucose transport in muscle but not in adipose tissue [47]. If TNF-α acts in a paracrine or autocrine fashion, rather than in an endocrine fashion, this could explain the ineffectiveness of TNF-α-neutralising antibody treatment in humans [15], and some reports in type 2 diabetic patients with insulin resistance but not showing elevated plasma TNF-α levels [44]. Here we have demonstrated by confocal microscopy and tissue subcellular fractionation that TACE, TIMP3, TNF-α and IR

are co-produced in the sarcolemma and this is contributing to the novel concept that locally generated TNF-α exerts paracrine effects to inhibit insulin signalling.

The paracrine effect of TNF-α is dependent on the reciprocal interaction between the stimulatory action of TACE or ADAM-17 and its endogenous inhibitor TIMP3. Recent evidence from animal models show that the actions of TNF-α on inflammation and metabolism are regulated through the TIMP3/TACE dyad [20,21,42,48].

The current study is the first to show in humans that insulin-resistant conditions, such as obesity and type 2 diabetes, are associated with elevated TACE activity in human skeletal muscle, while TIMP3 is diminished. These data are consistent with the hypothesis that TIMP3 down-regulation leads to an increase in TACE activity and an augmented release of TNF-α. Our study also shows that other specific TACE substrates like sIL-6R and TNFR1 are differentially shed in diabetes and insulin resistance. Also, we have shown for the first time in humans the relative contribution of fasting plasma glucose, HbA_{1c} and insulin sensitivity to circulating plasma TNF-α concentrations. Given the cross-sectional nature of the present study, it is not possible to determine whether elevated plasma TNF-α is a primary or secondary phenomenon. One could speculate that obesity leads to elevated TNF-α, which in turn can lead to systemic insulin resistance and hyperglycaemia. On the other hand, it is possible that hyperglycaemia and insulin resistance per se could lead to elevated TNF-α concentration.

Short lipid infusion leads to a physiological elevation in plasma NEFA concentrations, and causes insulin resistance in skeletal muscle and liver [35,49]. Therefore, we examined whether lipid infusion in lean healthy NGT individuals would cause an inversion in TACE/TIMP3 ratio resulting in increased TACE activity. As previously shown, a short-term elevation of the plasma NEFA concentration caused marked insulin resistance in the skeletal muscle [35], and we now demonstrate reciprocal regulation of considerable magnitude of *TACE* and *TIMP3* expression with increased *TACE* and decreased *TIMP3* gene expression of fourfold and threefold, respectively (Fig. 4). Furthermore, within 2 h of the start of lipid infusion, sIL-6R increased 1.3-fold, indicating that the change in *TACE* expression was associated with an increase in TACE activity (Fig. 4). In keeping with our in vivo observations, we found in vitro that palmitate caused an imbalance in *TACE/TIMP3* ratio that could favour increased TNF-α shedding. NEFAs are known to cause insulin resistance and induce TNF-α secretion as a result of JNK activation via a paracrine modality, in cell culture studies [50], while treatment of C2C12 mouse myoblasts with palmitate leads to increased TACE activity (M. Federici, unpublished data). To understand whether increased TACE activity directly impaired insulin metabolic actions we analysed primary myotubes infected with TACE, finding a significant decrease in AKT phosphorylation, a major contributor to regulation of insulin metabolic actions. In summary, our results show that local generation of TNF-α in skeletal muscle via the TIMP3/TACE dyad contributes to the pathogenesis of insulin resistance in type 2 diabetic obese patients. We propose that local targeting of this pathway represents a novel pharmacological approach to the inhibition of inflammation and reversal of insulin resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ACC	Acetyl-CoA carboxylase
ADAM-17	A disintegrin and metallopeptidase domain 17
AMPK	5′-AMP-activated protein kinase

Ch	Channel
ERK 1/2	Extracellular signal-regulated kinases 1 and 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HOMA-IR	Homeostasis model assessment of insulin resistance
ICA	Intensity correlation analysis
IKK-alpha/beta	I kappa-B kinase alpha/beta
IL-6R	IL-6 receptor
IR	Insulin receptor
IRα	IR alpha subunit
JNK	Jun N-terminal kinase
M	Insulin-stimulated glucose disposal rate
NGT	Normal glucose tolerant
PDM	Product of differences of the mean
Rr	Pearson's co-localisation coefficient
sICAM	Soluble intercellular adhesion molecule-1
sIL-6R	Soluble IL-6 receptor
sTNFR1	Soluble TNF-α receptor 1
sTFNR2	Soluble TNF-α receptor 2
sVCAM	Soluble vascular cell adhesion molecule-1
TACE	TNF-α converting enzyme
TIMP3	Tissue inhibitor of metalloproteinase 3
TNFR1	TNF-α receptor 1
TNFR2	TNF-α receptor 2

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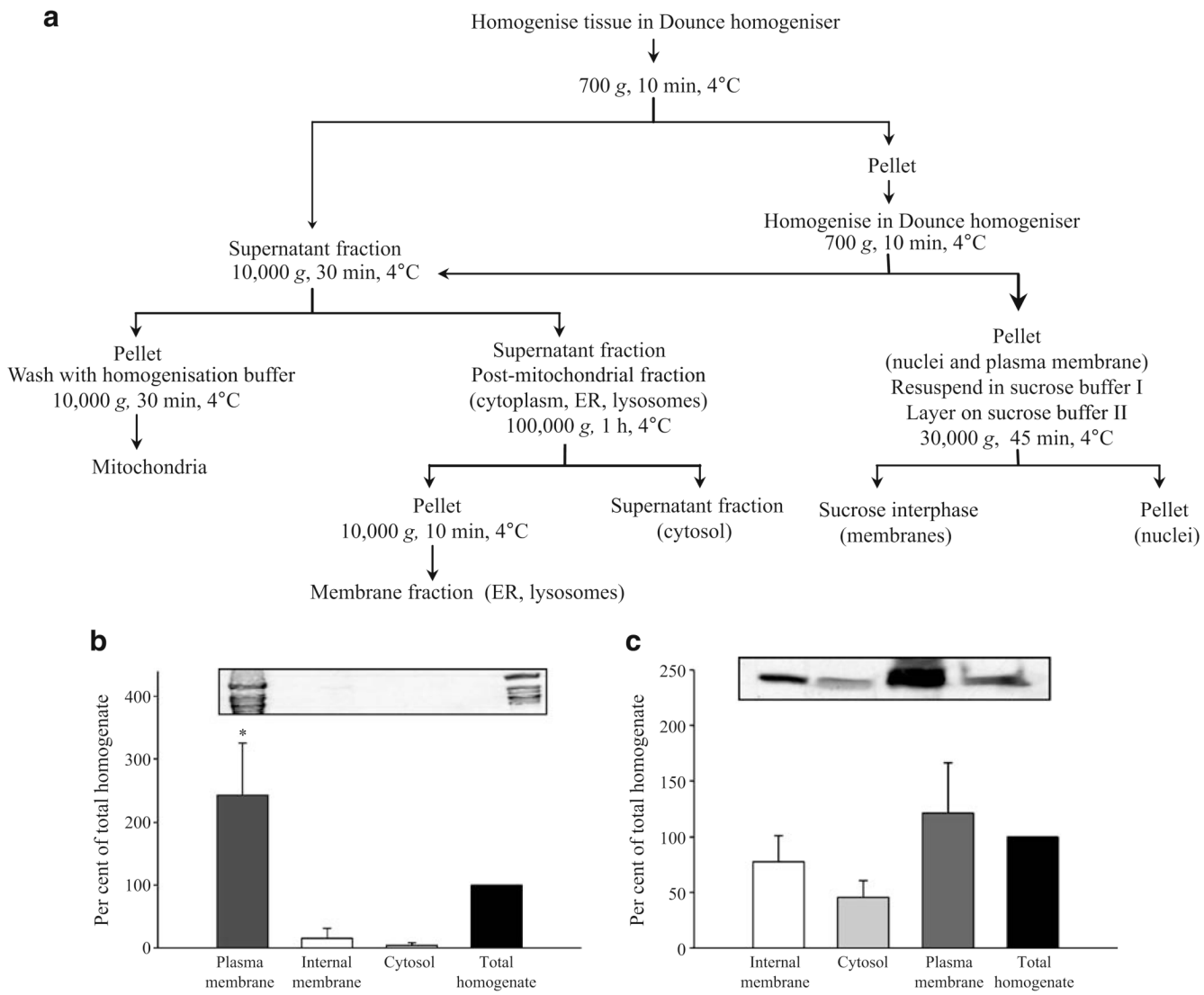

Fig. 1.

Subcellular distribution of TACE and TIMP3 in skeletal muscle of NGT individuals. **a** Subcellular fractionation method outline. **b**, **c** Immunoblot analysis of human skeletal muscle subcellular fractions employing anti-TACE (**b**) and anti-TIMP3 (**c**) antibodies. *p<0.05 (n=3–4 for **b** and **c**). Data are means ± SEM. ER, endoplasmic reticulum

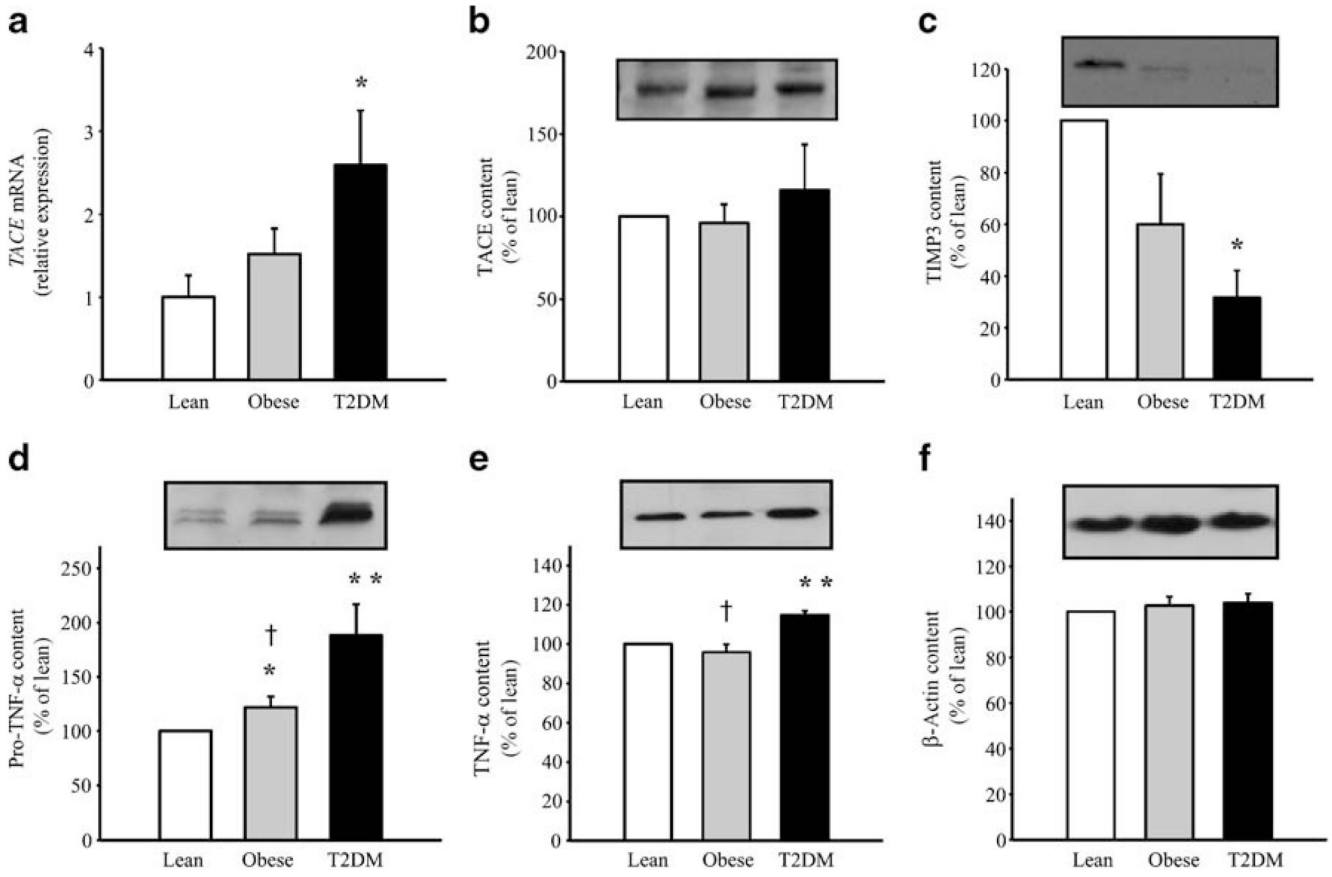

Fig. 2.

mRNA for *TACE*, and TIMP3, pro-TNF-α and TNF-α protein abundance in human skeletal muscle. **a** Expression of *TACE* was measured by quantitative real-time RT-PCR and normalised for 18S ribosomal RNA in vastus lateralis (skeletal) muscle. Relative quantification was calculated by the $2^{-\Delta\Delta C_t}$ where ΔC_t is C_t–18S C_t. Each reaction was done in duplicate. **b** Western blot analysis of TACE protein in vastus lateralis muscle. Protein was extracted from total homogenate, solubilised and separated on SDS-PAGE, transferred to nitrocellulose membranes and incubated with rabbit polyclonal anti-TACE antibody. Abundance of TIMP3 (**c**), pro-TNF-α (**d**), TNF-α (**e**) and β-actin (**f**) in vastus lateralis muscle. Data are means ± SEM. **p*<0.01 vs lean, ***p*<0.01 vs lean, †*p*<0.05 vs type 2 diabetes (*n*=10–12 per group). T2DM, type 2 diabetes mellitus

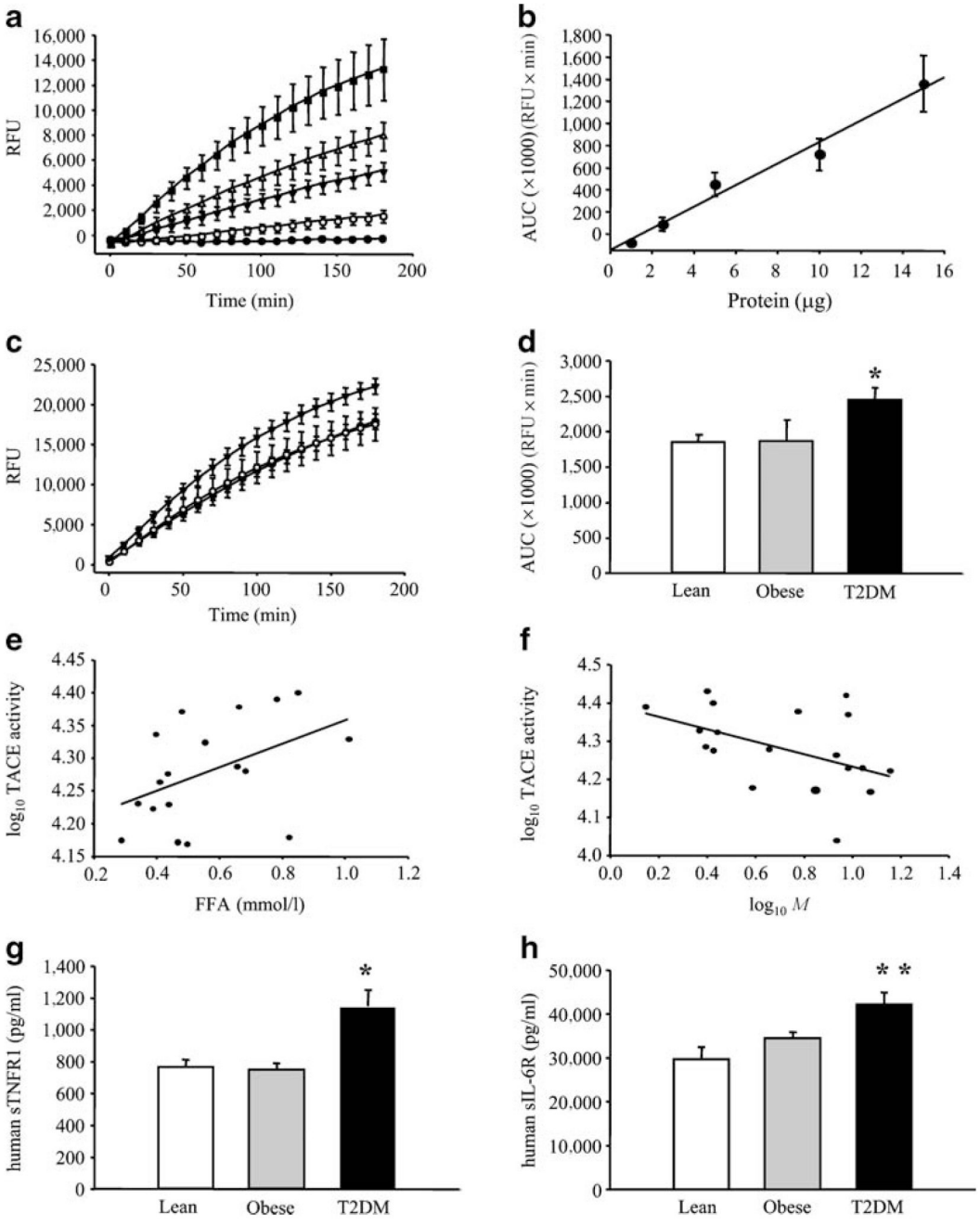

Fig. 3.

TACE activity in skeletal muscle in vitro and circulating TACE substrates in lean and obese individuals and type 2 diabetes patients. **a**, **b** TACE peptide cleavage was measured by a fluorimetric assay with different quantities of skeletal muscle protein: **a** relative fluorescence units (RFUs; black circles, 1 μg; white circles, 5 μg; black inverted triangles, 10 μg; white triangles, 15 μg; black squares, 30 μg); **b** AUCs. **c** TACE peptide cleavage (RFUs) at 10 μg muscle homogenate protein from lean NGT (black circles) and obese NGT individuals (white circles) and type 2 diabetes (T2DM) patients (black inverted triangles). **d** AUCs for TACE activity in vitro in lean NGT, obese NGT and type 2 diabetes patients. **e**, **f** Correlations between $\log_{10}$ TACE activity and NEFA (**e**, r=0.460, r^2=0.211, p=0.05) and $\log_{10}$ TACE activity and

$\log_{10} M$ (**f**, r=0.471, r^2=0.222, p=0.036). **g**, **h** Serum concentration (measured with ELISA) of sTNFR1 (**g**) and sIL-6R (**h**) in lean NGT and obese NGT individuals and type 2 diabetic patients. Data are means ± SEM. *p<0.05, **p<0.01 (n=20 for TACE activity and 8–10 for sTNFR1 and sIL-6R)

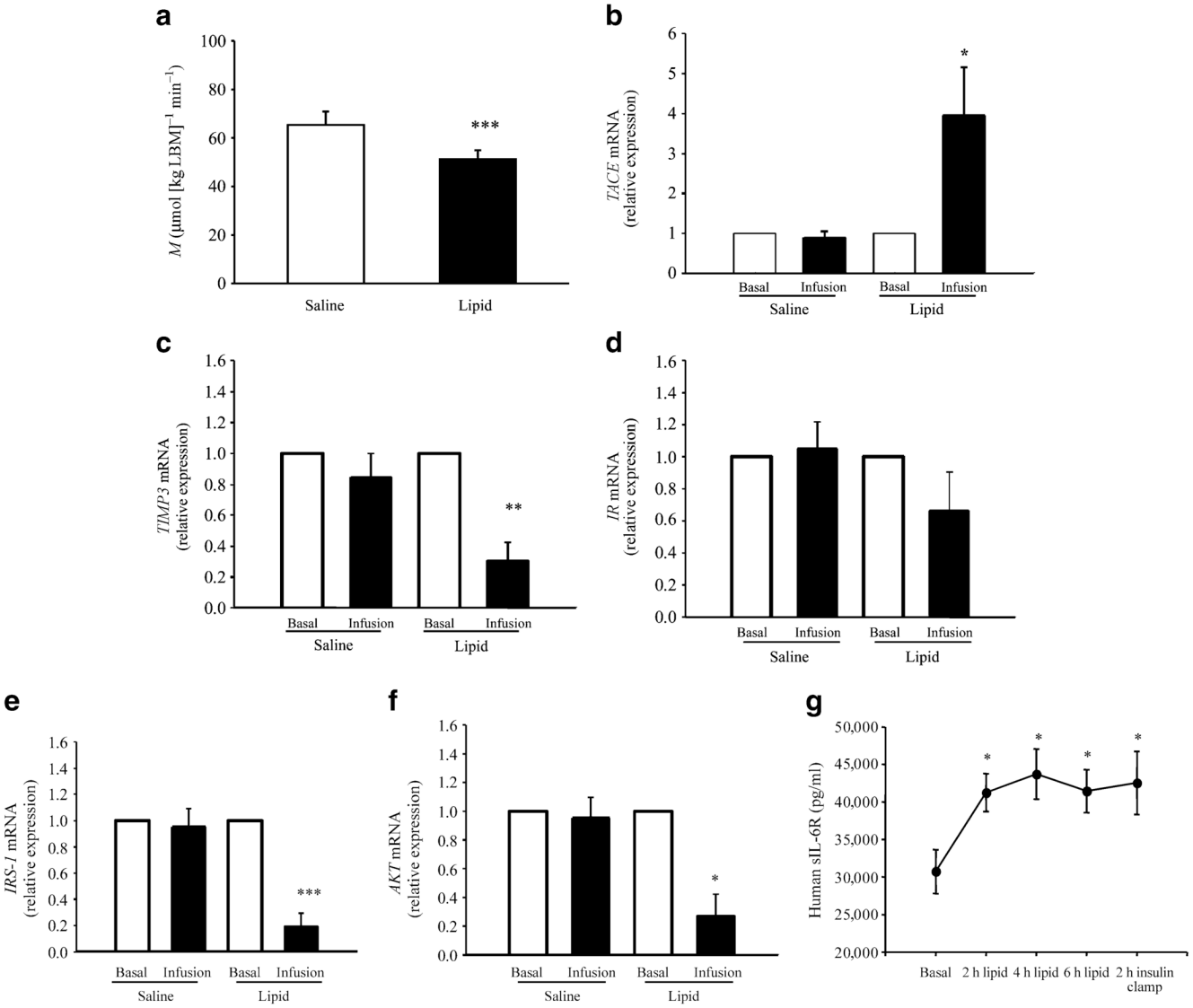

Fig. 4.

a Insulin-stimulated glucose metabolism (*M* value) was measured with the euglycaemic insulin clamp during saline and lipid infusion. **b–f** mRNA expression for *TACE* (**b**), *TIMP3* (**c**), *IR* (**d**), *IRS-1* (**e**) and *AKT* (**f**) was measured in human vastus lateralis (skeletal) muscle before and after a 6 h lipid or saline infusion in lean NGT individuals. **g** Serum concentrations of sIL-6R before and after a 2–6 h lipid infusion, and after a 2 h euglycaemic insulin 80 mU m^{-2} min^{-1} clamp. Data are means ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, all compared with basal (*n*=10–12 individuals per group). LBM, lean body mass

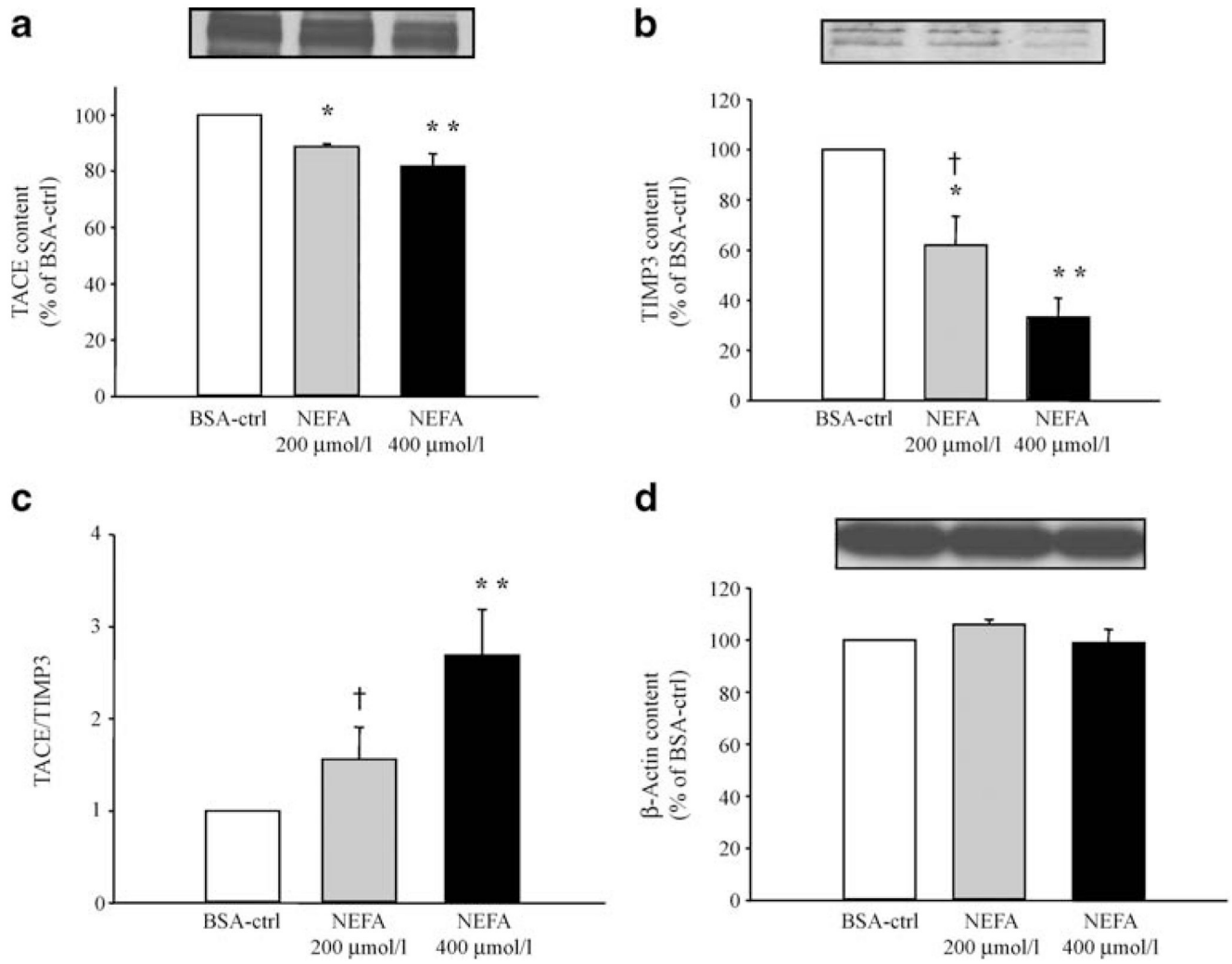

Fig. 5.

Expression in human myotubes of TACE (**a**), TIMP3 (**b**), TACE/TIMP3 ratio (**c**) and β-actin (**d**). **p*<0.05, 200 µmol/l NEFA vs BSA control (BSA-Ctrl); ***p*<0.05, 400 µmol/l NEFA vs BSA. †*p*<0.05, 200 µmol/l NEFA vs 400 µmol/l NEFA. *n*=4–6 per group. Data are means ± SEM

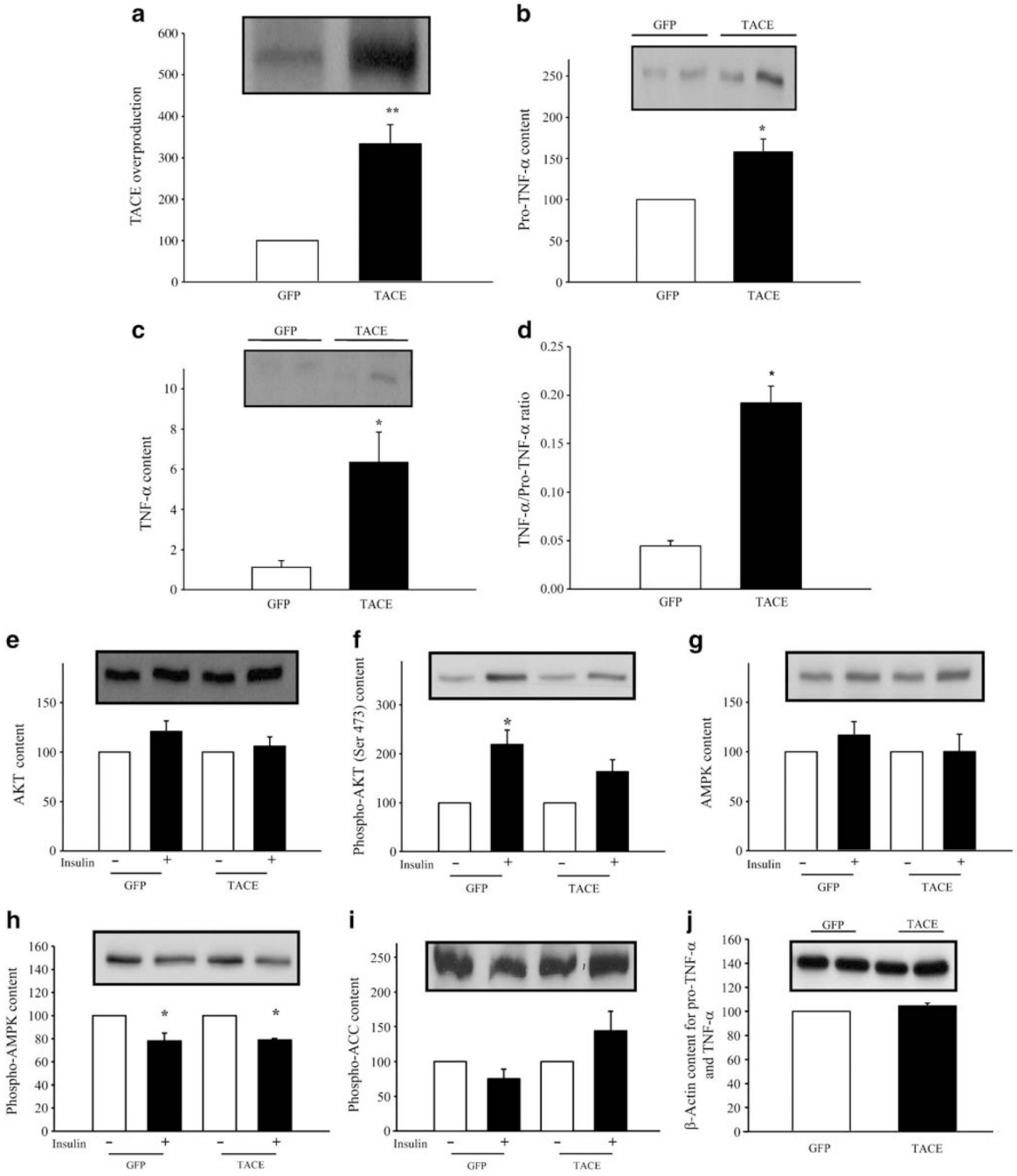

Fig. 6.

a TACE levels in human myotubes infected with adenovirus encoding *TACE* or *GFP* for control. **b–d** Increased TNF-α shedding in the presence of increased abundance of both pro-TNF-α and TNF-α. **e**, **f** AKT and phospho-AKT (Ser 473) levels in myotubes in the presence (+) and absence (−) of insulin (10 nmol/l). **g**, **h** AMPK and phospho-AMPK levels in the presence (+) and absence (−) of insulin. **i** Phospho-ACC levels in human myotubes in the presence (+) or absence (−) of insulin. **p*<0.05 vs GFP and absence of insulin; ***p*< 0.01 vs GFP. *n*=4–6 per group. Data are means ± SEM. **j** β-Actin as a protein loading control for pro-TNF-α and TNF-α levels. *n*=4 per group. Data are means ± SEM. Values are expressed as a percentage of control

Table 1

Clinical, laboratory and metabolic characteristics of participants

Characteristic	Lean	Obese	Type 2 diabetes
Sex (M/F)	7/7	7/7	7/7
Age (years)	37±3	39±2	42±3
BMI (kg/m^2)	22.3±0.5	30.1±0.7 *b*	31.9±1.8 *b*
HbA_{1c} (%)	5.1±0.1	5.2±0.1	8.4±0.3 *b, d*
M value (μmol kg^{-1} min^{-1})	644±50	411±28 *b*	189±22 *b, d*
Total cholesterol (mmol/l)	4.6±0.2	4.4±0.2	5.0±0.2 *d*
LDL-cholesterol (mmol/l)	2.9±0.2	2.9±0.2	2.8±0.2
HDL-cholesterol (mmol/l)	1.3±0.1	1.1±0.1	0.9±0.1 *b*
Triacylglycerol (mmol/l)	1.0±0.3	1.1±0.1	2.5±0.4 *a, d*
Fasting plasma glucose (mmol/l)	4.7±0.1	5.2±0.2 *a*	9.0±0.7 *b, d*
Fasting plasma insulin (pmol/l)	21±7	49±7 *a*	118±14 *b, d*
HOMA-IR	0.7±0.2	1.7±0.3 *a*	6.9±1.0 *b, d*
NEFA (μmol/l)	0.47±0.05	0.57±0.05	0.69±0.07 *a*
TNF-α (pg/ml)	0.9±0.15	0.88±0.18	1.44±0.19 *a, c*

Data are means ± SEM

a $p<0.05$,

b $p<0.001$ vs lean;

c $p<0.05$,

d $p<0.001$ vs obese

M, male; F, female

Table 2

Correlation of circulating TACE substrates and of NEFA with BMI, HbA_{1c} and *M* values

Substrate	BMI		HbA_{1c}		*M* value	
	r	*p* value	*r*	*p* value	*r*	*p* value
sTNFR1	0.502	0.001	0.6245	<0.0001	−0.519	0.003
sTNFR2	0.2038	0.22	0.2829	0.09	−0.262	0.16
sIL-6R	0.3406	0.04	0.3661	0.02	−0.441	0.015
sVCAM	0.1269	0.45	0.22	0.23	−0.271	0.15
sICAM	0.2347	0.16	0.058	0.75	−0.215	0.25
TNF-α	0.467	0.009	0.672	<0.001	−0.451	0.014
NEFA	0.117	0.479	0.331	0.04	−0.384	0.017

Table 3

Determinants of insulin sensitivity and HbA_{1c}

Variable	*M* value		HbA_{1c}	
	r	***p* value**	***r***	***p* value**
TNF-α	−0.451	0.007	0.672	<0.0005
sTNFR1	−0.516	0.002	0.656	<0.0005
sTNFR2	−0.309	0.05	0.399	0.02
sIL-6R	−0.401	0.02	0.373	NS
sVCAM	−0.241	NS	0.201	NS
sICAM	−0.246	NS	0.07	NS
Total explained variance (%)	46		71	

Data are correlation coefficients and their *p* values in multivariate models with insulin sensitivity (*M* value) and HbA_{1c} as the dependent variables