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Release of TNF-α during myocardial reperfusion depends on oxidative stress and is prevented by mast cell stabilizers☆

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Abstract

Objectives: Our study sought to elucidate the role of oxidative stress for shedding of tumor necrosis factor-α (TNF-α) and for activating TNF-α-converting enzyme (TACE). **Background**: TNF-α, a central inflammatory cytokine, is discussed as one of the mediators of reperfusion injury. Shedding of membrane-bound pro-TNF-α is thought to be largely due to TNF-α-converting enzyme (TACE). **Methods**: Release of TNF-α and TACE dependency were studied in isolated rat hearts and in the human mast cell line HMC-1. **Results**: In reperfused hearts, interstitial release of TNF-α occurred in two phases (2–10 and >45 min). It depended on the presence of oxygen during reperfusion and was attenuated by reduced glutathione. Infusion of the oxidants H_2O_2 or HOCl elicited release in non-ischemic hearts. TNF-α release was inhibited in hearts treated with degranulation inhibitors ketotifen or cromoglycate, suggesting mast cells as major source for myocardial TNF-α. This was confirmed by tissue staining. Post-ischemic release of histamine, however, did not parallel that of TNF-α. Heart tissue contained mainly mature TACE. HMC-1 expressed abundant pro-TACE and cleaved the pro-TNF-α-peptide Ac-SPLAQAVRSSSR-NH_2. However, cleavage was nonspecific and only partly inhibited by TACE inhibitor TAPI-2 (10–100 μmol/l), while it was stimulated by H_2O_2 and HOCl and fully blocked by the nonspecific metalloprotease inhibitor *o*-phenanthroline. **Conclusions**: The mechanism underlying TNF-α release from post-ischemic myocardium is oxidation-dependent but largely independent of activation of TACE. Mast cell stabilizers may be useful in preventing TNF-α release during reperfusion.

Keywords: Cytokines; Free radicals; Ischemia; Reperfusion

1. Introduction

Reperfusion of ischemic myocardium leads to a number of inflammatory reactions, e. g. endothelial expression of adhesion molecules and chemokines, activation and emigration of leukocytes and degranulation of resident mast cells, with sequelae such as edema formation and tissue remodelling. Acute release of a number of proinflammatory cytokines, such as interleukines (IL-1, -6, -8) and tumor necrosis factor-α (TNF-α), has been demonstrated in reperfused myocardium of man and of various animal species [1–4]. Enhanced levels of TNF-α, for example, have been found in blood from the coronary sinus of patients undergoing CABG [1]. Although negative inotropic [5] and proinflammatory [6] as well as some cytoprotective effects [7] have been proposed for TNF-α, the role of the cytokine in reperfusion injury is controversial. Also, the cellular source and the mechanism of release of myocardial TNF-α are still rather speculative.

Proteolytic cleavage of the 26-kDa membrane-bound precursor pro-TNF-α yields the soluble, systemically active,

Abbreviations: TNF-α, tumor necrosis factor-α; TACE, TNF-α-converting enzyme; ADAM, a disintegrin and metalloproteinase; MMP, matrix metalloproteinase; TAPI-2, TNF-α protease inhibitor: HON-$HCOCH_2CH(CH_2CH(CH_3)_2CO$-*t*-Butyl-Gly-Ala-$NHCH_2CH_2NH_2$; KHB, Krebs–Henseleit buffer; GSH, reduced glutathione; HMC-1, human mast cell line-1; CABG, coronary artery bypass grafting; DSCG, disodium cromoglycate

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17-kDa cytokine. This "membrane shedding" is thought to be mainly accomplished by TNF-α-converting enzyme (TACE/ADAM17) [8], a metalloproteinase of the ADAM superfamily [9]. TACE is synthesized as proenzyme, in which a cysteine residue (Cys-184) within the prodomain interacts via its SH-group with the catalytic site Zn^{2+}, inhibiting the proteolytic function [10]. This "cysteine switch" has been identified for many members of the ADAM and MMP families [11,12]. A postulated mechanism of TACE activation is via proteolytic removal of the entire prodomain, presumably by a furin-like activity [13]. A different mechanism might involve oxidation of the SH-group of the inhibitory cysteine, as already described for some MMPs [14]. Recent evidence suggests that, under certain circumstances, pro-TACE can be activated in a similar fashion by reactive oxygen species [15].

Since different oxidants and reactive oxygen species (ROS) are generated in reperfused ischemic tissue [16], we hypothesized that an acute oxidative activation of TACE during reperfusion might be responsible for the post-ischemic release of TNF-α from myocardial tissue.

2. Methods

2.1. Reagents

The peptides Ac-Ser-Pro-Leu-Ala-Gln-Ala-Val-Ser-Ser-Ser-Arg-NH_2 (TNF-α-peptide) and Ac-Ser-Pro-Leu-Ala-Gln-Ala were synthesized by Biosource (Nivelles, Belgium). The TACE inhibitor *N*-(*R*)-(2-(hydroxyaminocarbonyl)-methyl)-4-ethylpentanoyl-L-*t*-butyl-Gly-L-alanine-2-aminoethylamide (TAPI-2) was purchased from Peptides International (Louisville, USA). Ketotifen, disodium cromoglycate and *ortho*-phenanthroline were supplied by Sigma (Taufkirchen, Germany); +Complete® protease inhibitor cocktail by Roche Diagnostics (Mannheim, Germany). The primary anti-TACE antibody for Western blotting was a rabbit polyclonal antibody against a peptide corresponding to amino acids 807–823 of human TACE C-terminus (Chemicon International, USA). Antibodies for colocalization of TACE and TNF-α in rat ventricular tissue were (i) an affinity-purified goat polyclonal antibody (C-15) against a recombinant peptide of human TACE C-terminus (Santa Cruz Biotechnology, USA) and (ii) a rabbit monoclonal antibody against rat TNF-α (Endogen, Woburn, USA); secondary antibodies for immunofluorescence were (i) AlexaFluor-488-coupled anti-goat and (ii) AlexaFluor-546-coupled anti-rabbit antibody (Molecular Probes, Eugene, USA). All other reagents used were of analytical grade quality and supplied by Merck (Darmstadt, Germany).

2.2. Cell culture

HMC-1 cells [17] were maintained in ISCOVE's medium (Biochrom, Berlin, Germany) plus 10% iron-supplemented FBS, 1.2 mmol/l monothioglycerol and 10 μg/ml gentamycin (all from Sigma).

2.3. Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.4. Perfusion of isolated rat hearts

Male Wistar rats (body weight 300–500 g) were anesthetized with ether and killed by incision into the carotid arteries. Hearts were rapidly excised and perfused via an aortic cannula at 100 cm H_2O in a Langendorff-mode with a modified KHB (pH 7.4, 5.6% CO_2/94.4% O_2). The pulmonary, caval and azygal veins were ligated and a cannula was inserted into the pulmonary artery. After an equilibration period of 30 min, ischemic control hearts were subjected to 15 min of global ischemia (immersed in Tyrode's buffer at 37 °C) and reperfused (5.6% CO_2/94.4% O_2) for 120 min. A subset of hearts was treated similarly except for the presence of 100 μmol/l reduced glutathione (GSH) in the perfusate throughout. The group termed "anoxia hearts" was subjected to 15 min of global ischemia, and then reperfused in the absence of oxygen (5.6% CO_2/94.4% N_2) for 90 min. Longer anoxic peperfusion was not meaningful, because contractility declined markedly after 90 min. "Low-flow hearts" underwent 15 min of global ischemia and then 120 min of reperfusion (5.6% CO_2/94.4% O_2) at a constant, low rate of flow of 1 ml/min (normal: 4–6 ml/min). Time control hearts were not subjected to ischemia. Infusions of 100 nmol/l ketotifen or 10 μmol/l DSCG were initiated 30 or 15 min prior to ischemia, respectively, in two subset of hearts and continued throughout reperfusion. Further subsets of hearts received an infusion of oxidants (10 min 1 μM HOCl or 30 min 100 μM H_2O_2) instead of undergoing ischemia.

2.5. Immunodetection of TNF-α and histamine

Transudate (i.e., interstitial/lymphatic fluid appearing at the epicardial surface) was collected quantitatively from the apex of the heart over periods of 60–90 s at various points of time and snap-frozen in liquid nitrogen. Sample aliquots were analyzed by ELISA according to the manufacturer's protocols (TNF-α: *rat TNFα ultrasensitive*, Biosource; histamine: Beckman Coulter Bioresearch, Krefeld, Germany). Release of TNF-α and histamine from myocardial tissue was calculated for each time point by multiplication of the respective concentration and flow rate values (collected weight/sampling time) of transudate. Aliquots of coronary venous effluent were collected from the cannulated pulmonary artery and similarly analysed.

2.6. Quantitation of TNF-α mRNA by RT-PCR

About 200 mg of frozen ventriclular tissue was homogenized by pestle in liquid nitrogen, and 2 ml of Trizol™ reagent (Life Technologies) was added. Total RNA (5 μg) was reverse-transcribed using 20U of RevertAid™ M-MuLV Reverse Transcriptase (MBI Fermentas). A 5-μl aliquot of the resulting cDNA then underwent PCR using a CytoXpress quantitative PCR© Detection Kit (Biosource). The thermocycler profile was: (1 min 96 °/4 min 58 °C) × 2; (1 min 94 °C/2.5 min 58 °C) × 30; 10 min 70 °C. The ethidium bromide-stained PCR products were visualized by means of a UV transilluminator (Biorad, Germany) on a 2% agarose gel.

2.7. Immunohistochemical staining of rat ventriclular tissue

Isolated hearts were perfusion-fixed for 2 min with 1% formaldehyde in PBS at a flow rate of 4 ml/min. Staining of paraffin-embedded tissue slices for the presence of mast cells and TNF-α was performed as described before [18]. Colocalization of TNF-α and TACE was investigated by immunofluorescence (for antibodies, see Section 2.1) and confocal microscopy (Zeiss LSM 410, Zeiss, Oberkochen, Germany). Samples were inspected with a 63 × oil immersion lens, and illuminated with 488 and 543 nm laser, respectively. A 510–525 nm bandpass and a 570 nm longpass served as emission filters. The size of the pinhole was 20 throughout.

2.8. Preparation of heart tissue homogenates

Hearts were snap-frozen in liquid nitrogen using precooled aluminium tongs. About 175 mg of frozen material was homogenized, first by pestle and then glass homogenizer in cold homogenization buffer (50 mM Tris–HCl; 1 mM DTT; 100 μM *o*-phenanthroline; 0.2% Nonidet P-40; pH 7.4) supplemented with Complete® protease inhibitor cocktail. Samples were spun at 3900 × *g* at 4 °C for 30 min and the supernatants collected.

2.9. Lysis of HMC-1 for Western blot

About 10^6 cells were lysed at 4 °C in 200 μl of lysis buffer (20 mM KH_2PO_4; 1 mM EDTA; 1 mM PefaBlock; 1 μM leupeptin; 1 μM pepstatin; 50 mM NaF; 40 mM $Na_4P_2O_7$; 1 mM Na_3VO_4; 1 mM *o*-phenanthroline; plus Complete® protease inhibitor cocktail). HMC-1 cell lysates were centrifuged at 12,000 × *g* for 5 min, the pellets discarded and the supernatants collected.

2.10. Determination of TACE protein by Western blotting

Samples (supernatant of heart homogenates and HMC-1 lysates) were boiled for 7 min with 1/4 volume of 4 × loading buffer (250 mM Tris–HCl, pH 6.8; 8% SDS; 40% glycerol; 0.02% bromophenol blue; 400 mM mercaptoethanol), and defined amounts of protein were loaded on 8% SDS polyacrylamide gel. For blotting after electrophoresis, primary (1:1000) and secondary antibodies were diluted in 3% dry milk powder in PBS. The immunoreactive proteins were visualized by chemiluminescence.

2.11. TNF-α-peptide cleavage assay with HMC-1 cells

Cells (10^7) were washed 1 × with calcium-free PBS and were then resuspended in 1 ml PBS. Incubation with 17 μM of the TNF-α-peptide Ac-SPLAQAVRSSSR-NH_2 occurred at 37 °C in cylindrical cups on a rotating device. To compare specificity of cleavage, TNF-α-peptide was also incubated with recombinant human TACE catalytic domain. Disappearance of the original substrate as well as the corresponding appearance of Ac-SPLAQA, one of the two TACE-specific cleavage products, were monitored by HPLC. Ninety-five to one hundred percent inhibition of degradation by recombinant TACE was achieved with the drug TAPI at concentrations of about 1 μmol/l (IC_{50}: 0.01 μmol/l, data not shown).

2.12. HPLC analysis

Cells were lysed by adding 4N $HClO_4$ to the suspension (volume ratio 1:10) and vortexing immediately. Incubated rhTACE was treated similarly with $HClO_4$. High-pressure liquid chromatography (HPLC) was used to simultaneously quantify the 12-amino-acid peptide substrate of TACE (Ac-SPLAQAVRSSSR-NH_2) and the 6-amino-acid fragment Ac-SPLAQA in the acidic extracts. The conditions used were as follows: C18-Aqua® column (Phenomenex, Aschaffenburg, Germany), isocratic elution with 32% $HClO_4$ (pH 2) in methanol (41%) and water (27%), peak detection at 200 nm (L-7400 detector, Hitachi-Merck, Darmstadt, Germany). Retention times at a flow rate of 1 ml/min were 10.1 min for Ac-SPLAQAVRSSSR-NH_2 and 15.9 min for Ac-SPLAQA.

2.13. Statistical methods

The sample size of each experimental group is given in the results section. TNF-α release from hearts is expressed as mean ± S.E.M., all other data as mean ± S.D. Data from two independent populations were analysed for significant differences using Student *t*-test (normally distributed data) or Mann–Whitney rank sum test (nonnormal distribution). To test for significant differences between data of two treatment groups at a given point of time, two-way repeated measures ANOVA was performed. In the case of significant differences, the post hoc Student–Newman–Keuls multiple comparison procedure was applied. Differences were considered statistically significant if $p \leq 0.05$. All calculating procedures were carried out by the statistical software SigmaStat™, version 1.0 (Jandel).

3. Results

3.1. Isolated rat hearts release TNF-α into the interstitium after ischemia

Of the two myocardial fluid compartments (coronary effluent and transudate), only the interstitial transudate collected at the epicardial surface contained detectable amounts of TNF-α. This finding corresponds to previous results obtained with rat hearts. While control hearts release at most 3 pg/min TNF-α into the transudate at the end of a perfusion period of 180 min, hearts that underwent 15 min of global ischemia produce up to 50 pg/min TNF-α during 2 h of aerobic reperfusion (Fig. 1). In these hearts, TNF-α release displays an early peak 2–10 min after the onset of reperfusion, decreases and then rises again until 120 min after ischemia. Pertinently, almost no TNF-α mRNA is found in pre-ischemic heart tissue, while after 1 h of reperfusion, transcription of the cytokine gene is enhanced (Fig. 1, insert).

3.2. Oxidative stress is required for the post-ischemic release of TNF-α

A group of hearts was equilibrated in the presence of the antioxidant GSH (100 μmol/l), then underwent global ischemia and was finally reperfused again in the presence of GSH. In this group, both the early and the late release of TNF-α were reduced, although the late (60–120 min) post-ischemic TNF-α release still differed significantly from the mean pre-ischemic value (Fig. 2A). Two further groups of ischemic hearts were subjected to modified reperfusion protocols: one was reperfused with KHB gassed with 95% N_2, instead of with 95% O_2 as in the ischemic control group, the other was reperfused with oxygen-gassed buffer but at a constant, reduced flow rate (1 ml/min compared to reperfusion at constant pressure in ischemic control hearts with a mean coronary flow of 4–6 ml/min). Post-ischemic transudates of low-flow and anoxically reperfused hearts both contained significantly lower levels of TNF-α than transudates of ischemic control hearts (Fig. 2A).

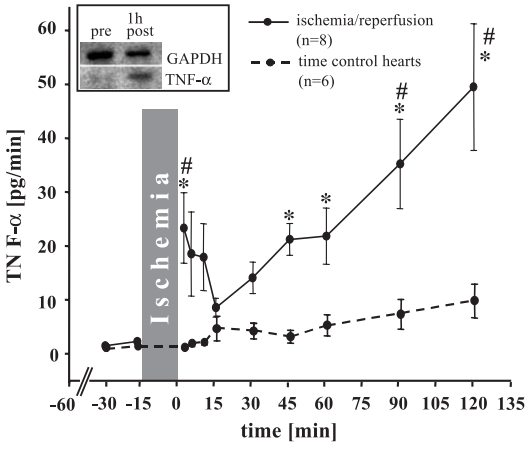

Fig. 1. TNF-α in transudate of rat hearts. Depicted are mean values (± S.E.M.). *Significant vs. pre-ischemic values. #Significant vs. time control hearts. Insert: RT-PCR of total RNA isolated from pre-ischemic (lane 1) and post-ischemic hearts reperfused for 1 h (lane 2), showing TNF-α and GAPDH (glyceraldehyde-phosphate dehydrogenase) mRNA.

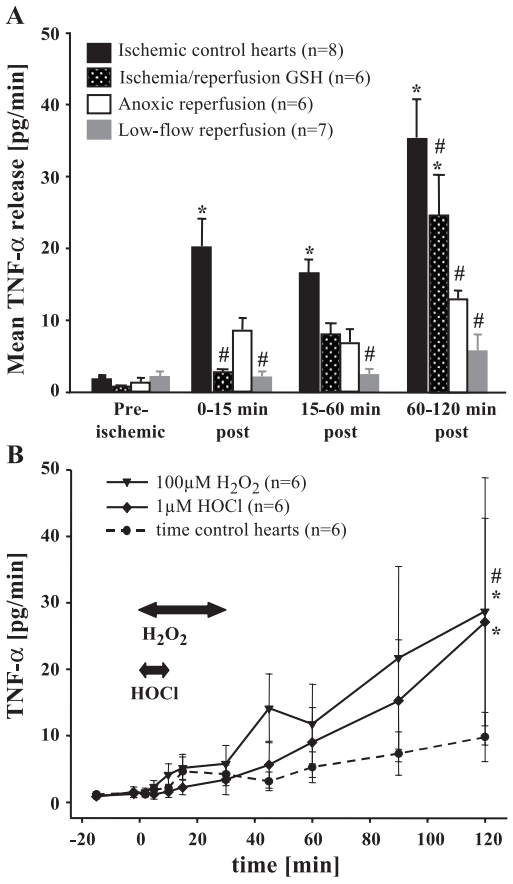

Fig. 2. (A) Mean TNF-α release (± S.E.M.) of ischemic rat hearts reperfused with different protocols. GSH = perfusate augmented with 100 μmol/l reduced glutathione. *Significant vs. pre-ischemic value. #Significant vs. ischemic control hearts. (B) Mean TNF-α release (± S.E.M.) of perfused rat hearts treated with oxidants. *Significant vs. pre-ischemic value. #Significant vs. time control hearts.

3.3. Infusion of oxidants mimics the effect of ischemia and reperfusion

Two groups of non-ischemic hearts, after an equilibration period of 30 min, were infused for another 30 min with 100 μmol/l H_2O_2 or for 10 min with 1 μmol/l HOCl, respectively. Both treatment groups showed elevated TNF-α levels as compared to the untreated control group (Fig. 2B), the TNF-α production of hearts treated with H_2O_2 already being significantly enhanced during the early phase of infusion. In contrast, the TNF-α release of HOCl-treated hearts was not significantly enhanced until 90 min after onset. Interestingly, release continued to rise even after cessation of infusion of either oxidant.

3.4. Ketotifen (100 nmol/l) or DSCG (10 μmol/l) inhibit release of TNF-α but not of histamine

A quantitatively identical suppression of post-ischemic release of myocardial TNF-α as seen in the absence of oxygen was observed in the presence of the mast cell stabilizers ketotifen or DSCG (Fig. 3A). Surprisingly, they inhibited both early and late release of the cytokine.

Histamine, like TNF-α, was restricted to the transudate of rat hearts, with no detectable release into the coronary effluent (data not shown). While some hearts released minor amounts of histamine even before ischemia, maximal levels were reached immediately after ischemia. Histamine was then rapidly washed out (Fig. 3B). In transudates of hearts treated with 100 nmol/l ketotifen, the post-ischemic histamine levels as well as the kinetics of histamine release remained unaltered (Fig. 3B). The same result was obtained with the degranulation inhibitor DSCG (Fig. 3B).

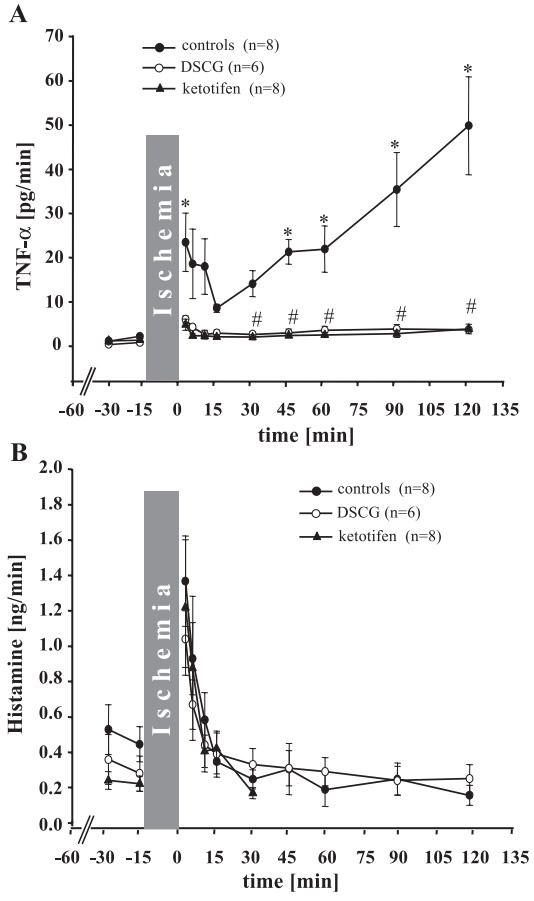

Fig. 3. (A) Mean TNF-α release (± S.E.M.) of ischemic rat hearts perfused in the absence or presence of ketotifen (100 nmol/l) or DSCG (10 μmol/l). *Significant vs. pre-ischemic values. #Significant vs. ischemic control hearts. (B) Mean histamine release (± S.E.M.) of ischemic rat hearts perfused in the absence or presence of 100 nmol/l ketotifen or DSCG (10 μmol/l).

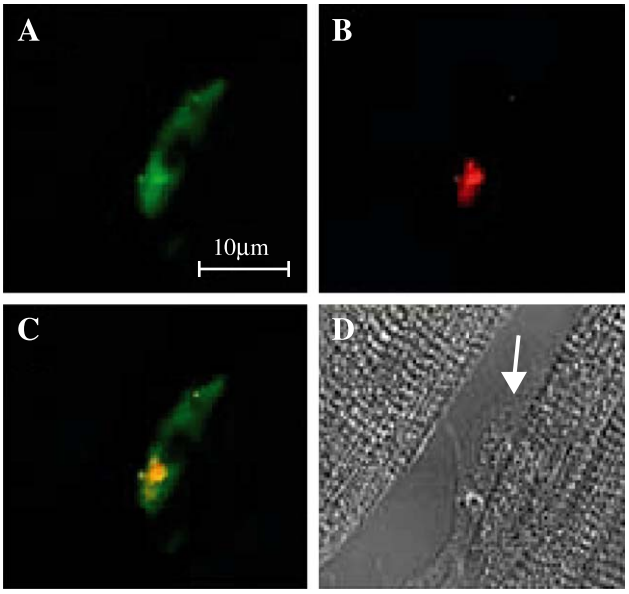

Fig. 4. Immunostaining of tissue sections through rat ventricle. (A) Immunoflourescent staining of TACE in a tissue mast cell. (B) Immunofluorescent staining of TNFα in the same cell. (C) Colocalization of TACE and TNF-α. (D) Phase-contrast image of the tissue containing the mast cell (arrow).

3.5. Tissue staining of rat ventricle

Mast cells, as detectable by Alcian blue staining, were frequent in rat, mouse and human myocardium (data not shown). In all three species, they exhibited a preferential perivascular localization. In the heart, mast cells are the major cell type showing positive staining for TNF-α (Fig. 4). Moreover, they showed a clear colocalization of TACE and TNF-α (Fig. 4, panels A–D). Especially bright TACE staining appeared perinuclearly and in some granules near the plasma membrane (Fig. 4A). The same peripheral granules also stained positive for TNF-α (Fig. 4B). Like TACE, most of the TNF-α was concentrated in the perinuclear space, possibly representing the trans-Golgi network.

3.6. Forms of TACE expressed in HMC-1 and rat heart tissue

As previously reported, TACE is located at the plasma membrane of HMC-1 [18]. In lysates of the same cells, TACE protein is abundant (Fig. 5A, lane 1), and two immunoreactive bands can be distinguished by Western blotting: a larger, 100-kDa and a shorter, 80-kDa form, likely representing the zymogen and the mature enzyme, respectively. Interestingly, HMC-1 predominantly express the unprocessed 100-kDa version of TACE.

In homogenates of rat hearts, the same two protein species can be found, even though the overall expression is lower than in HMC-1 lysates if normalized to the total protein amount loaded (Fig. 5A, lanes 2 and 3). In contrast to HMC-

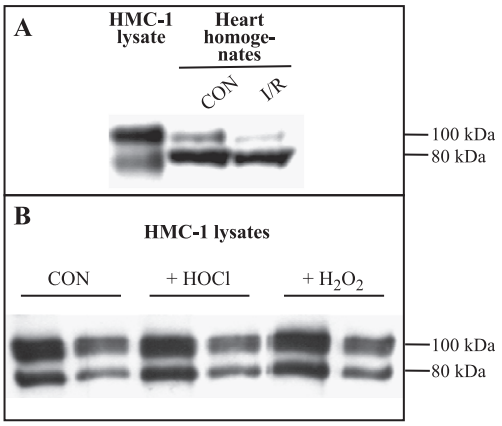

Fig. 5. TACE in Western blot of cell lysates and rat heart homogenates. (A) Lane 1 = HMC-1 lysate, lane 2 = tissue homogenate of a heart perfused for 180 min (time control), lane 3 = tissue homogenate of a heart equilibrated for 45 min, subjected to 15 min of ischemia and reperfused for 120 min. Amounts of total protein loaded per lane: 50 μg for cell lysate, 150 μg for heart homogenates. (B) Lanes 1 and 2 = lysates of untreated HMC-1 cells, lanes 3 and 4 = lysates of HMC-1 cells stimulated for 1 h with 50 μmol/l HOCl, lanes 5 and 6 = lysates of HMC-1 cells stimulated for 1 h with 1 mmol/l H_2O_2. Amounts of total protein loaded: 50 μg (lanes 1, 3 and 5) or 25 μg (lanes 2, 4 and 6). 100 kDa = pro-TACE, 80 kDa = mature TACE. All blots are representative of at least three independent experiments.

1, the 80-kDa form is the prevalent one in heart tissue. Although the intensity of the 100-kDa "immature TACE" band seems to decrease with ischemia/reperfusion in Fig. 5A (cf. lanes 2 and 3), the ratio of the two bands did not differ significantly beween homogenates of time controls and hearts reperfused for 120 min (ratio 80 kDa/100 kDa TACE = 2.99 ± 0.57 and 4.30 ± 2.33 for time control and ischemia/120 min reperfusion, respectively; mean ± S.D., $n = 4$ each). Similarly, the ratio of the two TACE bands in untreated HMC-1 cells (about 0.6) remained the same in lysates of cells treated for 1 h with 50 μmol/l HOCl or 1 mmol/l H_2O_2. A representative Western blot is shown in Fig. 5B.

3.7. TNF-α-peptide cleaving activity of HMC-1 cells

Degradation of the peptide Ac-SPLAQA*VRSSSR-NH_2 by HMC-1 cells was dependent on incubation time and cell number (Fig. 6A). About 100% degradation was seen after 1 h in the presence of 10^7 cells/ml. Although the 12-mer was readily degraded by HMC-1, there was never a trace of the TACE-specific cleavage product Ac-SPLAQA in supernatants or lysates. Incubation of HMC-1 cells with Ac-SPLAQA for up to 3 h revealed that the peptide is stable in supernatants and is not taken up by the cells (data not shown).

Incubation of TNF-α-peptide for 1 h with 10^7 HMC-1 cells/ml PBS together with Complete® inhibitor cocktail led to an approximately 40% reduction in nonspecific peptide degradation (Fig. 6B). A further reduction was achieved by adding 100 μmol/l of the TACE inhibitor TAPI-2, while 10 μmol/l TAPI-2 was less effective (Fig. 6B). An almost complete inhibition was accomplished by adding 1 mmol/l *o*-phenanthroline, a chelator of divalent metal ions and universal metalloproteinase inhibitor (Fig. 6B).

Exposure of cells to oxidants (300–500 μmol/l H_2O_2 or 50 μmol/l HOCl) stimulated TNF-α-peptide degradation

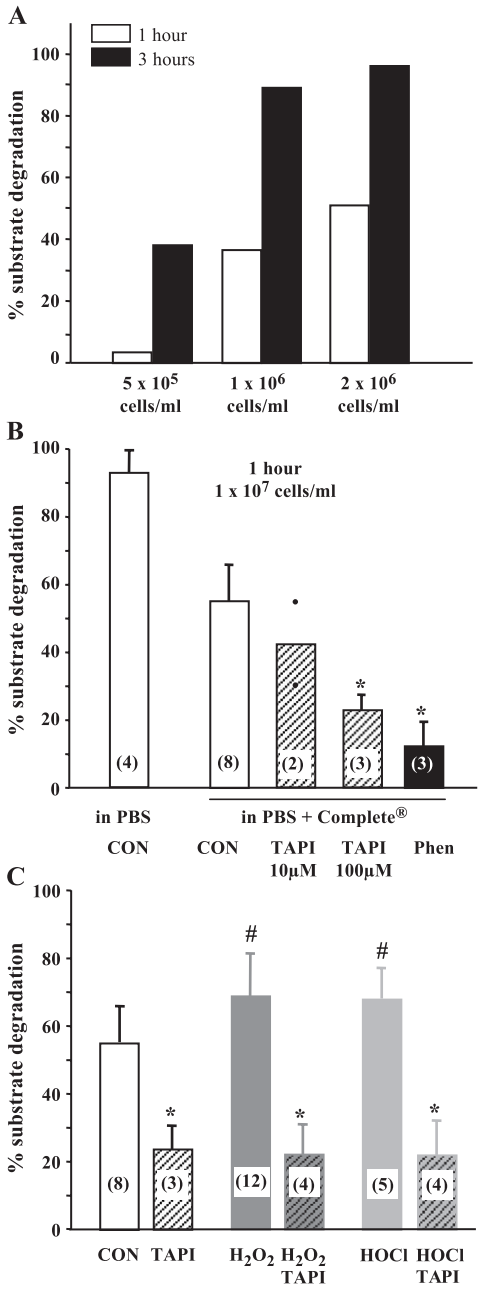

Fig. 6. TNF-α-peptide cleavage assay with HMC-1 cells. (A) Substrate degradation depending on cell number and incubation time in PBS (representative of two to three similar determinations per condition). (B) Substrate degradation in the absence or presence of different protease/metalloproteinase inhibitors. All incubations were carried out for 1 h at 1×10^7 cells/ml. CON = control; Complete® = protease-inhibitor cocktail; phen = 1 mmol/l *o*-phenanthroline. (C) Stimulation of cells with oxidants. Incubations were carried out for 1 h with 10^7 cells/ml PBS supplemented with Complete® protease inhibitor cocktail. Concentrations were 100 μmol/l for TAPI-2, 300–500 μmol/l for H_2O_2 and 50 μmol/l for HOCl. Depicted are mean values (± S.D.); #significant vs. control; *significant vs. value without TAPI-2.

significantly (Fig. 6C). In both cases, this increase was fully inhibitable by addition of 100 μmol/l TAPI-2 (Fig. 6C).

4. Discussion

4.1. Post-ischemic release of TNF-α depends on oxidative stress

The acute release of TNF-α from the heart has been observed previously in studies of patients after CABG and in work on reperfused isolated hearts [1,6,19]. However, neither the initiator of release nor the cellular source of the cytokine in the heart was identified. Our initial finding now was that ischemic rat hearts release TNF-α into the interstitial fluid during early as well as late reperfusion, the early release peaking at 2–10 min after ischemia. These rapid kinetics of TNF-α release make this cytokine an interesting candidate as a mediator of ischemic preconditioning [20]. The early peak could represent either a release of preformed TNF-α from granular stores or a rapid shedding of membrane-bound pro-TNF-α due to enhanced proteolytic cleavage. The late rise in TNF-α release, in contrast, involves de novo synthesis of the cytokine, as seen by the up-regulation of TNF-α m-RNA 1 h after ischemia.

The observations that ischemic rat hearts reperfused either anoxically, under low-flow conditions or in the presence of 100 μmol/l GSH produce less TNF-α than ischemic control hearts are strong indications of the importance of oxidative stress. The absence of the initial TNF-α-peak in transudate of the GSH-treated hearts suggests that rapid TNF-α release is particularly dependent on the extracellular redox status and that ischemia itself is not the direct trigger.

Reperfusion of ischemic tissue leads to the generation of a variety of oxygen free radicals and oxidants [16,21]. Zhang et al. [15] have recently demonstrated that H_2O_2 can activate recombinant TACE by oxidizing the sulfhydryl group of the inhibitory cysteine switch, and Fu et al. [14] have reported activation of an MMP by HOCl, proposing a similar mechanism of interaction. In our experiments, infusion of either HOCl or H_2O_2 into non-ischemic hearts led to enhanced TNF-α production as compared to untreated non-ischemic hearts. These results demonstrate that oxidants, even without ischemia, suffice to mediate release of TNF-α from myocardial tissue. Protracted up-regulation may occur via NFκB (intracellular redox stress); for early release, enhanced mast cell degranulation or acute activation of TACE and/or related metalloproteinases seem likely mechanisms (extracellular redox stress).

4.2. Role of mast cells

Immunohistochemical staining of rat hearts revealed TNF-α in high concentration localizing to perivascular mast cells. Similar localizations were found in tissue sections of human and mouse hearts (unpublished data). The proposed role of cardiac mast cells for post-ischemic release of TNF-α [4,7] could largely be confirmed in our model for ischemia/reperfusion. Notwithstanding, additional sources for TNF-α in the heart cannot be ruled out strictly by tissue staining. Besides $CD68^+$ tissue macrophages bearing TNF-α (results not shown), the cytokine might also be shed from cardiac myocytes, especially in hearts subjected to prolonged inflammatory stimulation [22,23].

Immunostaining of rat ventricle revealed that TNF-α of mast cells is lost within 10 min of reperfusion (unpublished data). Accordingly, at least in cardiac mast cells, a mechanism must exist by which TNF-α is rapidly released following reperfusion. The observed inhibition of early release by ketotifen or DSCG, at first sight, supports a role of degranulation. In contrast to TNF-α release, however, histamine release was not influenced by treatment with ketotifen or DSCG at the chosen concentrations.

There is evidence in the literature that the concentration of 100 nmol/l ketotifen, while fully sufficient to prevent release of TNF-α in our experiments, might simply not be high enough to inhibit discharge of the histamine granules [24]. Interestingly, ketotifen has been reported as an inhibitor of TNF-α release from peripheral blood mononuclear cells (PBMCs) and was successfully used on HIV-infected patients to treat malnutrition [25]. Furthermore, it has been shown to be beneficial in the treatment of some forms of physical urticaria (PU), where ketotifen reduced both cutaneous and serum expression of TNF-α in patients [26]. Additionally, a protective effect has been assigned to ketotifen in a model of experimental inflammation in the small intestine held to mimic Crohn's disease and which may involve TNF-α [27].

The fact that myocardial release of histamine in our model was also not suppressed by 10 μmol/l DSCG further supports the hypothesis that release of TNF-α and histamine from mast cells during early reperfusion are unlinked processes. While the post-ischemic TNF-α release is clearly triggered by reperfusion, histamine is already released in substantial amounts by mast cells during ischemia, leading to elevated histamine levels in the interstitium [28] and allowing rapid washout during reperfusion. Interestingly, even in the presence of 10 μmol/l DSCG, tissue histamine levels rose during the first 15 min of global ischemia [28]. The obvious difficulty in inhibiting histamine release from mast cells during myocardial ischemia might be due to ATP-depletion, leading to Ca^{2+} influx and piecemeal degranulation.

The protracted post-ischemic release of myocardial TNF-α observed in our experiments is presumably a result of enhanced TNF-α expression (Fig. 1, insert). The inhibition of the late phase obtained with ketotifen or DSCG indicates that mast cell stimulation seems to be vital for initiating this process. An autocrine stimulation of TNF-α expression has been described before [29]. Alternatively, as yet unidientified products of mast-cell degranulation might serve as trigger for late TNF-α release. Histamine alone obviously does not suffice.

4.3. Role of TACE

Both forms of TACE, the zymogen (100 kDa) and the mature enzyme (80 kDa), were detected in homogenized rat heart tissue, although the processed form (lacking the cysteine switch containing prodomain) was clearly the predominant one. Thus, there seems to be rather little scope for oxidative activation of pro-TACE. Furthermore, the ratio of the 100-kDa to the 80-kDa band did not shift significantly towards the smaller species after ischemia, which argues against acutely enhanced proteolytic processing of TACE during reperfusion. However, should oxidative or proteolytic activation of TACE occur only in the small subset of cardiac mast cells, it would be overlooked if whole heart homogenates are analysed.

HMC-1 served as model system for cardiac mast cells in order to better assess the role of oxidative stress in TACE-activation. Pro-TACE was not only abundant in lysates of HMC-1; it even predominated over the 80-kDa form. However, exposure to oxidants did not shift the relationship. Thus, one can imagine a rapid rise of enzyme activity via oxidation of the ''cysteine switch'' as likely alternative to its activation via conversion by a furin-like protease. Unfortunately, HMC-1 expressed hardly any TNF-α in the nonstimulated state. Hence, use was made of the TNF-α-peptide Ac-SLPA-QAVRSSSR-NH_2 as substrate for cleavage. HMC-1 rapidly degraded the peptide, but not to the TACE-specific hexamer Ac-SPLAQA, one of two products obtained after incubation of TNF-α-peptide with recombinant human TACE catalytic domain [8]. On the other hand, the rate of substrate degradation was increased by addition of 500 μmol/l H_2O_2 or 50 μmol/l HOCl to the cells and proved susceptible to inhibition by TAPI-2, but only at 100 μmol/l, at which concentration TAPI-2 is no longer specific for TACE. One ready explanation for these observations is the existence of (an)other substrate-degrading activity(ies), similar to but distinct from TACE. As degradation of the TNF-α-peptide was almost completely blocked by 1 mmol/l *o*-phenanthroline, the enzyme(s) in question must be (a) metalloproteinase(s). TNF-α-converting activity has been demonstrated previously for at least ADAM-10 and MMP-7 and -17 [30–32].

Taken together, our results clearly show that an oxidation-sensitive mechanism initiates post-ischemic myocardial release of TNF-α. Data obtained with HMC-1 cells and myocardial tissue make it unlikely that this is accomplished via enhanced processing of pro-TACE. In fact, shedding of TNF-α even seems to be largely independent of TACE, relying on other oxidatively activated metalloproteinases. In our heart model, early TNF-α release seems to stem from mast cell activation. Preliminary data using $Kit^{W}/Kit^{W/W\text{-}v}$ mice [33], a knockout line with very few myocardial mast cells, show that these animals lack the early post-ischemic peak of cytokine release (unpublished data). The later release, in contrast, requires de novo synthesis of TNF-α, possibly induced by TNF-α itself and/or intracellular oxidative stress. These conclusions have direct bearing on the situation in the human heart: After CABG, an absolutely identical time-course of coronary TNF-α release has been observed, with an early and a later phase, and both were mitigated by generating the radical scavenger nitric oxide [34]. Interestingly, a negative correlation exists between the amount of TNF-α released during the first 5 min of reperfusion and the *cardiac index* determined several hours after the intervention [19]. Owing to the early release of TNF-α, as well as the inflammatory potential of the cytokine, antioxidative interventions and mast cell stabilization in general will surely influence post-ischemic reperfusion damage. While TACE inhibitors may turn out to be ineffective, mast cell stabilizers could possess an unexpected potential in this regard.

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