

# Proteasome inhibition activates the transport and the ectodomain shedding of TNF- $\alpha$ receptors in human endothelial cells

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## Summary

Binding of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to its transmembrane receptors (TNFRs) mediates proinflammatory, apoptotic and survival responses in several cell types including vascular endothelial cells. Because ectodomain shedding of cell surface molecules can be modified by proteasome activity, we studied in human endothelial cells whether the TNF- $\alpha$ -TNFRs axis can be regulated by the cleavage of their transmembrane forms in a proteasome-dependent manner. We show that proteasome inhibition increases the release of TNF- $\alpha$  and TNFRs from human endothelial cells and decreases their cellular and cell surface expression. This phenomenon involves the transient activation of mitogen-activated protein kinase p42/p44 that triggers the dispersion of TNF- $\alpha$  and TNFRs from their intracellular Golgi-complex-associated pool towards the plasma membrane. This results in their enhanced cleavage by TNF- $\alpha$  converting enzyme

(TACE) because it is reduced by synthetic metalloprotease inhibitors, recombinant TIMP-3 and by a dominant negative form of TACE. In the presence of TACE inhibitor, proteasome inhibition increases the cell surface expression of TNFRs and enhances the sensitivity of these cells to the proapoptotic effect of recombinant TNF- $\alpha$ .

In conclusion, our data provide evidence that proteasome inhibitors increase TACE-dependent TNFR-shedding in endothelial cells, supporting the use of these molecules in inflammatory disorders. In association with TACE inhibitor, proteasome inhibitors increase the amount of TNFRs at the cell surface and enhance the sensitivity to the proapoptotic effect of TNF- $\alpha$ , which might be of interest in the antitumor therapy.

Key words: Endothelial cells, TNF receptors, Ectodomain shedding, Proteasome, TACE

## Introduction

Tumor necrosis factor- $\alpha$  (TNF) is one of the most important and pleiotropic cytokines in mediating inflammatory and immune responses (Aggarwal, 2003; Kollias and Kontoyiannis, 2002). Human TNF- $\alpha$  is synthesized as a transmembrane protein and is secreted after cleavage by the metalloprotease TNF- $\alpha$  converting enzyme (TACE) (Black et al., 1997; Moss et al., 1997). Both forms of TNF- $\alpha$  interact with two distinct receptors, TNFRSF1A (TNFR1) and TNFRSF1B (TNFR2) that exist as transmembrane and soluble forms (Engelmann et al., 1990) and genetic evidence identifies TACE as the enzyme involved in their ectodomain shedding (Peschon et al., 1998; Reddy et al., 2000). Soluble complexes of TNF- $\alpha$ -TNFRs can be formed, altering the availability of TNF- $\alpha$  for its cell-surface receptors (Peiretti et al., 2003a; Terlizese et al., 1996). Thus, the ectodomain-shedding of TNF- $\alpha$  and TNFRs participates in the fine tuning of the TNF- $\alpha$  biological activity. Once TNF- $\alpha$  binds to its cell-surface receptors, the complexes trigger diverse cell signals that depend on the receptor involved. Schematically, TNFR1 mediates both cell-death- and cell-survival signals, whereas TNFR2 primarily mediates cell-survival signals (Gupta, 2002). Cell survival mainly involves the activation of the nuclear

transcription factor NF- $\kappa$ B. In quiescent cells, NF- $\kappa$ B, bound to its specific inhibitor protein I $\kappa$ B, is sequestered in the cytoplasm. Under stressful conditions I $\kappa$ B is phosphorylated, ubiquitinated and degraded by the proteasome, which enables NF- $\kappa$ B nuclear translocation and the subsequent transcriptional activation of its target genes.

Continuous protein turnover is fundamental for cell viability by promoting the removal of damaged, abnormal or misfolded proteins and by the selective elimination of regulatory proteins. The primary component of the protein-degradation pathway in the cell is the proteasome (Rock et al., 1994) which degrades ubiquitinated and non-ubiquitinated proteins (Orlowski and Wilk, 2003). Amongst the key proteins modulated by the proteasome are those involved in the control of inflammatory processes, cell-cycle- and apoptosis-regulation (Adams, 2003b). Indeed, the proteasome-dependent degradation of I $\kappa$ B results in proinflammatory effects because of the role of NF- $\kappa$ B in upregulating the synthesis of numerous cytokines and cell-adhesion molecules. The inhibition of proteasome blocks the NF- $\kappa$ B-related signals, causes cell-cycle arrest and triggers apoptosis principally in tumor cells (Adams, 2003a; Adams, 2003b; Elliott et al., 2003).

Several signaling proteins are also regulated through their proteasome-mediated degradation. This is the case for the downmodulation of protein kinase C (PKC)- $\alpha$  and - $\epsilon$  (Junoy et al., 2002; Lee et al., 1997). Proteasome activity is supposed to be involved in the activation of mitogen-activated protein (MAP) kinase cascade because proteasome inhibitors induce sustained MAP kinase p42/p44 activation (Hashimoto et al., 2000). Proteasome inhibition was shown to increase the release of growth hormone receptor (GHR) in a PKC-dependent manner (Takagi et al., 2001). These data, combined with the fact that GHR was described to be a substrate for TACE (Zhang et al., 2000), give an indication of a potential regulatory effect of proteasome on the ectodomain-shedding of TACE substrates.

Here, we studied whether the TNF- $\alpha$ -TNFRs axis can be regulated by the shedding of its transmembrane components in a proteasome-dependent manner. We show that proteasome inhibition increases the ectodomain-shedding of TNF- $\alpha$  and TNFRs by TACE, an effect involving the transient activation of MAP kinase p42/p44, which triggered the redistribution of TACE substrates from their Golgi-associated pool towards the plasma membrane. In the presence of a TACE inhibitor, proteasome inhibition increases the amount of cell surface TNFRs which dramatically sensitizes cells to the proapoptotic effect of TNF- $\alpha$ .

## Materials and Methods

### Chemicals

Phorbol 12-myristate 13-acetate (PMA) was from Sigma. The cysteine protease inhibitor E-64d, proteasome inhibitors MG-262 and lactacystin, PKC inhibitors Gö6983, Gö6976 and Ro 31-8220, MAP-kinase-pathway inhibitors U0126 and PD98059 were from Biomol. Recombinant human TIMP-1 and TIMP-3 were from R&D Systems. The hydroxamic-based metalloprotease inhibitors Ro 32-7315 (Beck et al., 2002) and RU 36156 (Gallea-Robache et al., 1997) were donated by K. Walker (Roche Palo Alto, CA) and S. Roman-Roman (Avantis Pharma, Romainville, France), respectively. G 418 sulfate was from Invitrogen.

### Expression vectors

Expression vectors for TNF- $\alpha$ , TNFR1, TNFR2 were described elsewhere (Peiretti et al., 2003c). TACE dominant negative form, named DN, in which the pro and catalytic domains of TACE are deleted was previously described under the name of haTACE (Peiretti et al., 2003c). For stable transfection of TNF- $\alpha$ , its open reading frame was subcloned into pIRESneo (Clontech).

### Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were cultured as already described (Bernot et al., 2003). The human endothelial cell line ECV-304 (Suda et al., 2001; Takahashi et al., 1990) was maintained in M199 medium supplemented with 10% (v/v) fetal bovine serum, 100 i.u./ml penicillin and 100  $\mu$ g/ml streptomycin. For stable expression of TNF- $\alpha$ , ECV-304 cells were transfected with pIRES-TNF using Polyfect reagent (Qiagen) and 48 hours after transfection, 1 mg/ml of G 418 sulfate was added, selection pressure was maintained for 4 weeks. Remaining cells were then cultured in the presence of 300  $\mu$ g/ml of G 418. Transient transfections of ECV-304 cells were performed using Polyfect reagent (Qiagen) as described by the manufacturer.

### Flow-cytometry analysis

Cells were scraped off the culture dish and surface expressions of TNF- $\alpha$ , TNFR1, TNFR2, TACE and ICAM-1 were determined by flow cytometry by using monoclonal antibodies specific for their ectodomains (clones 1825.12; 16803; 22235; 111633 from R&D Systems, and clone 84H10 from Coulter Beckman) and the goat anti mouse FITC-conjugated antibody from Beckman Coulter. In some experiments, the FITC- or PE-conjugated forms of the monoclonal antibodies mentioned above were used. Labeled cells were analyzed on a XL-cytofluorograph (Coulter Electronics Inc.). Values shown in figures are the mean  $\pm$  s.d. of the mean fluorescence intensity (MFI), corrected for the value obtained with an irrelevant antibody.

### Protein and phosphorylation analysis by immunoblotting

TACE detection was realized after purification of N-glycosylated proteins as previously described (Peiretti et al., 2003b). To analyze the phosphorylation of MAP kinase p42/p44, cells were lysed in the presence of phosphatase-, protease- and metalloprotease inhibitors. Lysates were preclarified by centrifugation, diluted in Laemmli loading buffer, fractionated on a 10% SDS-PAGE and submitted to immunoblot analysis with specific antibodies to detect MAP kinase p42 and phosphorylated MAP kinase p42/p44 (Santa Cruz Biotechnology, D-2 and E-4, respectively).

### Immunocytochemistry

Endogenous ICAM-1 and ectopically expressed TNF- $\alpha$  and TNFR2 were localized in ECV-304 cells. Cells were washed three times with PBS, fixed 10 minutes in 4% paraformaldehyde, washed twice in PBS, incubated 5 minutes in cold acetone, washed again in PBS and incubated for 1 hour with the antibody diluted in a solution of BSA (1%) in PBS. ICAM-1, TNF- $\alpha$  and TNFR2 were detected with the same monoclonal antibodies as those used for flow cytometry. Human Golgin-97 was detected with the mouse monoclonal antibody CDF4 (Molecular Probes) and the goat anti-mouse FITC-conjugated antibody (Beckman Coulter). Observations were made with a Nikon E-800 epifluorescence microscope. For confocal microscopy analysis, TNF- $\alpha$  was detected with the rat monoclonal antibody (Caltag Laboratories, clone MP9-20A4) and the goat anti-rat Alexa Fluor<sup>®</sup> 546 conjugated antibody (Molecular Probes). Analyses were made with a Leica PCS SP2 microscope.

### Semiquantitative reverse transcriptase (RT)-PCR

Total RNA extraction and cDNA synthesis were performed as described previously (Lopez et al., 1999). The eukaryotic elongation factor  $\alpha$  (*E1 $\alpha$* ) was chosen as a stable housekeeping gene. The amplified fragment for TNF- $\alpha$  was generated by using specific oligonucleotides (5'-CTCTCTCCCCTGGAAAGGAC-3' and 5'-TTTGGGAAGGTTGGATGTTTC-3').

### Apoptosis measurement

Caspase 3 activity was measured with the colorimetric caspase 3 assay kit (Sigma Aldrich) as described by the manufacturer. Briefly, 10<sup>5</sup> cells were lysed in 5  $\mu$ l of lysis buffer and incubated with the caspase 3 substrate acetyl-DEVD-pNitroaniline for 5 hours at 37°C. Hydrolysis of the substrate by caspase 3 releases p-nitroaniline (pNA), whose concentration is calculated from the absorbance values at 405 nm and with a calibration curve prepared with defined pNA solutions. Caspase 3 activity is expressed in  $\mu$ M pNA/minutes/ml.

Phosphatidylserine exposure was measured through the binding of annexin V-FITC using the protocol outlined in the Apoptest<sup>™</sup>-FITC kit (Dako Cytomation, Trappes, France). Cells were also stained with propidium iodide (100  $\mu$ g/ml) before flow-cytometry analysis.

## Protein assays

Total proteins of cell lysates were assayed using a bicinchoninic acid protein assay kit (Sigma). Total amounts (free and bound forms) of soluble TNF- $\alpha$ , TNFR1, TNFR2 and soluble ICAM-1 were assayed in culture media and in cell lysates according to the specification of their respective enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems) and expressed as pg/ $\mu$ g of total cellular proteins.

## Statistical analyses

Experiments were performed in triplicate and repeated at least three times unless stated otherwise in the figure legends. All data are expressed as the mean  $\pm$  s.d. Treatments were compared with their respective controls and statistical calculation of the mean differences was performed with the two-tailed *t*-test. A value of  $P < 0.05$  was considered statistically significant (\*); \*\* indicates that  $P$  is at least  $< 0.001$ .

## Results

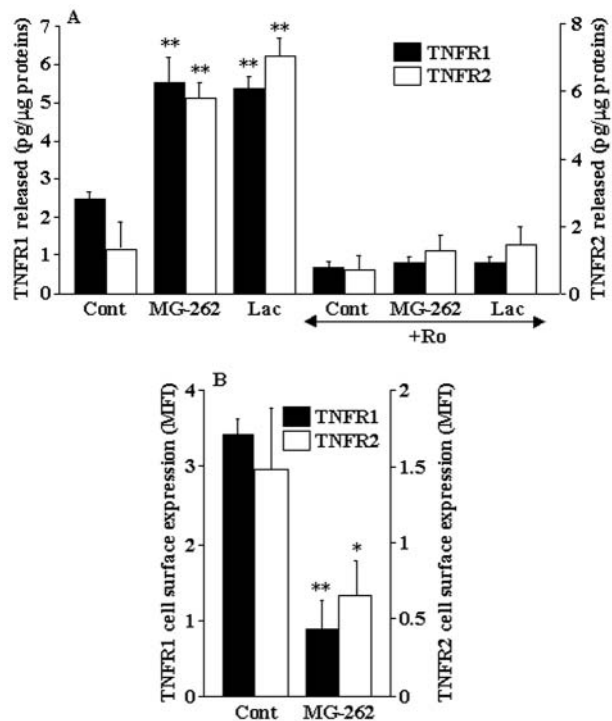
### Proteasome inhibition increases the release of TNFRs

As already reported, HUVECs do not produce detectable amounts of soluble TNF- $\alpha$  under basal culture conditions (Imaizumi et al., 2000). Here, TNF- $\alpha$  was undetectable in conditioned media after 24 hours of culture (data not shown), whereas soluble TNFR1 and TNFR2 were readily detectable (Fig. 1A,B) even after only 4 hours of accumulation. The ectodomain-shedding of TNFRs has been described to involve TACE activity (Peschon et al., 1998; Reddy et al., 2000). Expectedly, treatment of HUVECs with TACE-selective hydroxamic-acid-based metalloprotease inhibitors Ro 32-7315 (Fig. 1A) and RU 36156 (data not shown) reduced the accumulation of TNFRs in the cell culture medium.

Because proteasome activity seems to be involved in the ectodomain-shedding of cell surface molecules (Takagi et al., 2001), we investigated whether proteasome inhibition altered TNFR release. HUVECs were treated with two structurally unrelated proteasome inhibitors, the boronic acid peptide proteasome inhibitor MG-262 (Adams et al., 1998) and the *Streptomyces* metabolite lactacystin (Fentany et al., 1995). These treatments increased the accumulation of TNFR1 and TNFR2 in the cell culture medium by a factor of 2.2 and 3.8, respectively. This increase was prevented by Ro 32-7315 pretreatment (Fig. 1A). Proteasome inhibition decreased the fluorescence values associated to the flow-cytometric detection of the cell-surface TNFR1 and TNFR2 by a factor of 3.5 and 2.3, respectively (Fig. 1B). The inhibitory effect of Ro 32-7315 on MG-262-stimulated release of TNFRs, and the fact that as the level of released TNFR increases, the amount of cell-associated TNFR decreases suggested that activation of ectodomain-shedding following proteasome inhibition involves TACE activity.

### Proteasome inhibition increases TACE-dependent ectodomain-shedding processes

TNF- $\alpha$  is the sole TACE substrate for which the TACE-dependent ectodomain-shedding has been proposed by both, strong genetic and biochemical evidence (Black et al., 2003; Black et al., 1997; Mohan et al., 2002; Moss et al., 1997). Thus, to confidently study the TACE-dependent ectodomain-shedding, we decided to generate an endothelial cell line that

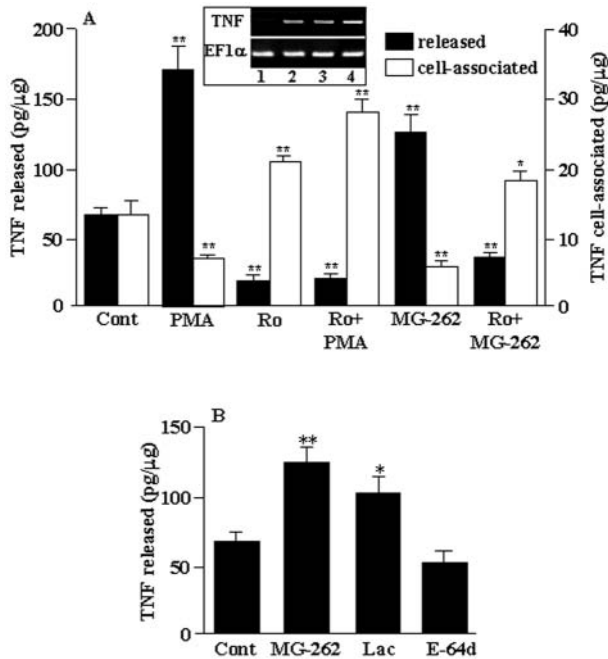


**Fig. 1.** In HUVECs, the ectodomain-shedding of TNFRs involves metalloprotease activity and is increased by proteasome inhibition. Ro 32-7315 (5  $\mu$ M) was added or not to HUVECs 15 minutes before the cells were incubated for 4 hours with MG-262 (600 nM) or lactacystin (40 mM) as indicated. (A) TNFR1 and TNFR2 that had accumulated in the culture media was measured by ELISA. (B) Cell surface expression of TNFR1 and TNFR2 was analyzed by flow cytometry. Cont, control; Lac, lactacystin; Ro, Ro 32-7315. Flow-cytometry experiments were performed in duplicate and repeated three times. Significance of difference between treated and control cells: \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .

stably expresses human TNF- $\alpha$ . The effect of proteasome inhibition on NF- $\kappa$ B activation was previously shown to be comparable in HUVECs and in the human endothelial cell line ECV-304 (Kalogeris et al., 1999), making this cell line a suitable model to match our criteria.

In ECV-304 cells, the release of stably expressed TNF- $\alpha$  was mainly under the control of a TACE-like activity, because short treatment with PMA – which is known to stimulate the TACE-dependent ectodomain-shedding process (Doedens and Black, 2000; Doedens et al., 2003) – increased the release of TNF- $\alpha$  (+150%) in the culture medium (Fig. 2A). Because fluorescence values associated to the flow-cytometric detection of cell-surface TNF- $\alpha$  were not significantly different from values obtained with an irrelevant antibody (data not shown), we then measured the amount of cell-associated TNF- $\alpha$  in cell lysates. The amount of cell-associated TNF- $\alpha$  was reduced by 50% after PMA treatment (Fig. 2A). By contrast, the metalloprotease inhibitor Ro 32-7315 reduced the basal and the PMA-stimulated release of TNF- $\alpha$ , and increased the amount of its cell-associated form (Fig. 2A). Together, these results suggest that the release of ectopically expressed TNF- $\alpha$  is under the control of a TACE-like activity. The proteasome inhibitor MG-262 significantly increased TNF- $\alpha$  accumulation in the cell culture medium (+80%) and reduced the amount



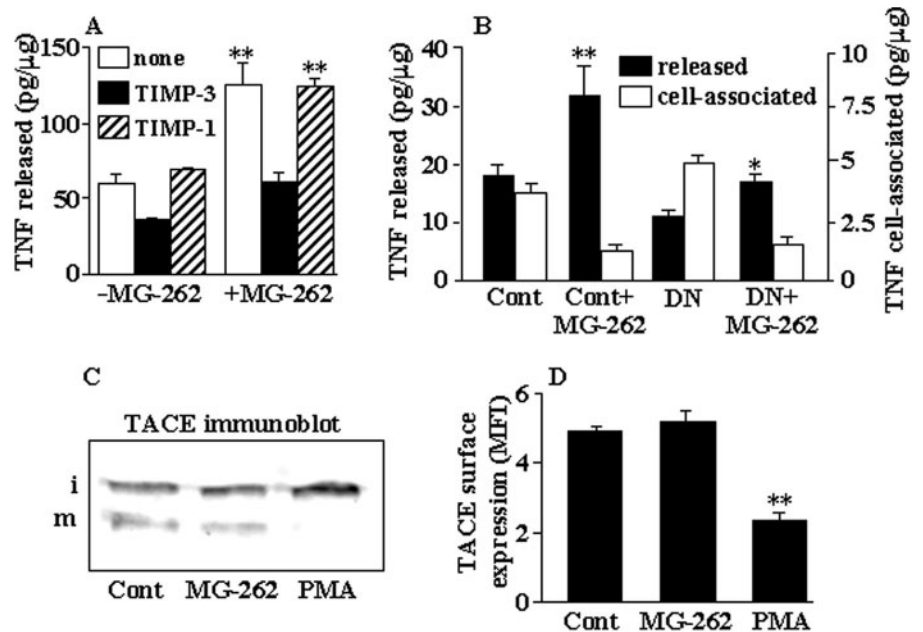


**Fig. 2.** In ECV-304 cells, the ectodomain-shedding of ectopically expressed TNF- $\alpha$  is under the control of a TACE-like activity and is stimulated by proteasome inhibition. (A) Ro 32-7315 (5  $\mu$ M) was added or not to ECV-304 cells stably expressing TNF- $\alpha$ , 15 minutes before the cells were incubated for 4 hours with PMA (20 nM) or MG-262 (600 nM) as indicated. TNF- $\alpha$  that had accumulated in the medium (black bars) and was present in the cell lysates at the end of the experiment (white bars) was measured by ELISA. Inset shows the mRNA levels of TNF- $\alpha$  and eukaryotic elongation factor (*EF1 $\alpha$* ) analyzed by RT-PCR in (lane 1) ECV-304 cells and (lanes 2-4) ECV-304 cells expressing TNF- $\alpha$  under basal conditions (lanes 1, 2), and after 4 hours of (lane 3) MG-262 or (lane 4) PMA treatment. (B) ECV-304 cells stably expressing TNF- $\alpha$  were treated for 4 hours with MG-262 (600 nM), lactacystin (40  $\mu$ M) or E-64d (10  $\mu$ M), and TNF- $\alpha$  that had accumulated in the medium was measured by ELISA. Cont: control; Lac: lactacystin; Ro: Ro 32-7315. Significance of difference between treated and control cells: \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .

of its cell-associated form (~50%) (Fig. 2A). MG-262, significantly increased the release of TNF- $\alpha$  at concentration as low as 10 nM. The plateau of accumulation was reached at 50 nM and persisted even at the highest concentration tested (1  $\mu$ M, data not shown). The metalloprotease inhibitor Ro 32-7315 abolished the MG-262-stimulated release of TNF- $\alpha$  and, by contrast, increased its cell-associated amount (Fig. 2A). The level of TNF- $\alpha$  mRNA was not altered when the cells were treated with MG-262 (Fig. 2A inset) suggesting that MG-262 does not increase the synthesis of TNF. However, PMA slightly increased TNF- $\alpha$  mRNA levels (Fig. 2A inset). The specific role of proteasome inhibition in the upregulation of TNF- $\alpha$

release is reinforced by the fact that lactacystin was also able to increase the release of TNF- $\alpha$ , whereas E-64d, a membrane permeable inhibitor of lysosomal cysteine proteases (McGowan et al., 1989), was without effect (Fig. 2B). The MG-262-stimulated shedding of TNF- $\alpha$  was abrogated by TIMP-3 but not TIMP-1, emphasizing the specific role of TACE in this shedding process (Fig. 3A). Moreover, transient co-transfection of cells with TNF- $\alpha$  and a dominant negative form of TACE (DN) reduced the stimulatory effect of MG-262 on the release of TNF- $\alpha$  (+83% in control cell compared with +60% in DN-transfected cells) (Fig. 3B), without reducing the intracellular amount of TNF- $\alpha$ . This confirms that TACE activity is specifically involved in the MG-262-stimulated release of TNF. However, this stimulated release of TNF- $\alpha$  was not the consequence of an increased amount of active TACE because its total and its cell-surface expression (Fig. 3C and D, respectively) were not altered by proteasome inhibition. A treatment with PMA reduced the amount of mature TACE

**Fig. 3.** Proteasome inhibition triggers TACE-dependent release of TNF- $\alpha$  without altering the amount of TACE antigen. (A) ECV-304 cells stably expressing TNF- $\alpha$  were incubated for 20 minutes without (white bars) or with 300 nM of TIMP-3 (black bars) or TIMP-1 (striped bars) before MG-262 (100 nM) was added or not. TNF- $\alpha$  that had accumulated in the culture medium over a period of 4 hours was measured by ELISA. (B) ECV-304 cells were co-transfected with TNF- $\alpha$  and a dominant-negative form of TACE (DN), or a control vector (Cont). Forty-eight hours after the transfection, cells were treated or not with MG-262 as described for Fig. 2. TNF- $\alpha$  that had accumulated in the medium over a period of 4 hours (black bars) and was present in the cell lysates at the end of the experiment (white bars) was measured by ELISA. (C) The total cellular amount of TACE was immunoblotted as described in Materials and Methods. The immature form of TACE is indicated by 'i', the mature form by 'm'. (D) The cell surface expression of TACE was measured by flow cytometry. Experiments were performed in duplicate and repeated three times. Significance of difference between treated and control cells: \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .



exposed at the cell surface, as already described (Doedens and Black, 2000).

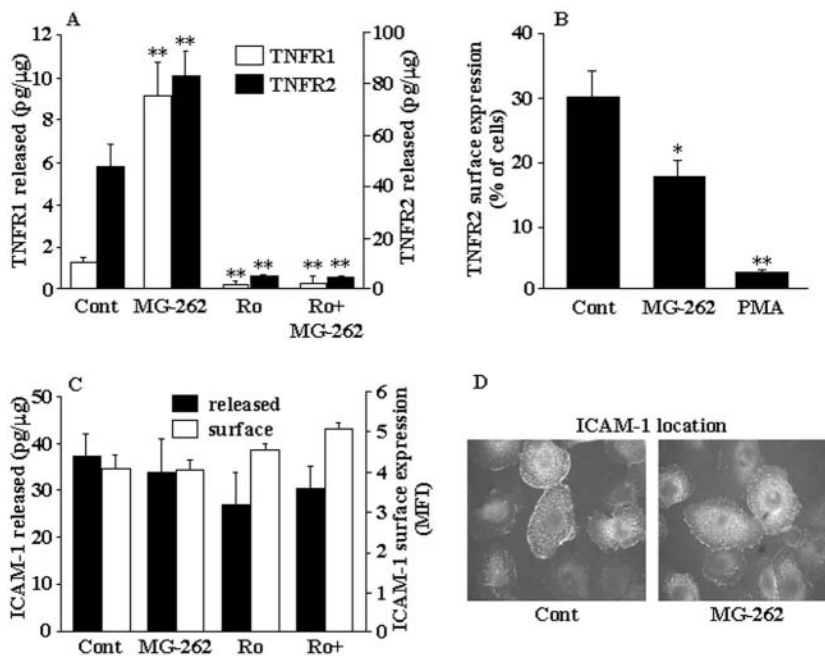
The release of endogenous TNFR1 from ECV-304 cells was increased by MG-262 and decreased by Ro 32-7315 (Fig. 4A). However, its basal cell surface expression was not detectable by flow cytometry (see below). TNFR2 was undetectable in the culture medium of the ECV-304 cell line (data not shown) which is in accordance with previous data (Bjornberg and Lantz, 1998). Proteasome inhibition increased the release of ectopically expressed TNFR2, which was impaired by Ro 32-7315 pretreatment (Fig. 4A). Flow-cytometry analysis showed that the number of cells expressing ectopic TNFR2 at their surface was reduced by proteasome inhibition (-42%) and by PMA treatment (-92%) (Fig. 4B). The stimulating effect of MG-262 on the release of TACE substrates was also observed using Cos-7 cells that expressed ectopic TNF- $\alpha$ , TNFR1 and TNFR2 (data not shown), suggesting that this effect is not limited to endothelial cells and not related to an alteration of their endogenous expression.

To investigate the specificity of MG-262 on TACE-dependent shedding of transmembrane substrates, we studied the release and the cell-surface expression of ICAM-1, which is not recognized as a TACE substrate. The amount of soluble ICAM-1 that accumulated in ECV-304 cell culture medium and at the cell surface was neither significantly altered by TACE inhibition (confirming that ICAM-1 is not a TACE substrate) nor by proteasome inhibition (Fig. 4C). In TNF- $\alpha$ -stimulated conditions, soluble ICAM-1 concentration increased up to 40-fold and was not altered by TACE inhibition (data not shown). Clearly, the intracellular localization of ICAM-1 was not modified by MG-262 treatment (Fig. 4D). Together, these results suggest that proteasome inhibition does not result in a general process of shedding of cell surface molecules.

#### Proteasome inhibition triggers the activation of a MAP kinase pathway that stimulates TACE-substrate ectodomain shedding

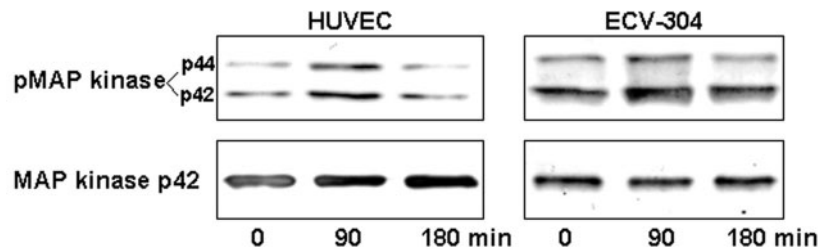
To dissect the signaling pathways responsible for MG-262-stimulated TACE activity, we used MAP-kinase- and PKC-pathway inhibitors in

**Fig. 4.** Proteasome inhibition specifically stimulates TACE-dependent ectodomain shedding. (A) ECV-304 cells were transiently transfected with TNFR2. Forty-eight hours post transfection cells were treated as described for Fig. 2. Ectopically expressed TNFR2 (black bars) and endogenous TNFR1 from untransfected cells (white bars) that had accumulated in the medium over a period of 4 hours were measured by ELISA. (B) Cells ectopically expressing TNFR2 at their surface were counted by flow cytometry. (C) Endogenous ICAM-1, released in the culture media of ECV-304 cells, was measured by ELISA and its cell surface expression was measured by flow cytometry. (D) Epifluorescence microscopy of the intracellular distribution of ICAM-1 in untreated and MG-262-treated ECV-304 cells. Flow-cytometry experiments were performed in duplicate and repeated three times. Significance of difference between treated and control cells: \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .



ECV-304 cells stably expressing TNF were treated for 15 minutes with U0126 (1 μM) or PD98054 (PD at 10 μM) or Gö6983 (2 μM) or not treated before the addition of MG-262 (600 nM) or PMA (20 nM). TNF that had accumulated in the culture medium over a period of 4 hours or that was present in the cell lysates at the end of the experiment was measured by ELISA. \*\*, significance of difference between treated and control cells ( $P < 0.001$ ); n.d., not determined.

ECV-304 cells that stably expressed ectopic TNF- $\alpha$ . The MAP-kinase-pathway inhibitor U0126 reduced the amount of TNF- $\alpha$  that was released after treatment with MG-262 and doubled its cell-associated amount (Table 1). Comparable results were obtained with another, structurally unrelated, MAP kinase pathway inhibitor, PD98059. By comparison, U0126 and PD98059 reduced the basal and PMA-stimulated release of TNF- $\alpha$  only insignificantly, without increasing its cellular level (Table 1). These results suggest that the MG-262-stimulated release of TNF- $\alpha$  involves a MAP kinase pathway. Accordingly, we detected a transient increase in the phosphorylation level of MAP kinase p42/p44 in HUVECs and



**Fig. 5.** Proteasome inhibition increases the phosphorylation of MAP kinase p42/p44. HUVECs and ECV-304 cells were serum-starved overnight and treated with MG-262 (600 nM). Phosphorylation of MAP kinase p42/p44 was analyzed by immunoblot after 0, 90 and 180 minutes of treatment (upper panels). Corresponding amounts of total p42 are shown in lower panels.

ECV-304 cells, which was clearly noticeable 90 minutes after proteasome inhibition and returned to basal level after 180 minutes (Fig. 5). The PKC inhibitor Gö6983 (efficient on the PMA-activated PKC isoforms  $\alpha$ ,  $\beta$ ,  $\delta$  and  $\gamma$ ) did not significantly alter the basal and the MG-262-stimulated release of TNF- $\alpha$ . However, Gö6983 abolished the PMA-stimulated TNF- $\alpha$  release (Table 1), which resulted in the cellular retention of TNF- $\alpha$  (+50%). The PMA-stimulated release of TNF- $\alpha$  was similarly inhibited by another PKC inhibitor, Ro 31-8220, but not by Gö6976 (mainly active on PKC isoforms  $\alpha$  and  $\beta$ ) (data not shown), suggesting the involvement of PKC isoforms  $\delta$  or  $\gamma$  in this process. These results show that the signaling pathways triggered by MG-262 and PMA are different. However, these pathways probably involve common downstream targets because the addition of PMA and MG-262 together resulted in an extracellular accumulation of TNF- $\alpha$  that was not significantly different from that obtained with PMA alone (Table 1).

#### Activation of MAP kinase p42/p44 triggered by proteasome inhibition increased the transport of TACE substrates to the cell surface

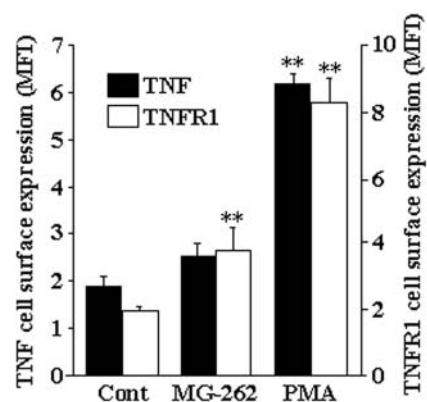
We then investigated the mechanism by which proteasome inhibition increased TACE-substrate release. In basal conditions, fluorescence values associated to the flow-cytometric detection of ectopically expressed TNF- $\alpha$  and endogenous TNFR1 at the surface of ECV-304 cells were not significantly different from values obtained with an irrelevant antibody, suggesting a low level of surface expression. However, the inhibition of TNF- $\alpha$  and TNFR1 cleavage by metalloprotease inhibitors allows their surface detection, as proposed earlier for TNF- $\alpha$  (Bueno et al., 2002). In these inhibitory conditions, treatment with MG-262 marginally but repeatedly increased the cell surface expression of TNF- $\alpha$  (31% increase of MFI,  $P=0.07$ ), and significantly that of TNFR1 (100% increase of MFI) (Fig. 6). PMA treatment increased the cell surface expression of TNF- $\alpha$  and TNFR1 by a factor of three and four, respectively, when Ro 32-7315 was present (Fig. 6). These data support the concept that MG-262 and PMA activate the transport of TNF- $\alpha$  and TNFR1 to the cell surface. To test this hypothesis, we analyzed the cellular distribution of TNF- $\alpha$  by confocal and epifluorescence microscopy when TACE was inhibited. In unstimulated conditions, TNF- $\alpha$  exhibited a compact juxtannuclear staining pattern that colocalized with Golgin-97, a marker of the Golgi complex (Fig. 7 and Fig. 8A). Treatments with MG-262, lactacystin or PMA (Fig. 8B,C,D) triggered the dispersal of the TNF- $\alpha$  from the perinuclear region throughout the cytoplasm, suggesting that these compounds can accelerate the exit of TNF- $\alpha$  from these internal pools. The accumulation of TNF- $\alpha$

at the plasma membrane was clearly noticeable after PMA stimulation (Fig. 8D). Comparable patterns of localization were observed with ectopically expressed TNFR2 (Fig. 8G,H). Interestingly, the dispersal of TNF- $\alpha$  induced by MG-262 was prevented by pretreating the cells with the MAP kinase pathway inhibitor PD98059 (Fig. 8E,F). The general Golgi architecture was not modified by MG-262 as judged by Golgin-97 staining (Fig. 8I,J).

#### The proapoptotic effect of TNF- $\alpha$ is increased by proteasome and metalloprotease inhibitors

To test whether the stimulatory effect of proteasome inhibition on TACE substrate ectodomain shedding has a potential impact on TNF- $\alpha$  signaling, we investigated a TNF- $\alpha$ -triggered cell-response that does not involve the activation of NF- $\kappa$ B because it is blocked by proteasome inhibition. The proapoptotic effect of TNF- $\alpha$  fulfills this condition: it is mainly triggered by TNFR1 and involves a signaling pathway independent of NF- $\kappa$ B (Gupta, 2002).

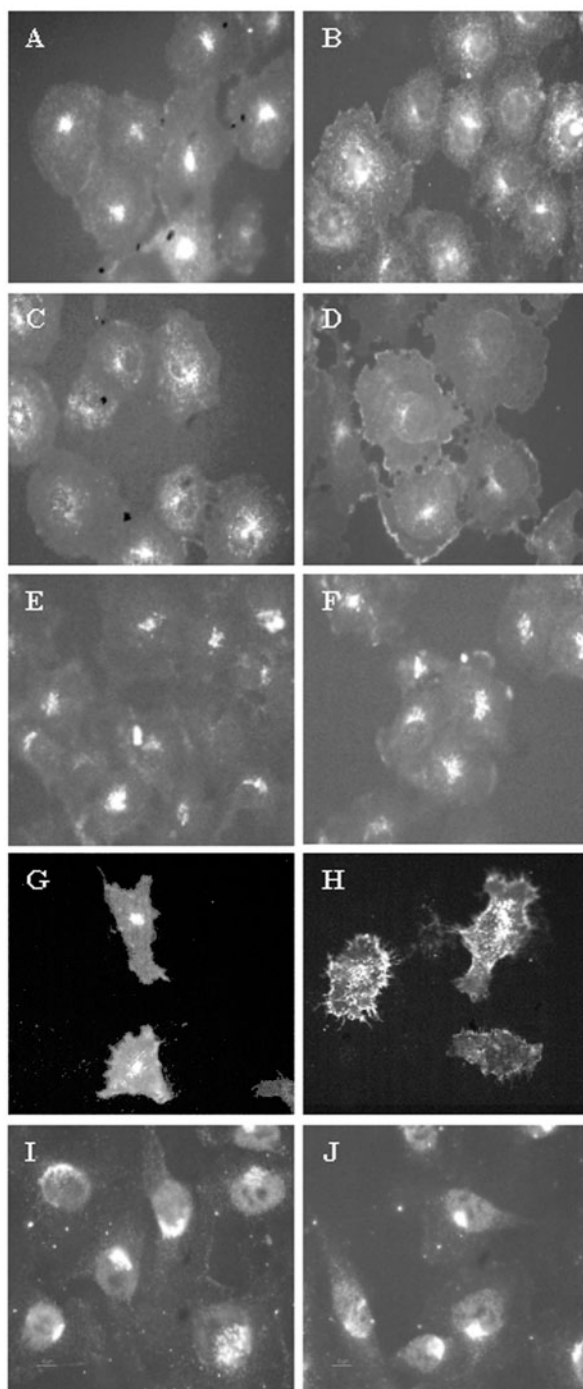
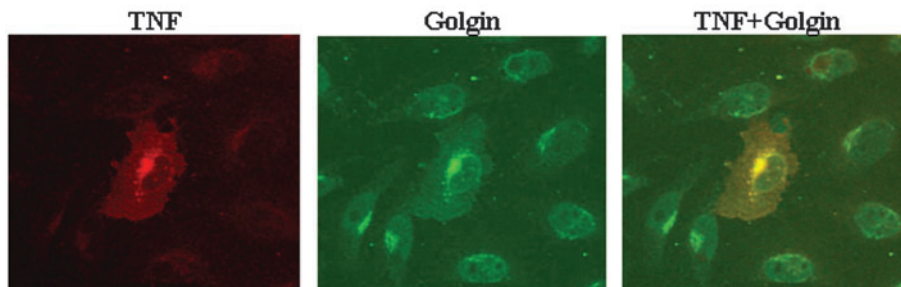
Here, we observed that MG-262 increased caspase 3 activity only in the ECV-304 cell line but not in primary HUVECs (Table 2). In MG-262-treated ECV-304 cells, caspase 3 activity was significantly increased by Ro 32-7315 only in presence of TNF- $\alpha$  (by a factor of two, see Table 2). In HUVECs, the basal caspase 3 activity was only significantly increased (by a factor



**Fig. 6.** Proteasome inhibition increases the amount of TACE substrates at the surface of TACE-inhibited cells. Native cells and ECV-304 cells stably expressing TNF- $\alpha$  were treated with Ro 32-7315 (5  $\mu$ M) 15 minutes before the addition of vehicle alone (Cont), MG-262 (600 nM) or PMA (20 nM) as indicated. Four hours later, cell surface expression of ectopic TNF- $\alpha$  (black bars) and endogenous TNFR1 on native ECV-304 cells (white bars) were measured by flow cytometry. Experiments were performed in duplicate and repeated three times. Significance of difference between treated and control cells: \*\*,  $P<0.001$ .



**Fig. 7.** In ECV-304 cells, ectopically expressed TNF- $\alpha$  is localized in the Golgi complex. Immunofluorescence analyses by confocal microscopy of ectopic TNF- $\alpha$  and endogenous Golgin-97 in ECV-304 cells that stably express TNF- $\alpha$ . TNF, localization of ectopic TNF- $\alpha$ ; Golgin: localization of endogenous Golgin-97; TNF + Golgin: superposition of the two previous images.



of two) in presence of TNF- $\alpha$ , proteasome and TACE inhibitors (Table 2). The proapoptotic effect of TNF- $\alpha$  on ECV-304 cells suggested by the measured caspase 3 activity was confirmed by Annexin V-FITC surface labeling (Fig. 9). In the presence of TNF- $\alpha$ , neither necrotic nor apoptotic cells were detected. However, in the presence of Ro 32-7315, MG-262 and Ro 32-7315+MG-262, the percentage of apoptotic (annexin V-FITC labeled cells) and secondary necrotic cells (prodium iodide and annexin V-FITC labeled cells) was increased by a factor of two, seven and 14, respectively. In the absence of TNF- $\alpha$ , the percentage of apoptotic cells was not altered by Ro 32-7315 alone and was increased by a factor of two by MG-262, independently of Ro 32-7315 (data not shown).

## Discussion

The potential therapeutic use of proteasome inhibitors is mainly justified by their ability to inhibit the activation of NF- $\kappa$ B. In the context of inflammation, NF- $\kappa$ B upregulates the synthesis of numerous cytokines, enzymes and cell adhesion molecules, and proteasome inhibition has been shown to be effective in several animal models of different inflammatory disease states to limit these processes (Elliott et al., 2003). In the pathogenesis of cancer, the activation of NF- $\kappa$ B promotes growth and in some cases prevents the benefit of chemotherapy. Moreover, proteasome inhibitors have been shown to possess anti-tumor activity in vivo (Adams, 2002; Almond and Cohen, 2002). However, the perturbation of the NF- $\kappa$ B signaling is not the only mechanism through which proteasome inhibitors act. For instance, they also induce apoptosis by allowing the accumulation of short-lived proapoptotic proteins (An et al., 1998; An et al., 2000). Here, we observed in HUVECs, that proteasome inhibition increased the release and concomitantly decreased the cell surface expression of TNFRs that are recognized as TACE substrates (Black et al., 1997; Moss et al., 1997; Peschon et al., 1998; Reddy et al., 2000). This finding motivated us to investigate the mechanisms and its impact on the reactivity of endothelial cells. We extended our study to TNF- $\alpha$ , the prototype of TACE substrate and publish

**Fig. 8.** Proteasome inhibition triggers the intracellular dispersion of TNF- $\alpha$  and TNFR2 from the Golgi-complex pool. ECV-304 cells that stably express TNF- $\alpha$  (A-F) or transiently express TNFR2 (G, H), or native ECV-304 cells (I,J) were treated with TACE inhibitor Ro 32-7315 (5  $\mu$ M) 15 minutes before the addition of vehicle alone (A,G,I), MG-262 (B,H,J), lactacystin (C), PMA (D), PD98054 (E) or PD98054+MG-262 (F). Four hours later, cellular expressions of TNF- $\alpha$  (A-F), TNFR2 (G,H) and Golgin-97 (I,J) were analyzed by immunocytochemistry and epifluorescence microscopy as described in Materials and Methods.

**Table 2. The association of TACE and proteasome inhibitors increases the caspase-3 activity**

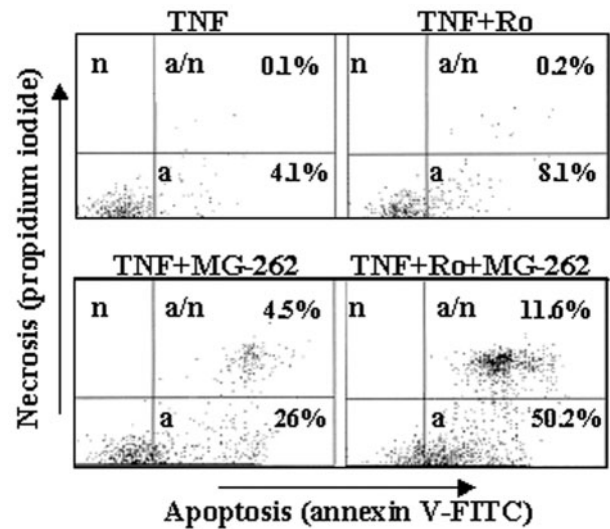
	ECV-304		HUVEC	
	-TNF	+TNF	-TNF	+TNF
Control	0.3±0.1	0.4±0.05	1.05±0.1	0.8±0.2
Ro	0.2±0.05	0.6±0.1	1.2±0.2	1.3±0.1
MG-262	1.1±0.3 <sup>†</sup>	3.1±0.4 <sup>**</sup> ; <sup>†</sup>	1.2±0.1	1.1±0.1
Ro+MG-262	1.4±0.5 <sup>†</sup>	6.5±0.5 <sup>**</sup> ; <sup>†</sup>	1.1±0.2	1.6±0.05 <sup>†</sup>

ECV-304 cells were incubated overnight in absence of serum with Ro 32-7315 (5 μM) and/or MG-262 (30 nM) in absence or presence of TNF (60 U/ml) as indicated. HUVEC were incubated like ECV-304 cells with the exception that 10% fetal calf serum was present in the culture media. Caspase 3 activity was measured as described in Materials and Methods section and expressed in (nmoles pNA/min/ml). \*\*, significantly different from values obtained without TNF ( $P<0.001$ ). <sup>†</sup>, significantly different from values obtained in control situation ( $P<0.001$ ); Ro: Ro32-7315.

compelling arguments in favor of an activating effect of proteasome inhibition on the ectodomain-shedding of TACE substrates. Clearly, synthetic (Ro 32-7315) and naturally occurring (TIMP-3) TACE inhibitors, and also a dominant negative form of TACE, reduced the stimulatory effect of proteasome inhibition on the release of ectopically expressed TNF- $\alpha$ , reinforcing the assertion that proteasome inhibition stimulates TACE-dependent ectodomain-shedding. It was therefore described that the ectodomain-shedding of the growth hormone receptor (GHR), another TACE substrate (Schantl et al., 2004; Zhang et al., 2000), was stimulated by proteasome inhibition (Takagi et al., 2001). However, we demonstrated that this phenomenon does not affect all transmembrane proteins, because the release of ICAM-1, as well as the cell surface expression levels of ICAM-1 and TACE were not modified by proteasome inhibition.

The presence of a TACE inhibitor increases the amount of TNF- $\alpha$  and TNFR1 at the surface of cells treated by proteasome inhibitor. Comparably, the cell surface expression of GHR, was shown to be increased by a proteasome inhibitor together with a metalloprotease inhibitor (van Kerkhof et al., 2003). This phenomenon suggests that the transport of TACE substrates to the cell surface is activated by proteasome inhibition. Consistent with this hypothesis, we as well observed that TNF- $\alpha$  and TNFRs colocalized with the proximal Golgi complex at basal conditions (Jones et al., 1999; Shurety et al., 2000), and that they redistributed towards the plasma membrane in response to proteasome inhibition. Our data are in accordance with those showing that in HUVECs, histamine-induced shedding of TNFR1 involves its mobilization from the Golgi complex (Wang et al., 2003). This mobilization of a pool of TACE substrate induced by proteasome inhibition is not the consequence of a general process because the intracellular distribution of ICAM-1 did not change. In the absence of a TACE inhibitor, the enhanced trafficking of TNFRs towards the cell surface triggered by proteasome inhibition is concomitant to a reduction in their cell-surface amount and an increase in their release, suggesting stimulation of TACE activity.

Our work demonstrates that activation of MAP kinase p42/p44 is a key event triggered by proteasome inhibition because it is crucially involved in the increased release of TACE substrate. This is consistent with previous data, showing that proteasome inhibitors promote MAP Kinase p42/p44 nuclear accumulation (Lenormand et al., 1998), and delay



**Fig. 9.** The association of TACE with proteasome inhibitors increases the cell susceptibility to the proapoptotic effect of TNF- $\alpha$ . ECV-304 cells were incubated overnight in the absence of serum with Ro 32-7315 (5 μM) and/or MG-262 (30 nM) in presence of TNF- $\alpha$  (60 U/ml) as indicated. Surface binding of annexin V-FITC and propidium iodide labeling were measured by flow cytometry. Apoptotic cells are in square 'a', necrotic cells in square 'n' and secondary necrotic cells in square 'a/n'. The percentage of apoptotic cells (a and a/n) is indicated. Ro, Ro32-7315. Experiments were performed in triplicate and repeated twice.

and/or sustain its phosphorylation state (Hashimoto et al., 2000; Vrana and Grant, 2001). The involvement of a MAP kinase pathway in the shedding of TACE substrates stimulated by growth factors was already documented (Fan and Derynck, 1999). However, our results help to delineate the mechanisms involved, because we demonstrate that the transport of TACE substrates, from their Golgi-complex-associated pool towards the plasma membrane, is stimulated by the activated MAP kinase p42/p44, which subsequently allows a higher rate of cleavage by TACE. In agreement to this, activation of MAP kinase p42/p44 was shown to be responsible for histamine-induced mobilization of TNFR1 from the Golgi complex (Wang et al., 2003). Proteasome inhibition does not modify the general architecture of the Golgi complex, as judged by the staining pattern of Golgin-97, supporting the proposal of a role of MAP kinase p42/p44 activation – specifically on the trafficking of Golgi-derived vesicles containing TACE substrates. However, Harada et al., described a fragmentation of the Golgi complex after a 24-hour proteasome inhibition (Harada et al., 2003). However, the role of the activation of MAP kinase p42/p44 in this phenomenon was not analyzed and the target of activated MAP kinase p42/p44 that triggers the intracellular redistribution of TACE substrates remains to be identified.

In our work, PMA treatment was used as the classic way to activate shedding of TACE substrate, although the underlying mechanisms are not clearly established. PMA treatment was reported to induce shedding by either activating a PKC pathway (Izumi et al., 1998), or a MAP kinase pathway following activation of PKC (Fan and Derynck, 1999; Gechtman et al., 1999). Montero et al., ascribed a major role



to the PKC pathway and a marginal role to the MAP kinase p42/p44 pathway (Montero et al., 2002). In our study, PMA-stimulated shedding of TACE substrates was only sensitive to PKC inhibitors but not to MAP kinase pathway inhibitors, suggesting that MAP kinase p42/p44 is not involved in the effect induced by PMA. Moreover, immunolocalization of TNF- $\alpha$  allowed us to establish that PMA treatment activated the transport of TACE substrates from their Golgi-associated pool towards the plasma membrane, which may account for their subsequent enhanced shedding by TACE. The fact that a PKC signaling pathway and a MAP kinase p42/p44 signaling pathway are independently involved in the intracellular mobilization of TACE substrates suggests this process as a prerequisite for the stimulation of TACE substrate-shedding. Because the addition of PMA and MG-262 together resulted in an extracellular accumulation of TNF- $\alpha$  not significantly different from that obtained with PMA alone, we suggest that a common substrate of PKC and MAP kinase p42/p44 might initiate the increased intracellular transport of TNF- $\alpha$ .

The use of proteasome inhibitors as an anti-inflammatory strategy (Elliott et al., 2003) is mainly based on the ability of these molecules to inhibit the activation of NF- $\kappa$ B. Our results, demonstrating an activation of the ectodomain-shedding of TNFRs in response to proteasome inhibition, support the concept of this anti-inflammatory strategy. First, the reduced number of TNFRs at the surface of endothelial cells could render them less sensitive to TNF- $\alpha$ . Second, released TNFRs could trap soluble TNF- $\alpha$ , therefore reducing its bioactivity. That clinical trials use proteasome inhibitors for cancer treatment is in part based on the fact that tumor cells have a higher sensitivity to the proapoptotic effects of these molecules than normal cells (Adams, 2002; Almond and Cohen, 2002). Moreover, it has been demonstrated that drug-induced proteasome inhibition increased TNF- $\alpha$ -related cell death (An et al., 2003; Chang et al., 2003; Delic et al., 1998; Kim and Kim, 2003). Our results clearly demonstrate that the proapoptotic effect of TNF- $\alpha$  is dramatically increased by the combination of proteasome and TACE inhibitors. This effect is probably the consequence of the increased cell-surface expression of TNFRs.

In conclusion, our data provide evidence that proteasome inhibitors increase TACE-dependent TNFR shedding in endothelial cells, justifying the use of these molecules in inflammatory disorders. In association with TACE inhibitor, proteasome inhibitors increase the amount of TNFRs at the cell surface and enhance the sensitivity to the proapoptotic effect of TNF- $\alpha$ , which might be of interest for antitumor therapy.

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