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Targeting neutrophil collagenase/matrix metalloproteinase-8 and gelatinase B/matrix metalloproteinase-9 with a peptidomimetic inhibitor protects against endotoxin shock

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Abstract

Gram-negative sepsis, bacterial meningitis and endotoxin shock are life-threatening disorders, associated with the rapid release of neutrophil enzymes. Neutrophil collagenase/matrix metalloproteinase-8 (MMP-8) and gelatinase B/matrix metalloproteinase-9 (MMP-9) are contained in granules, are quickly exocytosed upon granulocyte activation and efficiently cleave intact and denatured collagens, respectively. Genetic ablation of gelatinase B protects against endotoxin-induced mortality. Therefore, we designed and synthesized a peptidomimetic gelatinase B inhibitor Regasepin1, and compared the selectivity for the collagenases MMP-1, MMP-8 and MMP-13. Regasepin1 was found to inhibit, almost to the same degree, the neutrophil enzymes MMP-8 and MMP-9 and the monocytic tumor necrosis factor-α (TNF-α) converting enzyme (TACE/ADAM-17) in vitro. With the use of mass spectrometry analysis, the plasma half-life of inhibitor levels was determined after an intraperitoneal bolus injection in mice. Plasma peak levels of the inhibitor were reached at 50 min after intraperitoneal injection and the subsequent half-life in the circulation exceeded 40 min. Regasepin1 protected mice against lethal endotoxinemia by intraperitoneal and intravenous injection routes. This proofs the principle that early neutrophil MMP inhibition followed by TACE blockade may become a treatment strategy of gram-negative sepsis, endotoxinemia and other life-threatening inflammatory reactions.

Keywords: Matrix metalloproteinase; Lipopolysaccharide; Sepsis; Inflammation; Neutrophil; Endotoxin

1. Introduction

Bacteremia and septic or endotoxinemic shock are among the most frequent causes of mortality in modern hospitals. These clinical syndromes with multi-organ failure are caused by an excessive host inflammatory response to the invading microorganisms and their products [1,2]. Bacterial cell wall constituents, such as endotoxins/lipopolysaccharides (LPS) and peptidoglycans (PGN), are active agents in the inflammatory response and in the pathogenesis of septic shock. The biological effects of LPS and PGN are mediated by the activation of Toll-like receptors (TLR) on multiple leukocyte types [3]. In the case of sepsis, excessive TLR activation leads to exaggerated stimulation of leukocytes and excessive production of inflammatory mediators, including cytokines and enzymes. Genetic defects in the TLR, cytokine, enzyme and complement factor genes thus have been found to result in resistance against endotoxin shock. For instance, C3H/HeJ and C57Bl/10ScCr mice, homozygous for a mutation in the TLR4 gene, are resistant to endotoxin shock [3–5]. This role of TLR4 in the observed hyporesponsiveness to LPS challenge was confirmed in TLR4-deficient mice [6]. Similarly, mice deficient in cytokine signalling [7–9] and the enzyme gelatinase B/matrix metalloproteinase-9 (MMP-9) [10] have an increased resistance to LPS-induced toxicity, whereas mice deficient in protease inhibitors are

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; LPS, lipopolysaccharide; PGN, peptidoglycan; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TACE, TNF converting enzyme; DMSO, dimethylsulphoxide; ip, intraperitoneal; iv, intravenous

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more susceptible to LPS shock [11]. Similarly, the complement cascade [12] and the plasminogen activator–plasmin system [13] have been implicated in the pathogenesis of sepsis. These data imply that TLRs, cytokines and enzymes are not unique targets in such disease and that the pathophysiology of endotoxin shock is quite complex.

Since neutrophils are the most abundant cell type in the human circulation, LPS will mainly, directly and immediately act on these cells in the event of endotoxinemia. This interaction results in the release of various categories of neutrophil effector molecules, including enzymes, reactive oxygen intermediates which contribute to the activation of MMPs [14] and release of lysozyme which further generates PGN fragments. An important aspect of septic shock is its acuteness which may be based on the fast release of mediators by degranulation. In this respect it was demonstrated that the release of gelatinase B/MMP-9 precedes the production of chemokines and TNF-α in baboons with sepsis [15] and that in human volunteers MMP-9 plasma levels were already maximal at 1.5–3 h after LPS challenge [16], a time point which is too early for mRNA transcription, translation and secretion of proteins, but which is compatible with degranulation of preformed enzymes. In the study of the roles of MMPs in endotoxin shock pathology, clear inductions of MMP-9 and MMP-8 mRNAs by LPS were noticed [17]. To prove the principle that pharmacological inhibition of neutrophil proteinases and TNF-α production may be of therapeutic use, we recently selected a novel peptidomimetic inhibitor of gelatinase B, Regasepin1. Here we demonstrate the in vitro properties and in vivo effects of this molecular probe on LPS-induced morbidity and mortality in mice and document a novel way to combat excessive inflammation as it occurs in sepsis and endotoxin shock.

2. Materials and methods

2.1. Inhibition of MMP-9 by Regasepin1

Gelatinase B/MMP-9 was purified from human neutrophils and activated with 0.01 μM MMP-3 in assay buffer (100 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$ and 0.01% Tween-20) at 92 ng/μl (1 μM) as stock solution as described [18].

Hydrolysis of the substrate (7-methoxycoumarin-4-yl) acetyl Pro-Leu-Gly-Leu-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropyl-Ala-Arg-NH_2 (R&D Systems Europe Ltd.) was monitored by measuring the increase in fluorescence (λ_{ex} = 328 nm, λ_{em} = 392 nm) as previously described [19]. All assays were performed at 37 °C in 2 ml assay buffer. A substrate concentration of 2 μM was applied so that the fluorescence increase has a linear relationship with the enzyme incubation time for at least 20 min. The reaction was started by adding 1 μl gelatinase B stock solution to the 2 ml reaction system after which the rate of substrate hydrolysis was monitored continuously for 5 min. Then the appropriate amount of Regasepin1 solution was added (the final concentration of Regasepin1 varied from 0.1 to 28 μM) and the rate of substrate hydrolysis was monitored for another 5 min. The decrease of reaction rate is proportional to the inhibition of gelatinase B by Regasepin1.

Alternatively, the inhibitory effect of Regasepin1 against human gelatinase B was tested with another peptide substrate (Alexa-GSTSTSGPP′GPQGATGEP′G, P′ is hydroxyproline) which was N-terminally labelled with Alexa Fluor 488 carboxylic acid, succinimidyl ester (Molecular Probes Europe BV). Briefly, 10 μl reactions were performed in assay buffer by incubating 0.5 μl human neutrophil gelatinase B stock solution (1 μM or 92 ng/μl), 1 μl fluorescently labelled peptide substrate (36 μM) and varying amounts of Regasepin1 (final concentration from 0.05 to 54 μM) at 37 °C for 8 min. Reactions were terminated by adding 1 μl 100 mM 1,10-phenanthrolin. The substrate and reaction products were separated by capillary electrophoresis with online fluorescent detection. Enzyme activity was calculated by comparing the peak heights for the intact substrate and reaction product. The inhibitory effect of Regasepin1 was calculated by measuring the decrease of enzyme activity compared with that of a control reaction without inhibitor.

2.2. Inhibition of MMP-9 activity in neutrophil degranulate

Purified human neutrophils, derived from healthy human blood donors, were washed gently and suspended in degranulation buffer (20 mM Tris/HCl, pH 7.5, 117 mM NaCl, 15 mM $CaCl_2$) at a concentration of 10^7 cells/ml. Neutrophil degranulation was performed by incubating neutrophils with fMLP (final concentration 0.5 μM) for 20 min at 37 °C. Then 1 μl 1% Tween-20 was added to 100 μl degranulate. The proenzymes in the degranulation solution were activated by incubation with human stromelysin-l/MMP-3 (R&D system, at a final concentration 10.7 nM) for 2 h at 37 °C as described [20].

The fluorescent peptide substrate (Alexa-GSTSTSGPP′-GPQGATGEP′G) was used to detect gelatinase B activity from the activated human neutrophil degranulate. A substrate cleavage versus incubation time standard line was generated by incubating 1 μl fluorescently labelled peptide substrate stock solution (36 μM) with 4 μl activated neutrophil degranulate at 37 °C for 0, 8, 16, 24 and 32 min. Reactions were terminated by adding 1 μl 100 mM 1,10-phenanthrolin.

To test the inhibitory effect of Regasepin1 on the activated neutrophil degranulate, 1 μl fluorescently labelled peptide substrate stock solution (36 μM) and 4 μl activated neutrophil degranulate were incubated with 2 μl of Regasepin1 (final concentration from 0 to 30 μM)

at 37 °C for 32 min. Reactions were terminated by adding 1 μl 100 mM 1,10-phenanthrolin and analysed as above.

2.3. Inhibition of MMP-1, MMP-8 and MMP-13 by Regasepin1

MMP-1, MMP-8 and MMP-13 were purchased as recombinant human enzymes from R&D Systems. Native bovine collagen type II (5 μl at 2 mg/ml, a kind gift of Prof. D. Brand, University of Tennessee) was mixed with 5 μl of serial dilutions of Regasepin1 (dissolved in assay buffer with 4% DMSO) at final concentrations ranging from 1 μM to 1 mM. Enzymatic cleavages were performed by addition of 5 μl of collagenase stock solutions and incubation at 30 °C for 4 h. The reactions were terminated by adding 10 μl reducing SDS-PAGE loading buffer. The collagen/gelatin samples were heated for 3 min at 95 °C before separation by SDS-PAGE (7.5% or 12% separating gel). The protein bands of intact and two cleaved collagen II fragments were visualized by silver staining [21]. The relative intensities of the intact substrate and the largest reaction product (on 7.5% separating gel) were scanned by densitometry. MMP-8 was used at 2 ng/μl in assay buffer, whereas MMP-1 and MMP-13 were used from stock solutions of 34 and 15 ng/μl, respectively. The final concentration for MMP-8 in the cleavage mixture is 12.5 nM, whereas those for MMP-1 and MMP-13 were 210 and 92 nM, respectively.

2.4. Inhibition of neutrophil elastase

Human leukocyte elastase was purchased from Sigma-Aldrich and dissolved in assay buffer at 0.295 ng/μl (10 nM) as stock solution. Hydrolysis of the fluorogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (Sigma-Aldrich NV) was monitored by measuring the increase in fluorescence (λ_{ex} = 370 nm, λ_{em} = 460 nm) as previously described [22]. All assays were performed at 37 °C in 2 ml buffer (0.05 M Tris/HCl, pH 7.5, 0.5 M NaCl, 0.1 M $CaCl_2$ with 10% DMSO) with 0.2 mM substrate. The reaction was started by adding 2 μl human leukocyte elastase stock solution to the 2 ml reaction system (final concentration 10 pM) after which the rate of substrate hydrolysis was monitored continuously for 5 min. Then the appropriate amount of Regasepin1 solution was added (the final concentration of Regasepin1 varied from 0.25 to 1 mM) and the rate of substrate hydrolysis was monitored another 5 min. A similar procedure for control inhibition of neutrophil elastase was performed in which 1 mM or 2 mM PMSF was applied instead of Regasepin1.

2.5. Inhibition of TACE by Regasepin1

Recombinant human TACE was purchased from R&D Systems and was dissolved in 25 mM Tris, pH 8.0 at 100 ng/μl (1.43 μM) as stock solution. Hydrolysis of the substrate (7-methoxycoumarin-4-yl) acetyl Pro-Leu-Ala-Gln-Ala-Val-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropyl-Arg-Ser-Ser-Ser-Arg-NH_2 (R&D Systems) was monitored by measuring the increase in fluorescence (λ_{ex} = 320 nm, λ_{em} = 405 nm). All assays were performed at 37 °C in 2 ml buffer (25 mM Tris, pH 9.0, 2.5 μM $ZnCl_2$, 0.005% Brij-30). A substrate concentration of 10 μM was applied so that the fluorescence increases in a linear relationship with the enzyme incubation time for at least 20 min. The reaction was started by adding 2 μl recombinant human TACE stock solution to the 2 ml reaction system (final concentration 1.43 nM) after which the rate of substrate hydrolysis was monitored continuously for 5 min. Then the appropriate amount of Regasepin1 solution was added (the final concentration of Regasepin1 varied from 1 to 26 μM) and the rate of substrate hydrolysis was monitored another 5 min. The decrease of reaction rate is proportional to the inhibition of recombinant human TACE activity by Regasepin1.

2.6. Determination of inhibitor half-life in plasma

Heparinized blood samples were taken at the orbital sinus at various time intervals: before injection and at 0.5, 1, 2, and 3 h after intraperitoneal injection of 3.5 mg Regasepin1 per NMRI mouse. Blood was immediately cooled on ice. Peripheral blood cells were removed by centrifugation (10 min, 250 × *g*, 4 °C) and equal volumes of plasma samples were processed. Acetonitrile solution was used to remove proteins and other macromolecules. With five incremental steps of 10% acetonitrile (2 min, room temperature) and short centrifugation (10 min, 20,000 × *g*, 10 °C), the samples were made 50% in acetonitrile without removing the supernatant or pellet. Then, the solution was changed to a final concentration of 75% acetonitrile. After another centrifugation (10 min, 20,000 × *g*, 10 °C), equivalent volumes of supernatant were collected from each sample and diluted five times with 0.1% trifluoroacetic acid. These samples were subjected to reversed-phase HPLC on an Aquapore C8 column (RP-300, diameter 1 mm, length 5 cm, Perkin Elmer). For separation, a gradient of 16–32% acetonitrile was formed from 5 to 25 min by mixing 0.1% TFA and 80% acetonitrile in 0.1% TFA, the flow rate was 200 μl/min. Regasepin1 was detected in the eluent by online mass spectrometry analysis on an EsquireLC ion trap apparatus (Bruker Daltonik). The mass of Regasepin1 (*m*/*z* at 926 Da for the singly protonated molecule) was automatically traced during elution. For all the RP-HPLC separations, we detected a similar elution time between 6.0 and 13.0 min. After deconvolution, the uncharged intact molecule of 925 Da was reproducibly observed and the absolute abundance of this form was used to determine the relative amount of Regasepin1.

2.7. Induction of shock and treatment with Regasepin1

Adult NMRI or C57BL/6 mice (7 or 8 weeks) were acquired from commercial sources and injected intravenously on day 0 with indicated doses of LPS from *E. coli* serotype 0111:B4 (prepared by phenol-extraction; Sigma) dissolved in 0.9% saline. Prior to the experiments, the doses yielding 50% lethality (LD50) was determined to be 200 μg LPS per mouse. In the survival analysis of mice after LPS challenge, two administration pathways were compared. For intraperitoneal injection of Regasepin1, the inhibitor was dissolved in DMSO at 77 mM (or 71.4 mg/ml) and then further diluted in sterile pyrogen-free 0.9% saline solution to 3.1 mM (or 2.9 mg/ml). 1.25 ml was injected per mouse preventively 100 min before LPS challenge. Control animals were injected with saline containing equivalent amounts of DMSO as the treatment group. For intravenous injection of Regasepin1, the inhibitor (77 mM or 71.4 mg/ml in DMSO) was diluted to 7.5 mM (or 7 mg/ml) with sterile pyrogen-free 0.9% saline and 100 μl was injected per mouse 5 min after LPS challenge. Control animals were injected with saline containing an equivalent amount of DMSO. All animal experiments, including blood sampling (see previous section), were approved by the local ethics committee (Licence LA 1210243, Belgium).

3. Results

3.1. Design of Regasepin1 and inhibition of purified and neutrophil degranulate gelatinase B in vitro

Combinatorial chemical synthesis and high-throughput screening methodology were applied to screen more than 3000 peptide analogs for inhibition of gelatinase B. Peptide length and the position of Zn^{2+}-binding groups were experimentally optimized. The peptidomimetic sequences were essentially based on the consensus sequence of cleavage sites in denatured collagen type II [23]. From one sublibrary of compounds, the peptidomimetic Pro-Arg-Cys-Bip-Cys-Gly-Glu (Regasepin1, Bip corresponds to biphenylalanine, Fig. 1A) was isolated. Regasepin1 inhibited gelatinase B/MMP-9 activity in a dose-dependent way (Fig. 1B and C) with a 50% inhibitory dose (ID50) in the low micromolar range. Inhibition of MMP-9 activity by Regasepin1 was also confirmed in a cell-based assay system. After fMLP stimulation of neutrophils and activation of the neutrophil degranulate by stromelysin-l/MMP-3 (details see Section 2.2), the degranulate showed an enzyme activity capable to cleave the fluorescently labelled peptide substrate for MMP-9. A substrate cleavage versus incubation time standard line was generated (with a correlation coefficient $R = 0.99$, data not shown). The

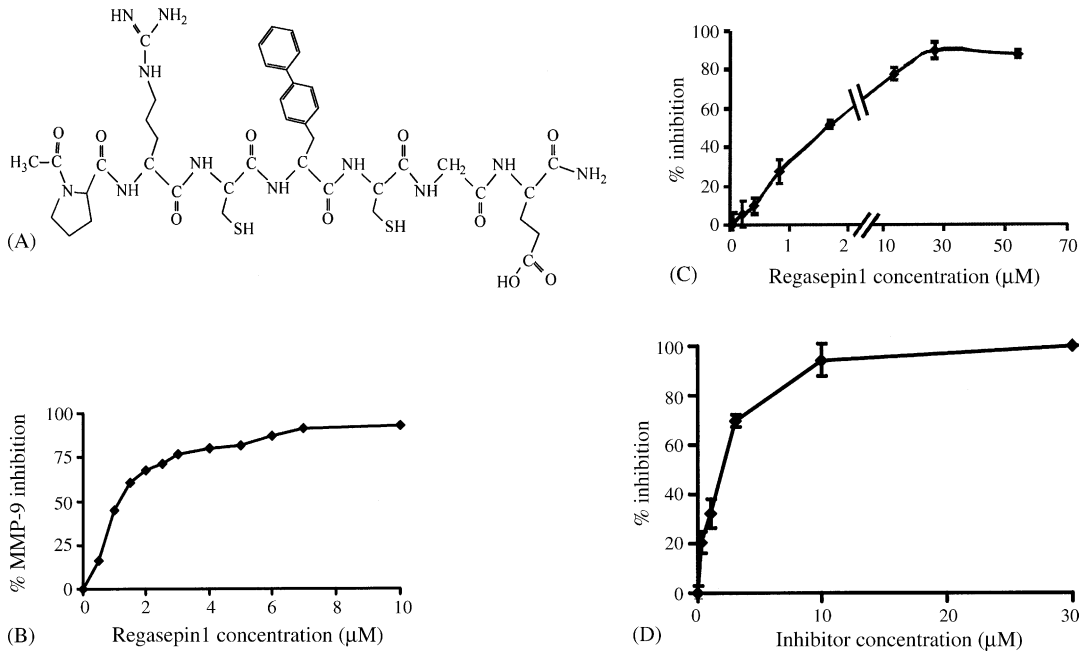

Fig. 1. Regasepin1 inhibits gelatinase B/MMP-9. (A) Structure of the peptidomimetic MMP inhibitor Regasepin1. (B) Purified gelatinase B from human neutrophils was tested in two substrate conversion assays in the absence or presence of increasing doses of Regasepin1. The percentage inhibition of enzymatic activity is represented as a function of inhibitor concentration. The dose yielding 50% inhibition (ID50) can be readily deduced from these graphs. The inhibition of MMP-9 by Regasepin1 is demonstrated with a commercially available fluorescent substrate McaPLGLDpaAR [19] (2 μM substrate, 0.5 nM gelatinase B). In panel (C) the inhibition is shown with the use of the cleavage of a fluorescent substrate Alexa-GSTSTSGPP′GPQGATGEP′G (Alexa indicates the dye molecule Alexa Fluor 488, P′ indicates hydroxylproline, 3.6 μM substrate, 50 nM gelatinase B). In panel (C), the data points are represented as means ± S.E.M. ($n = 3$). (D) The gelatinase B activity present in neutrophil degranulate, after activation by stromelysin-l/MMP-3, was inhibited by Regasepin1 in a dose-dependent way. The same methodology, as used in panel (C), was applied. Fifty percent inhibition of substrate conversion was achieved with a Regasepin1 concentration comparable to that used for ID50 determination of pure gelatinase B.

cleavage of the fluorescently labelled peptide substrate by the activated neutrophil degranulate was inhibited by Regasepin1 in a dose-dependent way (Fig. 1D). The 50% inhibition level was reached at approximately 2 μM which is comparable to the ID50 for the inhibition of the pure gelatinase B. After stimulation with fMLP or IL-8, neutrophils degranulate within 30 min and produce various forms of gelatinase B: monomers, homodimers and heterodimers with neutrophil gelatinase B-associated lipocalin [18]. These three molecular forms in neutrophil gelatinase B preparations are all inhibited by Regasepin1 (Fig. 1D). In contrast with the fact that many other leukocyte types constitutively synthesize gelatinase A/MMP-2, neutrophils do not. Therefore, MMP-9 is thus the major gelatinolytic enzyme from granulocytes [24]. Neutrophil degranulate MMP-9 is targeted by Regasepin1.

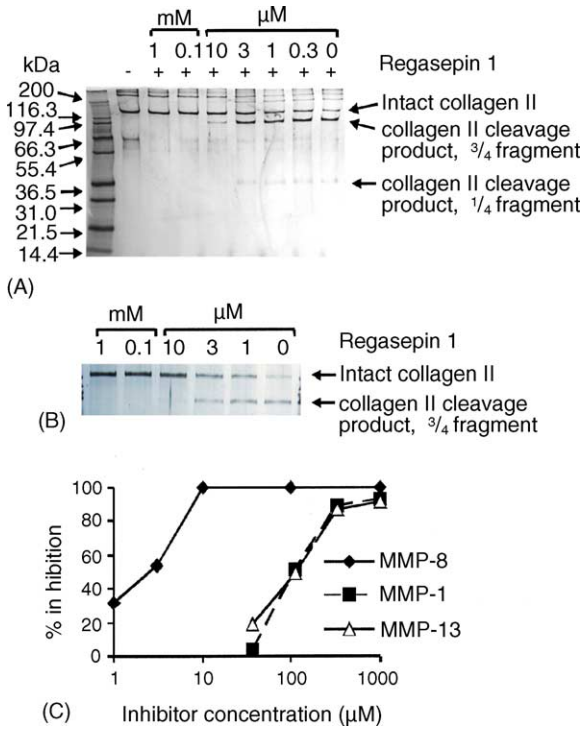

Fig. 2. Regasepin1 inhibits neutrophil collagenase/MMP-8. (A) Intact native type II collagen was incubated with MMP-8 in the absence or presence of increasing concentrations of Regasepin1, as indicated on top of each lane. After incubation, the residual intact collagen, as well as cleaved fragments were separated by gel electrophoresis (12% separating gel) and silver-stained. Because of its smaller size the 1/4 collagen II fragment precipitates much less silver nitrate and consequently is less pronounced than the larger 3/4 fragment. Molecular weight standard proteins are shown at the left and their masses indicated in kilodalton (kDa). (B) A similar experiment was performed and the bands of intact collagen II and the 3/4 fragment, which were separated with a 7.5% separating gel, were analysed by scanning densitometry. (C) The three collagenases, MMP-1, MMP-8 and MMP-13, were compared as in panel (B) and the percentage inhibition as a function of Regasepin1 concentration was calculated. The ID50 values were: 3 μM for neutrophil collagenase/MMP-8, and 100 μM for both MMP-1 and MMP-13. The final concentrations for MMP-1, -8, -13 in the cleavage mixtures are 210, 12.5 and 92 nM, respectively.

3.2. Regasepin1 is a specific neutrophil collagenase inhibitor

Whereas neutrophils do not produce MMP-2, they secrete neutrophil collagenase/MMP-8 together with MMP-9. Collagenases are necessary and sufficient to perform a single cleavage in intact native collagens before MMP-9 can act on the large (3/4) and small (1/4) reaction products. Three different soluble collagenases exist in humans and exert the physiological function of denaturation of the collagen triple helices: collagenase 1/MMP-1 also called interstitial collagenase, neutrophil collagenase or collagenase 2/MMP-8 and collagenase 3/MMP-13. In view of the selection of Regasepin1 as inhibitor of gelatinase B, it was important to evaluate its activity against the three collagenases. Fig. 2 illustrates that with native collagen II as substrate the peptidomimetic inhibited efficiently MMP-8 (ID50 = 3 μM), but not MMP-1 (ID50 = 100 μM) and MMP-13 (ID50 = 100 μM). Since the ID50 for MMP-8 is in the same order as that of MMP-9, it is concluded that Regasepin1 targets the two MMPs, which are present in and secreted by neutrophils and which predominate in endotoxinemia. Hence, Regasepin1 may be useful as molecular probe for neutrophil-mediated inflammatory processes.

3.3. Regasepin1 does not inhibit neutrophil elastase

Neutrophil elastase (NE) is a serine proteinase stored in azurophil granules. It has a broad spectrum of activities, including interstitial collagenase and gelatinase activities [25]. In addition, neutrophil elastase was found to have important antimicrobial roles in the mouse [26]. The potential of Regasepin1 as a neutrophil elastase

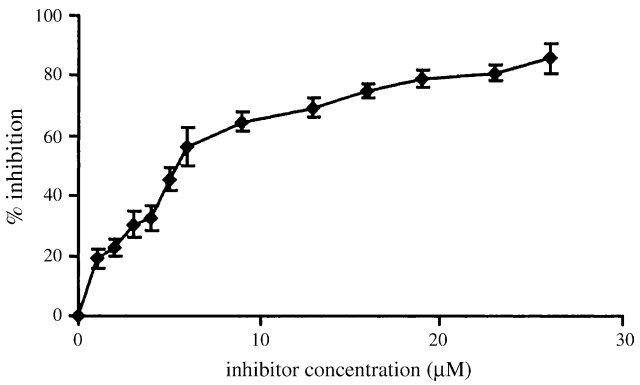

Fig. 3. Regasepin1 inhibits TACE. The activity of recombinant human TACE was tested using a commercially available fluorescent substrate McaPLAQAVDpaRSSSR (10 μM substrate, 1.43 nM TACE). The substrate conversion assays were performed in the absence or presence of increasing doses of Regasepin1. The percentage inhibition of enzymatic activity is represented as a function of inhibitor concentration and the dose yielding 50% inhibition (ID50) can be deduced as 5.3 μM. Each point represents the average ± S.E.M. of the inhibitory effect tested in three independent experiments.

inhibitor was tested at 0.25, 0.5, 0.75 and 1 mM concentrations. Regasepin1 did not inhibit neutrophil elastase at any of these concentrations. In parallel, PMSF (phenyl methyl sulphonyl fluoride) was used as a control inhibitor and blocked at 1 and 2 mM the activity of neutrophil elastase by more than 80 and 99%, respectively.

3.4. Inhibition of TACE

TNF-α signalling has also been demonstrated to be critical as a pathogenetic mechanism in endotoxin shock [7], although in vivo TNF-α levels increase later than those of gelatinase B [15]. Since proTNF-α is processed by a metalloproteinase, TNF-α converting enzyme/TACE, also called ADAM-17, it was relevant to investigate the possible inhibition of TACE by Regasepin1. Fig. 3 shows the inhibitory effect of Regasepin1 on the activity of TACE. Clearly, low micromolar concentrations (5 μM) of Regasepin1 were sufficient to inhibit 50% of TACE activity. Before pharmacological in vivo testing in an animal model of endotoxin shock, we verified that low micromolar concentrations of Regasepin1 can be reached in vivo.

3.5. In vivo distribution of Regasepin1

The peptidomimetic Regasepin1 as a rather hydrophobic inhibitor has the physicochemical property to precipitate in aqueous solution. Starting from a stock solution in dimethylsulphoxide (DMSO), an aqueous solution of the peptide was made, up to high concentrations (3.1 mg/ml, 3 mM). In order to evaluate the distribution of Regasepin1 after intraperitoneal (ip) injection, a micromethod was developed. This is based on precipitation of the macromolecules from heparinized plasma while keeping this hydrophobic peptide in solution under organic solvent conditions and subsequent HPLC purification of the peptidomimetic with detection by on-line mass spectrometry. Fig. 4 illustrates this methodology. In panel (A), the elution position at which the correct masses of Regasepin1 were detected and the intensities of the Regasepin1 signals were indicated as superposition on the total signals of all masses detected in the chromatogram. Panel (B) shows the mass over charge data of a typical Regasepin1 spectrum with a singly charged oxidized form at 926.3 and a doubly charged form at 463.6 Thomson. In panel (C) the mass deconvolution spectra are shown for the isotopes. When 100 μl plasma samples were collected after consecutive bleedings from individual mice and analysed, averaged peak plasma levels of Regasepin1 were observed at 1 h post-injection (data not shown). Since repetitive bleedings may result in artefacts by hemodilution, we also analysed smaller plasma samples pooled from groups of five mice. Fig. 4D shows that plasma levels of Regasepin1

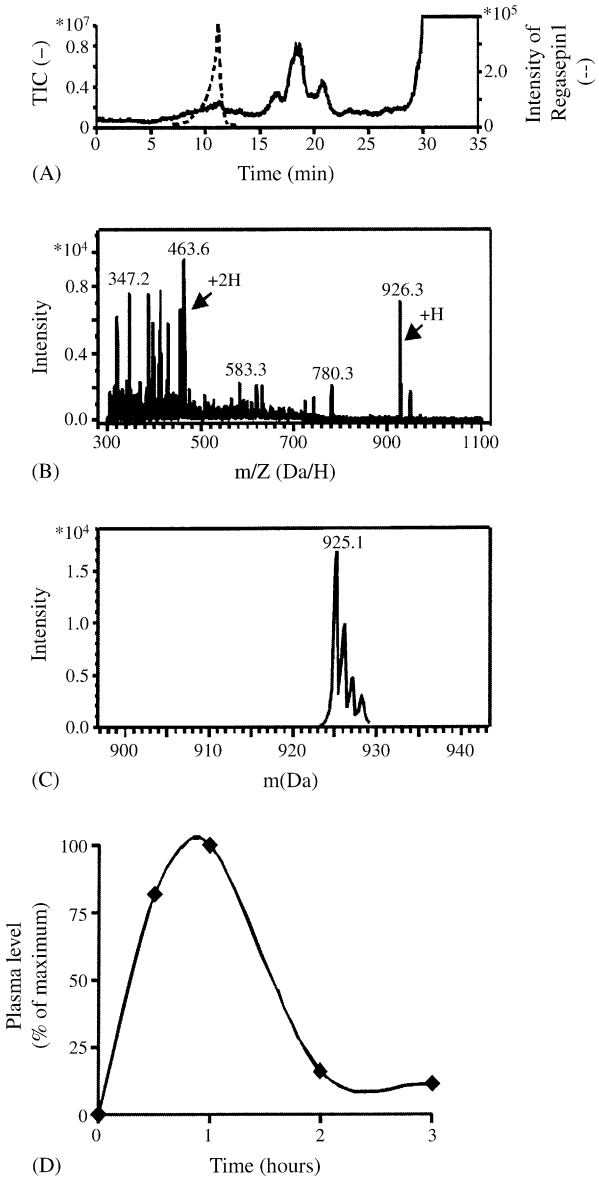

Fig. 4. Mass spectrometry analysis of Regasepin1 in mouse plasma. A single bolus injection of Regasepin1 was injected intraperitoneally and consecutive heparinized plasma samples were collected and processed. (A) The components including the hydrophobic inhibitor peptide in the supernatant were separated by RP-HPLC with continuous monitoring of mass intensity signals and software-based detection of the location and amount of Regasepin1. This peptide was eluted between 6.0 and 13.0 min. TIC represents the total ion count intensity in the HPLC chromatogram of the mass spectrometry analysis. (B) The mass spectra of the Regasepin1 peptidomimetic with singly and doubly charged mass peaks at 926.3 and 463.6 Da, respectively. (C) The mass peaks after deconvolution of the average mass spectra with major peaks of the intact peptidomimetic isotopes. (D) The plasma levels of Regasepin1 after a bolus injection. Groups of five mice were injected ip with a single bolus of 3.5 mg Regasepin1 and plasma samples (20 μl per mouse) were collected at different time intervals, pooled and analysed as indicated above. Average levels were calculated and plotted in function of time. Peak levels were obtained around 50 min and the maximal plasma levels declined with a calculated half-life of 41 min.

(from pooled samples) again peaked at about 1 h and that the subsequent half-life was about 40 min. On the basis of these pharmacological results, further in vivo experiments were done.

3.6. Regasepin1 protects against endotoxin shock

To document the in vivo effect of Regasepin1 in a clinically relevant setting, we used a mouse model of endotoxin shock. In parallel with the current mortality rates of endotoxin shock in humans, which exceed 50%, we predetermined the LD50 of LPS to be 200 μg per adult mouse and used higher doses of LPS to test the effect of Regasepin1. Groups of five mice were injected ip – 100 min before LPS challenge – with the inhibitor (3.5 mg per mouse) or with the used diluent, i.e. sterile pyrogen-free saline solution with an equivalent amount of DMSO. Then LPS was injected ip at predetermined doses and the animals were observed at regular intervals. Regasepin1 protected NMRI mice significantly against endotoxin shock challenges of 300 μg LPS (Fig. 5A). Similar results were obtained with the challenge of 400 μg LPS, i.e. 100% survival rate of the Regasepin1-treated group and lethality in the control group (data not shown). These data illustrate that pharmacological metalloproteinase inhibition, specifically of MMP-8, MMP-9 and TACE, is effective to prevent endotoxin shock lethality in vivo in NMRI mice. To reinforce these results and to demonstrate that the inhibitor is active independently of the used mouse strain, we performed similar experiments in C57BL/6 mice. First, we used a similar setting as described above. In two independent experiments, groups of five mice were injected ip – 100 min before LPS challenge – with the inhibitor (3.5 mg per mouse). Since the results of both experiments were similar the pooled data ($n = 10$ per group) are shown in Fig. 5B. As an alternative approach, in which the plasma concentration of Regasepin1 might be deduced, we also injected mice intravenously (iv) with the inhibitor and studied the biological effects. In this experimental setting, we injected only 0.7 mg per mouse in a volume of 100 μl. The control mice received saline with an according amount of DMSO. The inhibitor was injected 5 min after LPS challenge. In view of the calculated plasma level of approximately 350 μg/ml (375 μM) and a half-life of 40 min, we expected a therapeutic dose exceeding 5 μM at 240 min after administration. Accordingly and even with this five-fold lower doses than the ip doses, Regasepin1 was found again to improve the survival rate of LPS challenged C57BL/6 mice (Fig. 5C). The results of the survival analyses (Fig. 5) showed in all instances statistically significant differences between experimental groups and the corresponding control groups (for ip injection in Fig. 5A, $p < 0.05$; in Fig. 5B, $p < 0.002$ and for iv

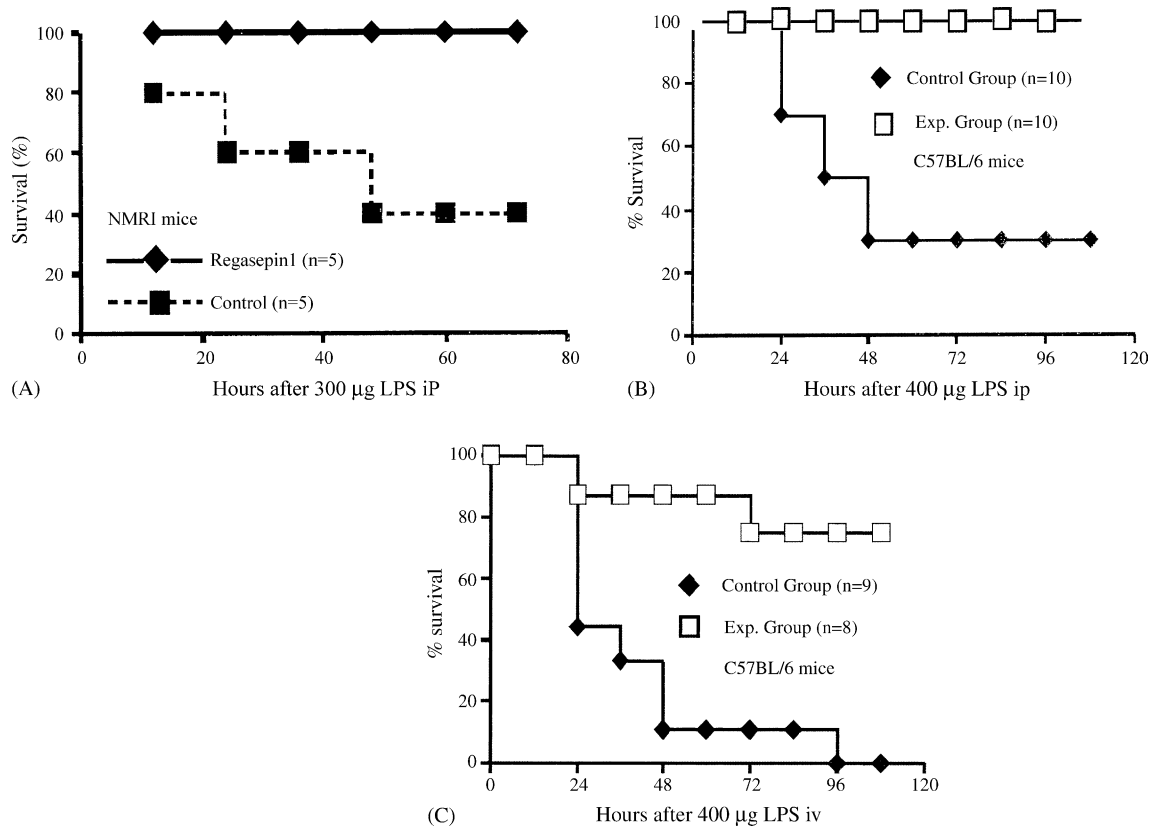

Fig. 5. Regasepin1-treated mice are resistant to endotoxin shock. (A) Hundred minutes after ip treatment with Regasepin1 (3.5 mg per mouse), groups of five control and five treated NMRI mice were injected intravenously with LPS in a model of endotoxin-induced shock. Kaplan–Meier survival curves were generated. With an LPS challenge of 300 μg, more than 50% of control mice died, whereas the Regasepin1-treated group completely survived. $p < 0.05$, compared with the vehicle-treated group. (B) Similar experiments were performed in C57BL/6 mice. The results of two parallel and independent experiments (with five mice in each group) were pooled. $p < 0.002$. (C) Treatment of endotoxin shock by intravenous injection. An iv bolus injection of 0.7 mg Regasepin1 also improved the survival rate of LPS challenged mice significantly. $p < 0.001$.

injection in Fig. 5C, $p < 0.001$). These data illustrate that pharmacological metalloproteinase inhibition, specifically of MMP-8, MMP-9 and TACE, is effective to prevent endotoxin shock lethality in vivo.

4. Discussion

Although various indirect and direct evidences indicated that neutrophil-derived factors play a pivotal role in the early phases of the physiopathology of gram-negative sepsis and endotoxin shock, only recently the possible role of MMPs has come to the forefront. Indirect evidence for a function of gelatinase B in sepsis may be provided by IL-8, which has been shown to be elevated in sepsis [27–29]. IL-8 is a potent neutrophil chemoattractant and activator. Neutrophil activation leads to almost immediate degranulation of gelatinase B [30]. Furthermore, a positive feedback was recently demonstrated between IL-8 and MMP-9 in neutrophils. Gelatinase B cleaves IL-8 into a 10-fold more potent chemokine [18] and, in addition to being a matrix remodeling enzyme, it is a tuner and amplifier of immune functions [24].

Also direct evidence links MMPs with sepsis and endotoxin shock. Because LPS induces virtually immediately the secretion of gelatinase B by neutrophils in the course of endotoxinemia, one can speculate that early therapeutic blocking of gelatinase B activity might ameliorate the course of the disorder. Serum gelatinase B levels increase immediately after LPS challenge, well before increases in chemokine and TNF-α levels are observed [10,15,17]. This indicates that the MMP-9 increase is a direct effect of LPS on leukocyte degranulation [30]. Gelatinase B-deficient mice are relatively resistant against endotoxin shock, i.e. the animals survive higher doses of LPS than wild type controls [10]. These studies incited us to develop novel MMP inhibitors and to investigate whether pharmacological inhibition of neutrophil MMPs may lead to protection against endotoxinemia.

Several classes of molecules with inhibitory activity against MMPs have been identified, e.g. tetracyclines, D-penicillamine and hydroxamates. Tetracyclines and hydroxamates have already shown protective effects against septic shock [31,32]. Since the target molecules were not defined in these reports, we aimed in this study to delineate the target enzymes of a novel peptidomimetic inhibitor.

Out of a series of more that 3000 peptidomimetic inhibitors we identified Regasepin1 as an MMP inhibitor and used this molecule here as a molecular probe to show relative selectivity against neutrophil MMP-8 and MMP-9 and monocytic TACE. Regasepin1 possesses an ID50 of about 3 μM against MMP-8 and 1.5 μM against MMP-9, whereas the ID50 for MMP-1 and MMP-13 is around 100 μM. These data indicate that physiological processes mediated by collagenase 1/MMP-1 and collagenase 3/MMP-13 can still take place while the neutrophil MMPs are inhibited. Furthermore, Regasepin1 inhibited TACE/ADAM-17 with an ID50 of approximately 5 μM (vide infra). Preliminary experiments indicate that Regasepin1

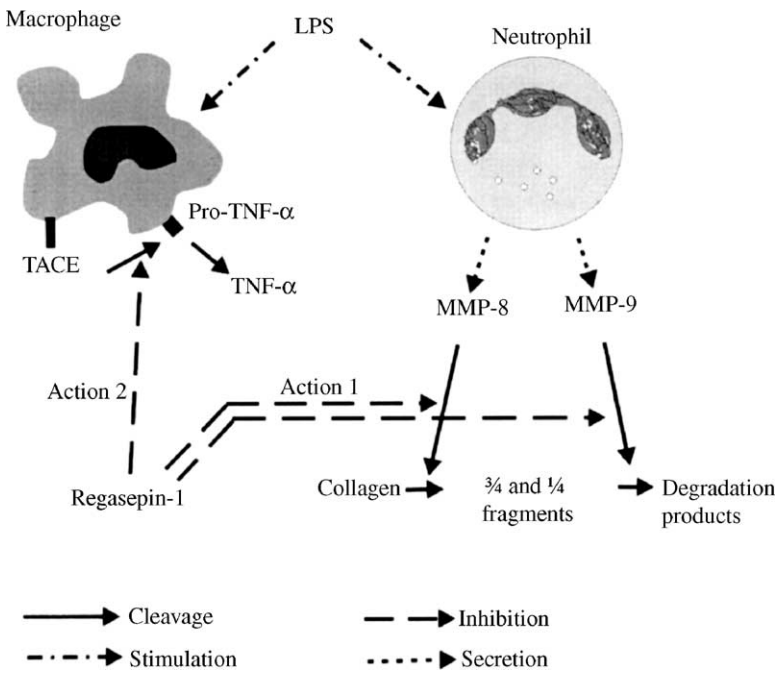

Fig. 6. Mechanisms of action of Regasepin1. In the circulation, LPS induces rapid (within 30 min) degranulation of MMP-8 and MMP-9 from neutrophils and de novo (hours to one day) production of TACE and proTNF-α from monocytes. MMP-8 and MMP-9 lead to rapid and efficient degradation of collagens. This process precedes TNF-α production which further enhances the inflammatory response. Regasepin1 inhibits both the immediate phase of collagen breakdown (action 1) as well as the later effect on TNF-α production (action 2).

does not interfere with intracellular signalling cascades (NF-κB), but rather acts extracellularly on secreted enzymes.

In view of the hydrophobic nature of Regasepin1 it was essential to study its pharmacological behaviour in vivo. Being a peptidomimetic, Regasepin1 will be destroyed after peroral administration. Therefore, and to control the dosage of the compound, it was injected intraperitoneally and intravenously. Parenteral administration is compatible with the treatment of sepsis and endotoxin shock, since most such patients are hospitalized in intensive care units and treated with intravenous drug injection. We first developed a method to determine whether and how efficiently the peptidomimetic is distributed in the circulation. With the use of mass spectrometry analysis, we were able to detect the peptide in plasma samples and to follow the pharmacodynamics after a single bolus injection. Irrespective of its hydrophobic nature, Regasepin1 was detectable in the circulation where peak levels were observed after 50 min and then the levels gradually decreased with a half-life of 40 min. It needs to be stressed that we observed two oxidized forms of Regasepin1 by mass spectrometry. One form contains an intramolecular disulphide bridge, whereas the other is a dimer form. We also evaluated the chemical dimer form by mass spectrometry analysis and observed similar pharmacodynamic results in vivo as for the monomer (data not shown).

Regasepin1 was tested in mouse models of lethal endotoxin shock and shown to protect against morbidity and mortality. An ameliorative effect of the synthetic broad spectrum hydroxamate-type MMP inhibitor GM-6001 was also demonstrated in LPS-induced endotoxinemia. This MMP inhibitor increased the survival of LPS-treated mice and inhibited plasma levels of TNF-α. However, the effects of this MMP inhibitor on gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 activity were not studied specifically [32]. Though Regasepin1 is a different molecule than GM-6001, it possesses at least the in vivo efficacy of the hydroxamate. Fig. 6 summarizes the present view how Regasepin1 interferes with the metalloproteinases MMP-8, MMP-9 and TACE, and how it may mediate protection against endotoxin shock. The important pharmacological issue addressed here is that mortality rates, presently over 50% in humans with septic shock, can be reduced with pharmacological agents that target neutrophil MMP effector molecules and monocytic TACE. It should be noted that endotoxin stimulates the almost immediate degranulation of neutrophils, whereas it also stimulates gene transcription and protein synthesis of TNF-α. The latter effect is slower and thus the inhibitory activity of Regasepin1 on TACE may be effective at a later time point in the disease, on the provision of sufficient dosage by e.g. continuous infusion. The possible in vivo effect of Regasepin1 against TACE must therefore be considered in relation to the pharmacokinetic data provided here. Our study is mechanistically important to demonstrate that efficient therapeutic effect may be achieved by targeting several MMPs at once. It also forms a positive stimulus to identify inhibitors with similar target spectra in the nanomolar range or below and to corroborate the present concepts. In summary, neutrophil MMP-8 and MMP-9 were defined as early targets for the treatment of gram-negative sepsis and endotoxin shock. This further substantiates the thesis that molecules such as MMP-9 and MMP-8, alongside TNF-α, are key regulators and effectors of innate and adaptive immune functions and that MMP inhibitors will become interesting pharmacological probes in basic and clinical immunology and, if given early in the disease course, may become important life-saving drugs.

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