

Short Communication

Application of Peptides Containing the Cleavage Sequence of Pro-TNF α in Assessing TACE Activity of Whole Cells

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Tumor necrosis factor- α (TNF α) is presumably shed from cell membranes by TNF α -cleaving enzyme (TACE). The peptides SPLAQAVRSSSR and Dabcyl-LAQAVRSSSR-Edans, each encompassing the cleavage sequence of pro-TNF α recognized by TACE, were applied to intact umbilical vein endothelium (HUVEC), peripheral blood leukocytes (PBL) and the mast cell line HMC-1, which express TACE, to homogenates of rat heart tissue and to membrane and cytoplasmic extracts of PBL. Formation of SPLAQA (specific cleavage) was determined by HPLC, while cleavage (specific plus non-specific) of Dabcyl-TNF α -Edans was followed over time by measuring fluorescence. Participation of TACE was assessed from inhibition due to the drug TAPI-2. Incubation with recombinant human TACE gave specific cleavage, fully inhibitable by TAPI-2 (IC₅₀<0.1 μ M). HUVEC rapidly degraded TNF α -peptide, but in a non-specific manner (no SPLAQA detectable) and 50 μ M TAPI-2 was without effect. Fluorescence was evoked when Dabcyl-LAQAVRSSSR-Edans was incubated with HMC-1 or PBL and also with cytoplasmic and membrane fractions of lysed PBL, but in no case was there significant inhibition by TAPI-2. However, marginal (10%) inhibition of fluorescence by 50 μ M TAPI-2 was observed with homogenized heart tissue. This contained TACE, about 75% of which was without the inhibitory cysteine switch (Western blot). In conclusion, simple peptide analogs of pro-TNF α cannot be employed as substrates for measuring membrane TACE activity, largely due to extensive non-specific proteolytic cleavage by whole cells and cell extracts.

Key words: Human umbilical vein endothelial cells/
Mast cells/Metalloproteinase/Oxidative stress/
Peripheral blood leukocytes/TAPI.

The shedding and liberation of the 17 kDa inflammatory cytokine TNF α requires for this cytokine to be first cleaved from its 24 kDa pro-form expressed in cell membranes at the scissile Ala76-Val77 bond. This process is presumed to rely largely on the action of metalloproteinase sheddases, foremost the TNF α -cleaving enzyme (TACE or ADAM17) (Black *et al.*, 1997). However, native TACE contains an inhibitory cysteine switch, removal of which seems a prerequisite for enzyme activity (Clarke *et al.*, 1998). We were puzzled, therefore, by the fact that an acute release of soluble TNF α from the myocardium begins immediately (0–30 min) upon post-ischemic reperfusion of the heart (Kupatt *et al.*, 1999; Massoudy *et al.*, 2000). Pertinently, this is a situation characterized by oxidative stress (Raschke *et al.*, 1993), and recent evidence suggests that oxidation of the crucial cysteine located at position 184 may acutely generate sheddase activity without a preceding enzymatic removal of the entire switch (Gilles *et al.*, 2001; Zhang *et al.*, 2001). However, there is uncertainty concerning oxidative activation of TACE *in situ* and which cells express TACE in the myocardium, *i.e.*, presumably serve as source for the liberated TNF α . Another confusing issue is that a number of other metalloproteinases, including MMPs-1, -7, -9 and -17 and ADAM10, have been reported to cleave pro-TNF α , and these proteases also are characterized by the presence of an inhibitory cysteine switch (Haro *et al.*, 2000; English *et al.*, 2000; Lunn *et al.*, 1997).

Candidate cells capable of acutely liberating TNF α (within minutes) *via* the action of TACE or related metalloproteinases under the inflammatory and oxidative conditions of post-ischemic reperfusion include coronary endothelial cells, resident mast cells (Frangogiannis *et al.*, 1998) and peripheral blood leukocytes (PBL), the latter becoming adherent and emigrating into the heart tissue in large numbers immediately upon reperfusion (Raschke *et al.*, 1993; Zahler *et al.*, 1999). Typification of suitable cells and elucidation of the mechanisms underlying activation of the sheddase(s) are confounded by the fact that pure cell cultures such as human umbilical vein endothelial cells (HUVEC) and HMC-1, a human mast cell-derived tumor cell line (Butterfield *et al.*, 1988) that expresses TACE at the cell surface (Becker *et al.*, 2002), do not generally express substantial amounts of pro-TNF α under non-inflammatory conditions. Indeed, initial hopes of being able to measure the release of TNF α into the medium (ELISA assay) or the shedding of TNF α from the cell

membrane directly (flow cytometry) were not realized (Becker *et al.*, 2002).

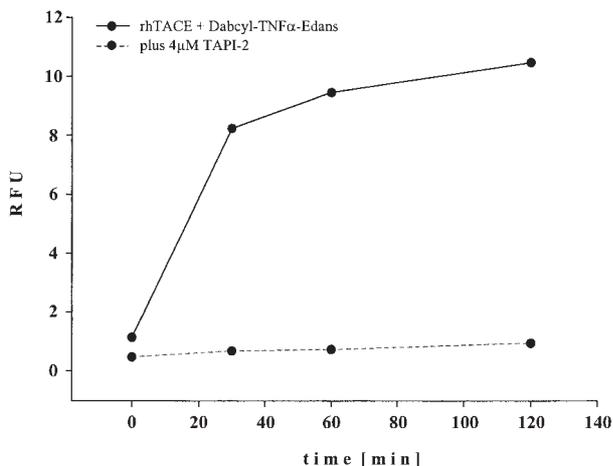


Fig. 1 Inhibition of Human Recombinant TACE by TAPI. Human recombinant TACE (Cat-Dis domains, from Arg 215 to Asn 671; 1 μ g/ml) was incubated at room temperature in 50 mM Tris (pH 7.4) with 10 μ M Dabcyl-TNF α -Edans (Dabcyl-Leu-Ala-Gln-Ala-Val-Ser-Ser-Ser-Arg-Edans; Bachem, Heidelberg, Germany) in the absence or presence of 4 μ M TAPI-2 [N-(R)-(2-(hydroxycarbonyl)methyl)-4-methylpentanoyl-L-*t*-butyl-Gly-L-alanine-2-aminoethylamide, Immunex, Seattle, USA and Peptides International, Louisville, USA]. The plot shows one of 4 similar experiments. RFU = relative fluorescence units.

Prolonged inflammatory prestimulation of the cells to induce TNF α was no option, because we needed to mimic the acute oxidative stress imposed on cells in myocardial tissue during early reperfusion. Frequently, *ersatz* markers of shedding of TNF α are determined, for instance shedding of TNF α -receptors or of L-selectin (Zhang *et al.*, 2001), but their kinetics of cleavage by TACE could be different from those of TNF α . Alternatively, employing peptide substrates with sequences identical to the scissile region of pro-TNF α in the absence and presence of known inhibitors of TACE (such as TAPI-2) to probe the activity of endogenous TACE could provide a possible solution (Black *et al.*, 1997; Zhang *et al.*, 2001). However, extension of this technique to whole cells and tissue homogenates has not been reported.

We first verified that the small peptide Ac-SPLAQA*VRSSSR-NH₂ and the cryptic fluorescent probe Dabcyl-LAQA*VRSSSR-Edans, each encompassing the scissile amino acid sequence of pro-TNF α , are proteolytically degraded by recombinant human TACE. Both substrates suffered the expected rapid cleavage when incubated with 'Cat-Dis' rhTACE (approx. 1 μ M). In the latter case, this is illustrated by increase of fluorescence (Figure 1). In the case of Ac-SPLAQAVRSSSR-NH₂ cleavage was specific, disappearance of the test peptide leading strictly to a 1:1 formation of Ac-SPLAQA (data not shown). TAPI-2

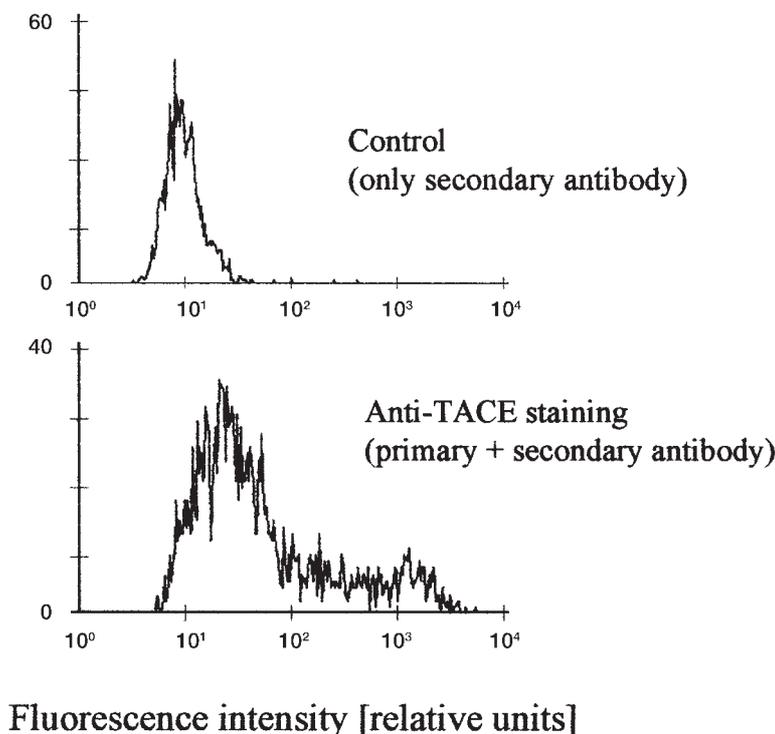


Fig. 2 Flow Cytometric Demonstration of TACE on HUVEC.

Shown is a representative experiment of three cultures. HUVEC were brought into suspension with citrate buffer and fixed with 1% formaldehyde diluted in PBS (10 min, room temperature). After washing with Tyrode's buffer, pH 7.4, and 3 rinsings with PBS, the cells were covered with 0.2% BSA in PBS for 10 min and labeled with primary antibody against human TACE (1 μ g/ml huTACE-M222 from Immunex, in blocking buffer, 45 min, room temperature) and secondary antibody carrying phycoerythrin (lower panel). Control with secondary antibody alone (anti-mouse IgG, 30 min incubation at room temperature, 3 washings in PBS, resuspension in Tyrode's buffer) is shown in the upper panel.

inhibited cleavage of the substrates by rhTACE, giving about 50% inhibition at 0.01 μM and 95–100% at concentrations $>1 \mu\text{M}$. The total inhibition of Dabcyl-TNF α -Edans cleavage caused by 4 μM TAPI-2 is exemplified in Figure 1.

Before commencing experiments with HUVEC and heart tissue, it was necessary to demonstrate the expression of TACE. Staining of non-permeabilized HUVEC with antibody against human TACE and subsequent analysis by flow cytometry revealed the presence of immunoreactive TACE on these cells (Figure 2). The intensity of expression was clearly non-homogeneous, some cells being without, and others with, high levels of TACE. Western blots of extracts of homogenized rat heart tissue using an antibody directed against the C-terminal (intracellular) end of the TACE molecule demonstrated two bands: the fainter one at a molecular mass of about 100 kDa, the far stronger band at approx. 80 kDa (Figure 3, top). These molecular masses correspond roughly to those expected of the rat pro-enzyme with the cysteine switch attached and the furin-processed enzyme, respectively (Milla *et al.*, 1999; Doedens and Black 2000; Swiss-Prot: www.expasy.ch/sprot/). Obviously, it is impossible to state from which cells or cell compartments this TACE is derived. The ratio of the two bands did not differ between hearts subjected to a protocol of ischemia and reperfusion and controls (Figure 3, bottom). These particular results are intriguing, since they do not support any extensive proteolytic activation of TACE in reperfused myocardial tissue, or even the need for any such conversion. However, facile cleavage of substrate peptides should be expected, both with HUVEC and homogenates.

Incubation of test peptides with whole cells (HUVEC, HMC-1, PBL) or heart tissue homogenates did not yield the same results as with rhTACE. Table 1 lists the extent of degradation of Ac-SPLAQAVRSSSR-NH₂ co-incubated for 1 or 24 h with confluent HUVEC at 37°C in PBS. While no peptide remained intact for detection by HPLC after 24 h of contact with HUVEC, about 45–55% of the peptide was still detectable after 1 h, also irrespective of oxidative (with H₂O₂ and HOCl) or inflammatory pre-stimulation of the endothelial cells (see Table 1). This came as a surprise, because TACE is reportedly activated by H₂O₂ (Zhang *et al.*, 2001). Moreover, TNF α shedding in the heart can be induced by intracoronary infusion of either HOCl or H₂O₂ (Gilles *et al.*, 2001). As an additional discrepancy, TAPI-2 did not attenuate degradation, even at 50 μM . Importantly, under no condition was it possible to find the specific cleavage product Ac-SPLAQA (Table 1). This did not seem to be due to selective cellular uptake, because Ac-SPLAQA co-incubated with HUVEC over 1 h remained at a constant level in the supernatant (result not shown). Uptake of TNF α -peptide into HUVEC cannot be excluded. Most likely, however, non-specific cleavage by other membrane proteases and peptidases may account for its disappearance. Preliminary experiments indicate that full inhibition of degradation can be achieved in the presence of protease inhibitor cocktail plus *ortho*-

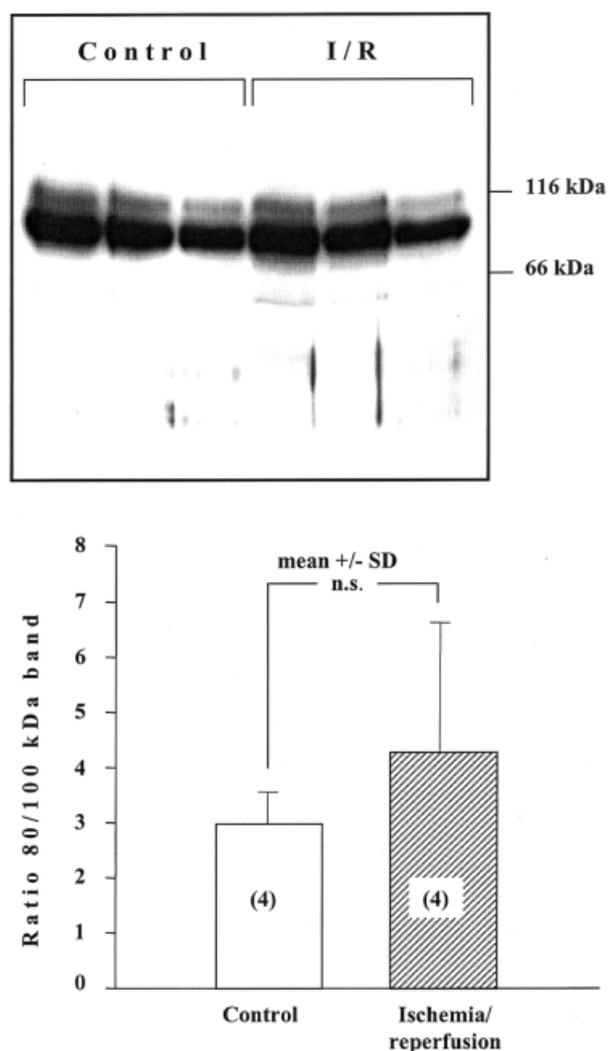


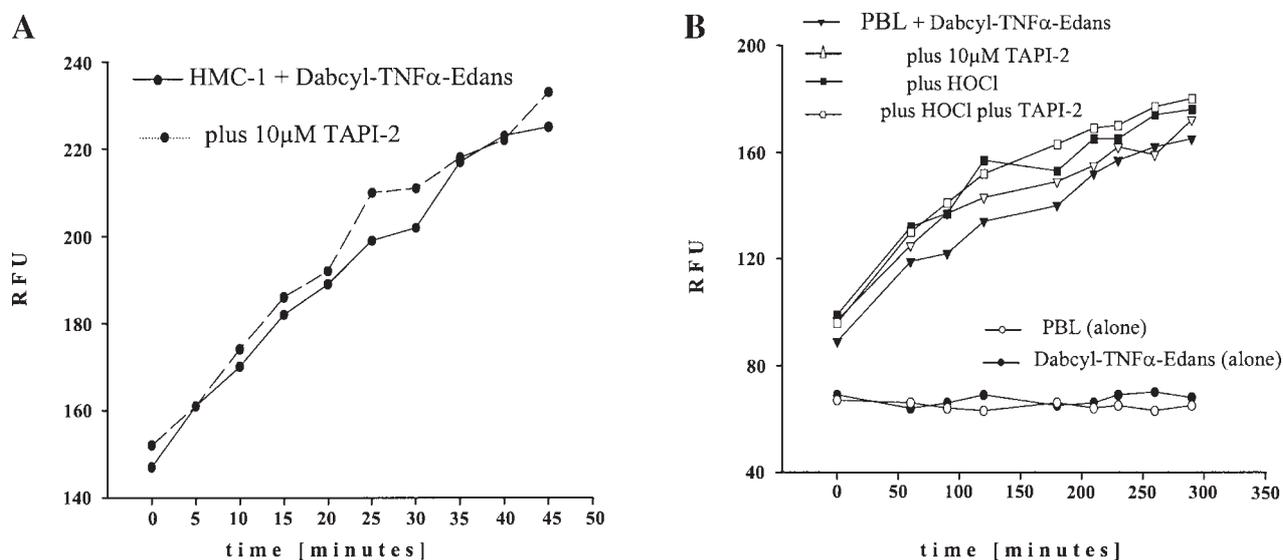
Fig. 3 Determination by Western Blotting of TACE in Homogenates of Isolated Perfused Rat Hearts.

Top panel: representative Western blot. Two bands were detected by TACE antibody/chemiluminescence. The upper (approx. 100 kDa) band presumably represents the unprocessed precursor (pro-TACE), while the lower (approx. 80 kDa) band corresponds to mature TACE (lacking the prodomain). Total protein loaded amounted to 50 μg (lanes 1 and 4), 33 μg (lanes 2 and 5) and 25 μg (lanes 3 and 6). Bottom panel: ratio of unprocessed to mature TACE in control hearts and hearts subjected to 15 min global ischemia (37°C) and 120 min of reperfusion. There was no significant difference between the two conditions (mean values \pm SD, $n = 4$, $p = 0.1$ by *t*-test). Heart tissue was homogenized in 50 mM Tris-HCl, 1 mM DTT, 100 μM phenanthroline, 0.2% Nonidet P-40, pH 7.4, mixed with Complete® protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The samples were boiled for 7 min with 1/4 volume of 4 \times loading buffer (250 mM Tris-HCl, pH 6.8; 8% SDS; 40% glycerol; 0.02% bromophenol blue; 400 mM mercaptoethanol), and loaded on a 10% SDS polyacrylamide gel. After blotting onto a nitrocellulose membrane with transfer buffer (39 mM glycine; 48 mM Tris base; 0.037% SDS; 10% methanol), proteins were blocked for 2 h (blocking buffer 50 mM Tris base, pH 7.5; 10% horse serum; 3% BSA; 200 mM NaCl; 0.05% Tween 20). Primary (rabbit anti-TACE/ADAM17 polyclonal; Chemicon; 1:1000) and secondary antibody were diluted in blocking buffer.

Table 1 Incubation of the TNF α Test Peptide Ac-SPLAQAVRSSSR-NH₂ with HUVEC.

Incubation condition	Amount of test peptide remaining (% of initial)	+ TAPI-2 (% of initial)	Formation of Ac-SPLAQA
1 h	59 ± 6.5	58 ± 1	Not detectable
1 h + H ₂ O ₂ (1 mM)	54 ± 11	66 ± 12	n.d.
1 h + HOCl (10 μ M)	54 ± 11	59 ± 2	n.d.
1 h + IFN γ	46 ± 9	–	n.d.
1 h + TNF α	51 ± 8	–	n.d.
1 h + LPS	45 ± 6	–	n.d.
1 h + PMA	52 ± 9	–	n.d.
24 h	0	0	n.d.
24 h + H ₂ O ₂	0	0	n.d.
24 h + HOCl	0	0	n.d.

TNF α test peptide (2–20 μ M) was incubated with HUVEC (confluent, first passage) at 37°C in the absence and presence of the TACE inhibitor TAPI-2 (50 μ M), added before test peptide (mean values \pm SD, $n = 3–6$, n.d. = not detectable). Interferon- γ (IFN γ , 250 ng/ml), TNF α (2.5 ng/ml) or lipopolysaccharide (LPS, 100 ng/ml) were added to the cultures 24 h before peptide, HOCl (10 μ M) and phorbolmyristate (PMA, 10 μ M) 10 min, and H₂O₂ (1 mM) 30 min prior to the test peptide. The supernatant was acidified with 0.4 M HClO₄, centrifuged and analyzed by HPLC simultaneously for the 12-amino acid peptide substrate and the 6-amino acid fragment Ac-SPLAQA, one of the 2 TACE-specific cleavage products. The conditions used were: C18-Aqua® column (Phenomenex, Aschaffenburg, Germany), isocratic elution with 32% HClO₄ (pH 2) in methanol (41%) and water (27%), peak detection at 200 nm (L-7400 detector, Hitachi-Merck, Darmstadt, Germany). Peak identification and calibrations were performed with substrate degraded by recombinant human TACE and with standard peptides (Biosource, Nivelles, Belgium). Retention times at a flow rate of 1 ml/min were 10.1 min for Ac-SPLAQAVRSSSR-NH₂ and 15.9 min for Ac-SPLAQA.

**Fig. 4** Peptide Cleavage Assay with Whole Cells.

(A) HMC-1 cells (10⁶/ml) incubated in Tyrode's buffer at 37°C with 10 μ M Dabcyl-TNF α -Edans in the absence or presence of TAPI-2. (B) PBL (10⁶/ml) in 250 mM Tris with 10 μ M Dabcyl-TNF α -Edans. HOCl (10 μ M) was added 10 min prior to peptide substrate. In each case, the Figure depicts one of 3 similar experiments. Development of fluorescence was monitored over a total period of 45 min (HMC-1) or 300 min (PBL) using a microplate reader (SPECTRAFluor plus, TECAN, Salzburg, Austria) with excitation at 355 nm and emission at 495 nm. RFU = relative fluorescence units. The buffy coat obtained by centrifugation of fresh, citrated venous blood at 190 g for 15 min was mixed with 6% HES solution. The suspension was then centrifuged at 50 g for 10 min, the supernatant transferred to a fresh tube and centrifuged again at 380 g for 10 min. The pellet was resuspended in 5 ml of calcium-free PBS and carefully layered over 5 ml percoll (Pharmacia, diluted to a final density of 1.077 g/ml). After centrifugation for 25 min at 380 g, the interphase (PBL) was collected, washed with, and resuspended in, calcium-free phosphate buffered saline (pH 7.4).

phenanthroline, a universal inhibitor of metal-containing proteases (data not shown).

Fluorogenic Dabcyl-TNF α -Edans peptide was cleaved by HMC-1 cells. However, the time-dependent increase in fluorescence was not influenced by TAPI-2, even when applied at the high concentration of 10 μ M (Figure 4A). Similarly, freshly isolated peripheral blood leukocytes (PBL) caused fluorescence when brought into contact with Dabcyl-TNF α -Edans, but neither prestimulation of the PBL with oxidant (HOCl) nor the presence of TAPI-2 (10 μ M) had any noticeable effect on the rate or extent of peptide cleavage (Figure 4B). No increase in fluorescence was observed when the reagent was kept in buffer without cells or when PBL were incubated alone. Previous studies with oxidants and HMC-1 cells had also failed to uncover TACE activation (Becker *et al.*, 2002). It should be kept in mind that fluorescence of Dabcyl-TNF α -Edans is elicited by peptide cleavage at any site of the molecule. The reaction could, thus, very well be due to any number of peptidases or proteases expressed on the surface of HMC-1 cells and PBL.

Since TACE should be present in the membrane of PBL, cells were lysed and various fractions prepared: cytoplasmic, soluble membrane and insoluble membrane. As demonstrated in Figure 5, fluorogenic substrate was cleaved by all 3 preparations at approx. equal rates. Again, TAPI-2 was without any significant influence. Fi-

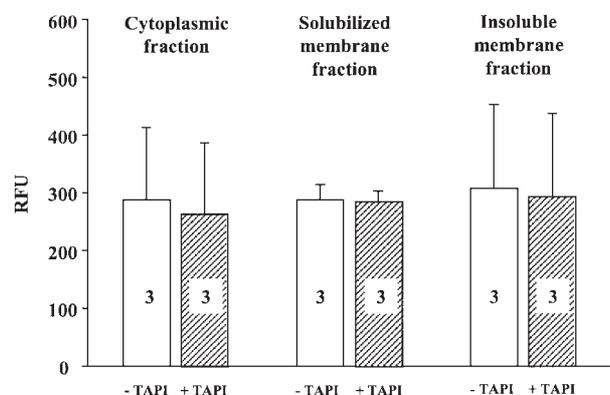


Fig. 5 TACE Activity in Cell Fractions of Lysed PBL.

Aliquots (15 μ l) of cytoplasmic, soluble or insoluble membrane fractions (suspension) were incubated with 10 μ M Dabcyl-TNF α -Edans at 37 $^{\circ}$ C in 200 μ l of 50 mM Tris (pH 7.4) in the absence or presence of 50 μ M TAPI-2. Fluorescence was measured after 60 min (mean values \pm SEM, RFU = relative fluorescence units). There were no significant effects of TAPI in any fraction (paired *t*-test). For lysis, 10⁷ PMN in 50 μ l of PBS were alternately frozen (5 min, -20 $^{\circ}$ C) and thawed (5 min, 37 $^{\circ}$ C) 5 times. Lysed cells were then centrifuged (20 000 *g*, 15 min, 4 $^{\circ}$ C). The supernatant corresponded to the cytoplasmic fraction, the pellet to membrane fractions. Pellets were resuspended in 30 μ l of solubilization buffer (10 μ g/ml leupeptin; 400 μ M benzamidine; 5 μ M pepstatin A; 2 μ g/ml aprotinin; 1% Triton X-100 in PBS) and stirred lightly for 2 hours at 4 $^{\circ}$ C. Finally, these samples were centrifuged (20 000 *g*, 4 $^{\circ}$ C, 30 min), solubilized membrane proteins being found in the supernatant, insoluble membrane components in the pellet.

nally, tissue homogenates were prepared from isolated perfused rat hearts that had been subjected to transient global ischemia (15 min) and then reperfused for 65 min, or that had been perfused without intermission (controls). As illustrated in Figure 6, fluorescence was emitted from Dabcyl-TNF α -Edans in both types of homogenate, but there was no significant difference in the rate of substrate turnover. TAPI-2 (50 μ M) did cause a slight (approx. 10%) reduction in fluorescence in each case (Figure 6), however, the effect was not statistically significant.

In conclusion, Cat-Dis TACE without the cysteine switch can readily cleave TNF α test peptides *in vitro*. Also whole cells can cleave the substrate peptides, but the contribution made by TACE seems marginal (non-specific cleavage, lack of inhibition by TAPI, avid cleavage by both membrane and non-membrane fractions of lysed PBL). Alternative processes, such as degradation by other metalloproteinases or peptidases, and perhaps uptake into cells, dominate substrate turnover. Activation of pro-TACE is presumed to require removal of the cysteine switch by a furin-type enzyme (Black *et al.*, 1997; Doedens and Black 2000; Milla *et al.*, 1999; Skovronsky *et al.*, 2001). Which form of TACE is present at cell membranes

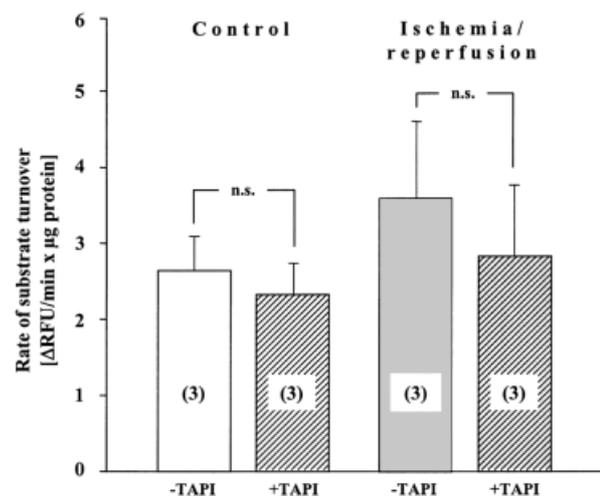


Fig. 6 TACE Activity in Rat Heart Homogenates.

Dabcyl-TNF α -Edans (10 μ M) was incubated with rat heart homogenates in assay buffer for 45 min in the absence and presence of 50 μ M TAPI-2 and fluorescence was measured every 5 min. Average rate of increase in fluorescence, normalized to amount of protein in the assay, is plotted on the ordinate (mean values \pm SD, n.s. = not significant). Ischemia/reperfusion (15 and 65 min, respectively) did not significantly increase substrate turnover vs. homogenates from control hearts (80 min perfusion without ischemia). TAPI-2 treatment had only a slight inhibitory effect in both groups (*p* = 0.1 for controls, *p* = 0.06 for ischemia/reperfusion by paired *t*-test). Homogenates were prepared in buffer containing 50 mM Tris-HCl, 320 mM sucrose, 1 mM DTT, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, 10 mM NaMoO₄, 5 mM NaF and 0.2% Nonidet P-40 (pH 7.4). Supernatants obtained by centrifugation (20 min, 3900 *g*, 4 $^{\circ}$ C) were diluted 1:10 in assay buffer (50 mM Tris-HCl; 1 mM DTT; 10 mM NaMoO₄; 5 mM NaF; 100 μ M ZnCl₂; pH 7.4); for details of heart perfusion, see Kupatt *et al.*, 1999.

is not quite certain (Schlöndorff *et al.*, 2000). The failure to uncover TACE activity by imposing oxidative stress on the perfused heart prior to homogenization, or on cells in culture, may relate to the fact that TACE is already present largely in the pre-cleaved, mature form (Western blot data) or that alternative, redox-insensitive peptidases predominate. Thus, liberation of 17 kDa TNF α from the post-ischemic heart may be more a question of acutely enhanced access of pro-TNF α to mature TACE. Certainly again open to question is whether TACE is really the sheddase primarily responsible for acute release of TNF α from the myocardium.

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