Auto-antigen expression of proteinase 3 on the membrane of neutrophils is dependent on phosphatidylinositol 3-kinase and p38 MAPKinase activity

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ABSTRACT

Introduction. Expression of the autoantigen proteinase 3 (PR3) on the membrane of neutrophils is a prerequisite for the activation process of neutrophils by PR3-ANCA. Translocation of PR3 to the membrane occurs via exocytosis of secretory vesicles, specific granules and azurophilic granules during priming or apoptosis. Little is known about the signaling events that translocate the autoantigen PR3 to the membrane in these processes. Therefore, we analyzed involvement of major regulatory cascades as the MAPkinases and phosphatidylinositol3-kinase (PI3-K) during priming and apoptosis in translocation of proteinase 3 to the membrane in order to explore new therapeutic targets in the management of ANCA-associated vasculitis.

Materials and methods. Neutrophils were primed with TNF-α or accelerated in their apoptosis by treatment with TNF-α and cycloheximide (CHX) or by UV-B irradiation. Pharmacological inhibitors interfering with p38 MAPK, ERK, JNK and PI3-K were added to study their involvement in mPR3 expression during priming or apoptosis. Apoptosis and mPR3 expression were measured using flow cytometry.

Results. mPR3 expression during TNF-α priming was dependent on p38 MAPK activation only. Furthermore, mPR3 expression induced during TNF-α/CHX-accelerated apoptosis was dependent on p38 MAPK and PI3K, but not on ERK or JNK. Blocking p38MAPK or PI3-K signaling did affect apoptosis itself, as induced by TNF-α/CHX. Also, UV-B-accelerated apoptosis resulted in mPr3 expression which was dependent on p38MAPK, but not on PI3-K, ERK or JNK. Interestingly, culture of neutrophils after priming resulted in gradually reduced expression of mPR3 in time.

Conclusion. mPR3 expression is transient during in vitro culture and pharmacological inhibitors interfering with p38 MAPK and PI3-K activity can block the induced expression of mPR3 during apoptosis, while not affecting apoptosis itself. As autoantigen expression plays such a dominant role in the pathophysiology of ANCA-associated vasculitis, pharmacological inhibition of p38 MAPK (or PI3-K) may be an effective therapeutic strategy in the management of ANCA-associated vasculitis.

Key words. proteinase 3, ANCA, p38 MAPK, PI3K, Wegener's granulomatosis
INTRODUCTION

The anti-neutrophil cytoplasm autoantibody (ANCA)-associated vasculitides (AAV) are characterized by necrotizing inflammation of mainly arterioles, venules, and capillaries. ANCA autoantibodies are in most cases directed against proteinase 3 (PR3) or myeloperoxidase (MPO), enzymes stored in the granules of neutrophils. Based on histopathological and clinical manifestations three major categories of AAV are defined: Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA), and Churg Strauss syndrome (CSS). In patients with Wegener's granulomatosis ANCA most frequently target proteinase 3 (PR3). A pathophysiological role of ANCA is supported by clinical observations and experimental animal studies. Furthermore, in vitro studies have shown that ANCA are capable of activating TNF-α-primed neutrophils. In this activation process, binding of ANCA to their target antigens on PMN is a prerequisite for activation of PMN by ANCA, as has been shown in many studies. In concordance with this hypothesis, activation does not occur in MPO-deficient neutrophils after stimulation with MPO-ANCA, despite normal activation by other stimuli.

Proteinase 3 (PR3) is a proteolytic enzyme, mainly stored in the azurophilic granules, and to a lesser extent in the specific granules and secretory vesicles of neutrophils. Based on membrane bound proteinase 3 (mPR3) expression, two subsets of neutrophils can be defined: neutrophils that do not express proteinase 3 (mPR3-negative) and neutrophils that do substantially express proteinase 3 (mPR3-positive) neutrophils. Proteinase 3 is translocated to the membrane by priming with low-dose of TNF-α or IL-8, but also GM-CSF and TGF-β have been described to translocate PR3 to the membrane. In addition, a number of reports have proposed upregulation of PR3 during apoptosis as an alternative mechanism for ANCA to interact with mPR3 on neutrophils. Proteinase 3 is translocated from various granules or vesicles of the neutrophils to the cell membrane. This translocation process is sequentially regulated, and starts with exocytosis of readily mobilizable secretory vesicles, followed by the specific granules, and, finally, limited exocytosis of azurophilic granules. Neutrophil degranulation has been reported to be under the control of Mitogen Activated Protein Kinases (MAPK) and Phosphatidylinositol-3-Kinases (PI-3K). In mammalian cells, three major MAPK signaling cascades exist: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. Especially the latter, p38 MAPKinase, has been demonstrated to be involved in exocytic processes in neutrophils. Kettritz et al have shown that translocation of proteinase 3 during priming with TNF-α is dependent on p38 MAPK, but independent of ERK or PI3-K signaling. However, little is known about the signaling events that translocate the autoantigen PR3 to the neutrophil membrane during apoptosis.

As autoantigen expression on the neutrophil membrane plays such a dominant role in the pathophysiology in ANCA-associated vasculitis, the search for target regulatory molecules that control translocation of PR3 to the membrane may reveal effective therapeutic strategies in the management of ANCA-associated vasculitis.

Therefore, we studied major regulatory cascades in exocytic processes, i.e. the MAPKinases and PI3-Kinase, during apoptosis and priming in relation to
translocation of proteinase 3 to the cell surface in order to explore potential therapeutic targets that control mPR3 expression.

MATERIALS AND METHODS

Materials
All chemicals used were from Sigma (Zwijndrecht, The Netherlands), unless otherwise indicated. Anti-coagulant tubes were from BD Vacutainer Systems, Plymouth, UK. Hanks Balanced Salt Solution (HBSS) and Gentamycin were purchased from Gibco/Life Technologies, Breda, The Netherlands. RPMI 1640 and FCS were from BioWhittaker Europe, Verviers, Belgium, and culture plates from Costar, Badhoevedorp, The Netherlands. The inhibitors SB203580 and PD98059 were purchased from Calbiochem (via Merck Eurolab, Amsterdam, The Netherlands). LY294002 was purchased from Cell Signaling (via Westburg BV, Leusden, The Netherlands). Wortmannin was obtained from Sigma. RWJ67657 (Johnson and Johnson, R.W.Johnson Pharmaceutical Research Institute, Raritan, New Jersey, USA) was kindly provided by dr. J. Westra (Groningen, The Netherlands).

Isolation, culture and priming of neutrophils
Neutrophils of healthy individuals who were bimodal in their expression for mPR3 were selected only. Bimodal expression was defined as the presence of 10% - 90% mPR3+ cells on the total neutrophil population. Neutrophils were isolated from EDTA-anticoagulated blood by centrifugation on Polymorphprep™ (Nycomed, Oslo, Norway) and hypotonic lysis of contaminating erythrocytes with ice-cold ammonium chloride buffer. Cells were washed with cold Hanks’ balanced salt solution (HBSS) without Ca2+/Mg2+ (GIBCO/Life Technologies) and resuspended in culture medium (RPMI 1640 supplemented with 10% FCS and streptomycin (60 µg/ml)) to obtain 2 × 10^6 cells/ml. For kinetic experiments on mPR3 expression, the following three groups of isolated neutrophils were studied: 1) non-primed neutrophils incubated in culture medium for 15 min without TNF-α, followed by washing and reconstitution of cells in culture medium for 0, 2, 3, and 4 hrs, 2) neutrophils primed with 10 ng/ml of recombinant TNF-α (Boehringer Mannheim, Germany) for 15 min, followed by washing and reconstitution of cells in culture medium for 0, 2, 3, and 4 hrs, 3) neutrophils continuously primed with TNF-α in culture medium for 0, 2, 3, and 4 hrs.

To study effects of pharmacological agents on mPR3 expression, isolated neutrophils, cultured for 4 hrs in order to have minimal expression of mPR3, were primed with TNF-α (10 ng/ml) for 30 min in the presence or absence of pharmacological inhibitors. Pharmacological inhibitors were added during culture, at least 30 minutes before priming.

Induction of apoptosis
Isolated neutrophils (2 x 10^6/ml) were reconstituted in RPMI 1640 medium supplemented with 10% FCS and streptomycin (60 µg/ml). Apoptosis of neutrophils was accelerated by culture in 24-wells plates (2 x 10^6 cells/well) for 4 hrs at 37°C in a humidified, 5% CO2 atmosphere in the presence of TNF-α (10 ng/ml) and Cycloheximide (CHX; 2.5 µg/ml), as described earlier. UV-accelerated apoptotic
neutrophils were generated by irradiating neutrophils \((2 \times 10^6 / 300 \mu l / \text{well})\) in 12-wells plates with ultraviolet-B light for 20 min. \((360 \text{ mJ} / \text{cm}^2)\) using a TL12 lamp (Philips, Best, The Netherlands), followed by culture for 4 hrs at 37°C in a humidified, 5% CO₂ atmosphere. After induction of apoptosis, neutrophils were incubated for 4 hrs in order to reveal apoptosis in these cells. Pharmacological inhibitors were added 30 min prior to addition of TNF-α / CHX or prior to irradiation with UV-B light.

Detection of membrane expression of mPR3 and apoptosis

Membrane expression of proteinase 3 and CD35 were measured using flowcytometry as previously described\(^37\). All steps were performed on ice. Briefly, samples containing \(10^6\) neutrophils were fixed with 0.5% paraformaldehyde for 10 minutes, washed with PBS/1% BSA by centrifugation at 1200 x g, 4°C for 3 min, and incubated with 0.5 mg/ml heat-aggregated goat immunoglobulins (IgG; Sigma) for 15 min to saturate Fcγ receptors. Next, cells were stained with a saturating dose of mouse monoclonal IgG\(_1\) antibody (MAb) directed against human PR3 (PR3.G-3) \(^38\), human CD35 (Ber-Mac-DRC, Dako Cytomation, Glostrup, Denmark) or with an irrelevant IgG\(_1\) control antibody (MCG1; IQProducts, Groningen, The Netherlands) for 30 min. Next, non-bound antibodies were washed off with PBS / 1% BSA. This step was followed by 30 min incubation with phycoerythrin (PE)-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc., Birmingham, USA) in the presence of 0.5 mg/ml heat-aggregated goat IgG. For apoptosis detection, cells were subsequently washed with a Ca\(^{2+}\) rich-binding buffer (10mM HEPES [pH 7.4], 140 mM NaCl, 5 mM CaCl\(_2\)), and incubated for 10 min on ice with 99 µl binding buffer to which 1 µl fluorescein isothiocyanate (FITC)-labeled annexin V (1:10 diluted, Nexins Research, Kattendijke, The Netherlands) was added. Apoptosis was confirmed by staining for cleaved caspase-3, according to the manufacturer’s instructions for FACS analysis (cleaved caspase-3 (Asp 175) antibody, Cell Signaling, Beverly, MA, USA). Fluorescence intensity was analyzed on an ELITE flowcytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and calibrated using the Flow-Set™ fluorospheres (Beckman Coulter, Hialeah, FL, USA). Bimodal mPR3 expression was defined as the presence of 10 - 90% mPR3\(^+\) cells \(^39\). The level of mPR3 was expressed in arbitrary units (AU) calculated as the mean fluorescence intensity (MFI PR3) of mPR3\(^+\) cells corrected for non-specific binding of the isotype control antibody (MFI NSB) and multiplied by the percentage of cells within the mPR3\(^+\) subset \(^40\). In some figures mPR3 expression was calculated as follows: (AU of pharmacologically inhibited sample – AU of 4 hrs medium sample) / (AU of stimulated sample – AU of 4 hrs medium sample) x 100. Stimulated samples were primed with TNF-α or accelerated in apoptosis using TNF-α and CHX or UV-B only.

Western blotting for activated kinases

Human neutrophils \((2.10^6/ml)\) were resuspended in RPMI supplemented with 10% FCS and streptomycin (60 µg/ml) and then stimulated with TNF-α or the combination of TNF-α and cycloheximide (CHX) at 37°C in a humidified, 5% CO₂ atmosphere, or irradiated with ultraviolet-B (UV-B) for 10, 15, 60 and 120 min. Afterwards cells were lysed with pre-warmed SDS buffer (containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris-HCl (pH=6.8) and 0.01 % bromephenolblue). This was
immediately followed by sonication for 5 - 10 seconds and boiling for 5 min. After centrifugation, samples were loaded onto a 10% SDS-PAGE gel and resolved by running at 200V and 15 Watt. Subsequently, semidry-blotting onto a nitrocellulose membrane was followed by immunoblotting with specific antibodies to p38 MAPK, phospho-p38 MAPK, ERK1/2, phospho-ERK1/2, JNK/SAPK, phospho-JNK/SAPK (Cell Signaling Technologies). Protein bands were detected by enhanced chemiluminescence (ECL) according to manufacturer's instructions (Lumi-Light plus, Roche Diagnostics, Mannheim, Germany).

STATISTICS
Results are expressed as mean ± SEM of the number of independent experiments. Statistical analysis was performed using the two-tailed Students t-test and Graph-Pad Prism, version 3.0 (GraphPad Prism Software, San Diego, CA, USA)

RESULTS
mPR3 expression of cultured neutrophils
First, we investigated mPR3 expression under culture conditions in time. Therefore, neutrophils in culture were analyzed for their mPR3 expression at 0, 2, 3 and 4 hrs after neutrophil isolation with or without priming with TNF-α. Due to priming events during the isolation procedure, neutrophils showed pronounced mPR3 expression after isolation (0 hrs) even without additional priming with TNF-α (Figure 1A). Furthermore, we found that culture of neutrophils resulted in a gradual reduction of mPR3 expression to minimally detectable levels (Figure 1A). Also, after priming with low dose of TNF-α (10 ng/ml), mPR3 expression gradually reduced in time (Fig 1a). Neutrophils that where continuously primed with TNF-α showed a strong decrease in mPR3 expression as well (Figure 1A). However, these continuously primed neutrophils still showed pronounced mPR3 expression after 4 hrs (Figure 1A).

Figure 1. mPR3 expression of cultured neutrophils. 1) Non-primed neutrophils were incubated for 15 min without TNF-α, followed by washing and reconstitution of cells in culture medium for 0, 2, 3 and 4 hrs, 2) neutrophils were primed with 10 ng/ml of TNF-α for 15 min, followed by washing and reconstitution of cells in culture medium for 0, 2, 3 and 4 hrs, 3) neutrophils were continuously primed with 10 ng/ml TNF-α in culture medium for 0, 2, 3 and 4 hrs. At the indicated time points neutrophils were fixed and stained for mPr3 or CD35. A) Expression of mPR3 is depicted as arbitrary units (AU, see materials and methods) on the y-axis. B) Expression of CD35 is depicted on the y-axis as the mean fluorescence intensity (MFI). Values are given as mean ± standard error of the mean (SEM).
In contrast to mPR3 expression, the secretory vesicle marker CD35 (Complement Receptor 1 (CR1)) showed no apparent decrease in time (Figure 1B).

**Upregulation of mPR3 expression during apoptosis**

Neutrophils in culture were accelerated in their apoptosis by treatment with TNF-α and cycloheximide, or by UV-irradiation. After 4 hrs, $70.6 \pm 9.9\%$ (n=6) of neutrophils were AnnexinV positive (Figure 2B) when apoptosis was accelerated by treatment with both TNF-α (10 ng/ml) and cycloheximide (2.5 µg/ml), whereas only $9 \pm 1.8\%$ (n=6) of untreated neutrophils were positive for Annexin V staining (Figure 2A) (n=6). UV-irradiation of neutrophils for 20 min, followed by culture for 4 hrs, resulted in comparable percentages of apoptotic neutrophils (63.5 ± 7.2%, n=6) (Figure 2C). In addition, TNF-α/CHX- and UV-B-induced apoptotic neutrophils showed comparable

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**Figure 2.** Upregulation of mPR3 during apoptosis. Neutrophils of donors bimodal in mPR3 expression were accelerated in apoptosis by combined treatment with TNF-α (10ng/ml) and Cycloheximide (2.5µg/ml), or by UV-B irradiation for 20 min, followed by culture for 4 hrs. (A,B,C) Representative dot blots of neutrophils from a bimodal mPR3 donor cultured for 4 hrs in medium only (A), following treatment with TNF-α and CHX (B), or following irradiation with UV-B light (C). After 4 hrs of culture, neutrophils were stained for mPR3 expression using a mouse anti-PR3 monoclonal antibody (PR3, G-3) and Annexin V binding. D) Overlay of anti-PR3 binding of TNF-α/CHX- induced apoptotic neutrophils (black line) and UV-B irradiated apoptotic neutrophils (dashed line). In grey, the non-specific isotype (IgG1) control antibody is shown. E) Effect of UV-B irradiation and treatment with TNF-α/CHX on mPR3 expression in the total population of neutrophils from bimodal donors. F) mPR3 expression induced by TNF-α / CHX or UV-B on the mPR3⁻ and mPR3⁺ subset separately. Values are given as mean ± standard error of the mean (SEM) of 6 independent experiments.
percentages of activated caspase-3 after 4 hrs (data not shown). Apoptosis of neutrophils induced by treatment with TNF-α / CHX or UV-B-irradiation resulted in a significant upregulation of mPR3 expression to 350 ± 130 AU in TNF-α / CHX-accelerated apoptotic neutrophils and to 117 ± 16 AU in UV-B accelerated apoptotic neutrophils as compared to neutrophils cultured in medium (29 ± 9 AU, p < 0.05) (Figure 2E). In addition, the substantial upregulation of mPR3 expression during apoptosis was only seen on mPR3⁺ PMN (Figure 2D and 2F). There were no differences in rate of apoptosis between mPR3⁻ and mPR3⁺ neutrophils.

**p-38 MAPK and PI3-K dependent expression of mPR3 during TNF-α / CHX-accelerated apoptosis**

To investigate the role of different MAPK's and PI3-K during induction of mPR3 expression in TNF-α / CHX-induced apoptosis, neutrophils were stimulated with TNF-α alone or with both TNF-α and CHX for different periods and assessed for phosphorylated and unphosphorylated p38 MAPK, ERK and JNK. Neutrophils stimulated with TNF-α alone or with TNF-α and CHX showed a clear phosphorylation of p38 MAPK and ERK (Figure 3), but not of JNK (data not shown). Interestingly, phosphorylation of p38 MAPK and ERK persisted longer in neutrophils treated with TNF-α and CHX than after treatment with TNF-α only (Figure 3).

![Phospho-p38 MAPK and p-38 MAPK](image)

**Figure 3.** Phosphorylation of p38 MAPK and ERK during TNF-α/CHX-accelerated apoptosis. Human neutrophils (2.10⁶/ml) were incubated without stimulation (5 min.) or stimulated with TNF-α or with TNF-α and CHX for 5, 10, or 20 min. When indicated this was followed by culture for 40 or 100 min at 37°C. Stimulation was terminated by lysing cells with pre-warmed SDS buffer, sonication and boiling for 5 min. Subsequently, samples were loaded onto a 10% SDS-PAGE gel, and semidry-blotted followed by immunoblotting with specific antibodies to p38 MAPK, phospho-p38 MAPK, ERK1/2 and phospho-ERK1/2.

Subsequently, neutrophils were pre-incubated for 30 min at 37°C with increasing concentrations of the specific p38 MAPK inhibitors SB203580 or RWJ 67657 to investigate effects on induction of mPR3 expression. Pre-incubation with increasing concentrations of 0.01; 0.1; 1 and 10 µM of SB203580 resulted in a significant dose-dependent inhibition (p < 0.05, n=3) of mPR3 expression on mPR3⁺ neutrophils during TNF-α / CHX-accelerated apoptosis (Figure 4). Nearly complete inhibition of mPR3 expression was achieved at concentrations of 1 µM SB203580, without decreasing
apoptosis itself as assessed by Annexin V binding (Figure 4, n=3). The more potent p38 MAPK inhibitor RWJ 67657 showed already significant inhibition (p < 0.05, n=3) of induced mPR3 expression at concentrations of 0.01 µM and nearly complete inhibition at 1 µM (Figure 4).

Figure 4. P38 MAPK-dependent upregulation of mPR3 expression during TNF-α/CHX- accelerated apoptosis. Neutrophils of bimodal mPR3 donors were pre-treated with increasing concentrations of pharmacological inhibitors of p38 MAPK or ERK, followed by culture in the presence of TNF-α (10 ng/ml) and Cycloheximide (2.5 µg/ml) for 4hrs. After 4 hrs neutrophils were stained for mPR3 expression using a mouse anti-PR3 monoclonal antibody (PR3.G-3) and Annexin V binding. MFI of anti-mouse IgG-PE staining and Annexin V-FITC staining are represented on the x and y-axis, respectively. A) Representative dot blot of neutrophils after 4 hrs in medium only B) Dot blot of TNF-α / CHX - accelerated apoptotic neutrophils after 4 hrs. C) Dot blot of TNF-α / CHX - accelerated apoptotic neutrophils pretreated with 1 µM SB203580 for 30 min. D) Dot plot of TNF-α accelerated apoptotic neutrophils pretreated with 2 µM PD98059 for 30 min. E) Dose- dependent inhibitory effect of SB203580 on mPR3 expression induced during TNF-α / CHX - accelerated apoptosis. F) Dose- dependent inhibitory effect of RWJ67657 on mPR3 expression induced during TNF-α / CHX- accelerated apoptosis. G) Lack of inhibition of 2 µM PD98059 on mPR3 expression during TNF-α / CHX - accelerated apoptosis. Results are expressed as percentages of basal mPR3 expression on TNF-α / CHX- accelerated apoptotic neutrophils. Values are given as mean ± standard error of the mean (SEM) of at least 3 independent experiments.
Next, we studied participation of ERK in the translocation of PR3 to the membrane of neutrophils during TNF-α / CHX-accelerated apoptosis. Pre-incubation of neutrophils for 30 min at 37°C with the ERK inhibitor PD98059 (2 μM) showed no significant inhibitory effect (n=3) on induced mPR3 expression during TNF-α / CHX-accelerated apoptosis (Figure 4). In these neutrophils, apoptosis was, again, not significantly reduced. Furthermore, pre-incubation with PD98059 at 50 μM (n=4) showed, also, no significant inhibitory effect (data not shown). Finally, involvement of phosphatidylinositol-3- Kinases (PI3-K) in mPR3 expression during TNF-α / CHX-accelerated apoptosis was studied. Pre-incubation of neutrophils for 30 min at 37°C with the PI3-K inhibitor LY 294002 showed significant inhibition (p < 0.05, n=3) of mPR3 expression induced during TNF-α / CHX-accelerated apoptosis at concentrations of 10 and 50 μM (Figure 5). When using another PI3-K inhibitor, Wortmannin, comparable inhibition of mPR3 expression was seen but at far lower concentrations (nanomolar range). Finally, apoptosis itself was not significantly reduced by treatment with PI3- Kinase inhibitors (data not shown).

**P38 MAPK dependent and PI3-K independent mPR3 expression during UV-B-accelerated apoptosis**

To investigate the role of the different MAPK’s and PI3-K in the induction of mPR3 expression in UV-B accelerated apoptosis, neutrophils were irradiated with UV- B light for different time points and cell extracts were blotted for phosphorylated and total p38 MAPK, ERK and JNK.

![Figure 5. PI3-K-dependent upregulation of mPR3 expression during TNF-α / CHX-accelerated apoptosis. Neutrophils of bimodal mPR3 donors were pre-treated with increasing concentrations of the pharmacological inhibitor LY294002 of PI3-K, followed by culture in the presence of TNF-α (10 ng/ml) and Cycloheximide (2.5 μg/ml) for 4hrs. After 4 hrs neutrophils were stained for mPR3 expression using a mouse anti-PR3 monoclonal antibody (PR3. G-3) and for Annexin V binding. A) Representative dot blot of mPR3 expression induced during TNF-α / CHX- accelerated apoptosis of neutrophils pretreated with 10 μM LY294002 for 30 min. MFI of anti-mouse IgG-PE staining and Annexin V-FITC staining are represented on the x and y-axis, respectively. B) Dose-dependent inhibitory effect of RWJ67657 on mPR3 expression induced during TNF-α / CHX-accelerated apoptosis. Results are expressed as percentages of basal mPR3 expression on TNF-α / CHX-accelerated apoptotic neutrophils. Values are given as mean ± standard error of the mean (SEM) of 3 independent experiments.**
Neutrophils irradiated with UV-B light showed a clear phosphorylation of p38 MAPK and ERK, but not JNK (Figure 6, data not shown for JNK). Phosphorylation of p38 MAPK and ERK was visible even after 100 minutes of culture. (Figure 6A). Dependence of mPR3 expression during UV-B accelerated apoptosis on p38 MAPK, ERK and PI3-K was examined by pharmacological inhibition of the respective proteins. Pre-incubation with 0.1 µM of the p38 MAPK inhibitor SB203580 resulted in a significant (p < 0.01, n=3) inhibition of mPR3 expression after UV-B-accelerated apoptosis (Figure 6), whereas apoptosis itself was not significantly decreased (data not shown). Although occasionally a slight tendency to reduced rates of apoptosis was observed. Also participation of ERK in mPR3 expression during UV-B-accelerated apoptosis was studied. In agreement with TNF-α-accelerated apoptosis, inhibition of ERK activation by the MEK-inhibitor PD98059 had neither a significant inhibitory effect on UV-B-induced mPR3 expression, nor on apoptosis itself (Figure 6). In contrast to TNF-α-accelerated apoptosis, pre-incubation of neutrophils with the PI3-K inhibitor LY 294002 or Wortmannin (data not shown) did not result in significant inhibition (n=3) of mPR3 expression during UV-B-accelerated apoptosis (Figure 6).

**Figure 6.** p38 MAPK independent upregulation of mPR3 expression during UV-B-accelerated apoptosis. A) Phosphorylation of p38 MAPK and ERK during UV-B-accelerated apoptosis. Human neutrophils (2.10^6/ml) were irradiated with UV-B for 5 or 10 min, or were irradiated for 20 min followed by 40 or 100 min culture at 37°C, and subsequently lysed and blotted for the respective proteins. B-D) Neutrophils of bimodal mPR3 donors were pre-treated with increasing concentrations of pharmacological inhibitors of p38 MAPK, ERK or PI3-K, followed by UV-B irradiation for 20 min and culture for 4 hrs. After 4 hrs neutrophils were stained for mPR3 expression using a mouse anti-PR3 monoclonal antibody (PR3.G-3) B) Inhibitory effect of SB203580 on mPR3 expression induced during TNF-α-accelerated apoptosis. C) Lack of inhibition of 2 µM PD98059 on mPR3 expression during TNF-accelerated apoptosis. D) Effects of increasing concentrations of LY294002 on mPR3 expression induced during UV-B-accelerated apoptosis. Results are expressed as percentages of basal mPR3 expression on UV-B-accelerated apoptotic neutrophils. Values are given as mean ± standard error of the mean (SEM) of at least 3 independent experiments.
At 10 and 50 µM of LY 294002 a tendency to increased apoptosis was seen, resulting in more leaky cells (data not shown). These leaky cells probably could have attributed to the higher levels of mPR3 expression observed at these molarities (Figure 6).

p38 MAPK dependent upregulation of mPR3 during priming

Regulatory pathways involved in induction of mPR3 expression during priming were studied by culturing neutrophils for 4 hrs at 37°C, followed by priming with TNF-α for 15 min at 37°C. 4 hrs pre-cultured neutrophils had minimal levels of mPR3 (Figure 1A), which was highly upregulated after post-culture priming with TNF-α. (Figure 7). Pre-incubation of neutrophils with the respective inhibitors SB 203580, RWJ 67657, LY 294002 and PD 98059 was done for 4 hrs during culture of the neutrophils, as well. Pre-incubation with the p38 MAPK inhibitors SB203580 or RWJ 67657 resulted in a significant reduction (p < 0.01) of mPR3 expression induced by TNF-α (Figure 7). As expected, pre-incubation of PD98059 did not result in a significant reduction on TNF-α-induced expression of mPR3. Furthermore, blocking the PI3-K activity with LY294002 did not result in a significant inhibition of mPR3 expression after TNF-α priming.

**Figure 7. p38 MAPK dependent upregulation during priming. Neutrophils of bimodal mPR3 donors were pretreated with various pharmacological inhibitors of p38 MAPK, ERK and PI-3K, and cultured for 4 hrs, followed by priming with TNF-α (10 ng/ml) for 30 min. Results are expressed as MFI (PR3) of mPR3+ cells corrected for the non-specific binding of isotype control antibody (MFI IgG1). Values are given as mean ± standard error of the mean (SEM) of 3 independent experiments. Pharmacological inhibitors were abbreviated as follows: SB203580 (SB), RWJ67657 (RWJ), LY294002 (LY) and PD98059 (PD).**

**DISCUSSION**

Expression of the autoantigen proteinase 3 (PR3) on the membrane is a prerequisite for activation of neutrophils by PR3-ANCA 8-11. Translocation of PR3 to the membrane is proposed to proceed via exocytosis of secretory vesicles, specific granules and azurophilic granules after priming or during apoptosis 17-23,25. The most widely used priming agent in vasculitis research is TNF-α, which already at low dosage induces translocation of PR3 to the membrane of neutrophils 15,20. However, next to other cytokines, also isolation procedures or even in vitro incubation of neutrophils of
neutrophils in whole blood has been recognized to result in priming \(^{25,41,42}\). In this study, we observed that, indeed, neutrophils express PR3 on their membrane after isolation procedures. However, to our surprise culturing isolated neutrophils for 4 hrs gradually reduced expression of membrane bound PR3, in contrast to CD35. These observations contrast with those of Yang et al, who observed increased mPR3 expression in time of neutrophils cultured in whole blood. The authors attributed this increase to minor trauma during in vitro incubation in whole blood \(^{25}\). However, we cultured neutrophils in FCS- enriched culture medium instead of whole blood in which neutrophils are apparently less prone to activation. Reduction in mPR3 expression was also seen for neutrophils temporarily or continuously primed with TNF-\(\alpha\).

Next to priming, apoptosis has been shown by various groups to result in translocation of PR3 to the membrane as well \(^{21-23,25}\). Gilligan et al showed that, in the absence of priming neutrophils can translocate PR3 or MPO to the membrane during ageing-related induction of apoptosis, as assessed by increased ANCA binding \(^{22}\). In another study, apoptosis in neutrophils was shown to be accelerated by using low dose of TNF-\(\alpha\) (10 ng/ml) in combination with the protein synthesis inhibitor cycloheximide (CHX). These neutrophils in which apoptosis was induced by TNF-\(\alpha\) showed increased PR3 and MPO expression compared to TNF-\(\alpha\)-primed PMN \(^{23,23}\). Here, we confirm that apoptosis is associated with an increase in mPR3 expression. However, only a fixed proportion of neutrophils from donors who are bimodal in their mPR3 expression showed induction of mPR3 expression, which is in concordance with the stability of bimodal expression for mPR3. So, also during apoptosis only a subpopulation of neutrophils, from donors bimodal in their mPR3 expression, will express PR3 on their surface. This subpopulation equals the proportion of mPR3\(^+\) neutrophils induced by priming.

As autoantigen expression on the neutrophil membrane plays a dominant role in the pathophysiology of ANCA-associated vasculitis, we searched for target regulatory molecules that control translocation of PR3 to the membrane in order to explore new therapeutic strategies. Translocation of PR3 is proposed to proceed via exocytosis. Exocytosis of granules and vesicles after stimulation with TNF-\(\alpha\) is mediated via p38 MAPK \(^{29,31-33}\). Four isoforms of p38 MAPK have been described, p38\(\alpha\), p38\(\beta\), p38\(\gamma\) and p38\(\delta\) \(^{43}\). Only p38\(\alpha\) and p38\(\delta\) are expressed in PMN \(^{44,45}\). Here, we demonstrate that translocation of PR3 during apoptosis is dependent on p38 MAPK activation, whereas apoptosis itself is not. We observed that apoptosis via combined treatment with TNF-\(\alpha\) and CHX, or by UV-B irradiation results in p38MAPK phosphorylation in neutrophils. Furthermore, blocking of p38MAPK in these apoptotic neutrophils via the pharmacological inhibitor SB203580 resulted in a clear reduction of mPR3 expression, whereas apoptosis was not reduced. However, blocking of p38MAPK only partly inhibited mPR3 expression induced by UV-B irradiation, suggesting a less pronounced role of p38MAPK in UV-B induced mPR3 expression as compared to TNF-\(\alpha\) / CHX-accelerated apoptosis. In addition, as SB203580 has been shown to be not specific at concentrations higher than 3\(\mu\)M \(^{46,47}\), we also used the more specific inhibitor RWJ 67657, which is approximately 10-fold more potent than SB203580 \(^{48}\). Both RWJ 67657 and SB203580 inhibit the enzymatic activity of recombinant p38\(\alpha\) and \(\beta\), but not of p38\(\gamma\) and p38\(\delta\) by interfering with ATP binding \(^{48,49}\). As such, phosphorylation of down-stream substrates of p38 MAPK, like MAPKAP2,
inhibited. Finally, we confirmed that priming of neutrophils with TNF-α in order to translocate PR3 to the membrane is dependent on p38 MAPK activation, which is in agreement with a study of Kettritz et al. 34,35.

Next, we demonstrated that ERK is not involved in translocation of PR3 either during priming with TNF-α or following apoptosis induced by TNF-α/CHX treatment or UV-B irradiation. While ERK was phosphorylated during priming or apoptosis, blocking ERK activation via inhibition of MEK by using the pharmacological inhibitor PD098059 showed no significant effect on mPR3 expression. This implicates that ERK is not involved in translocation of PR3 to the membrane. Various reports have confirmed that ERK is phosphorylated after TNF-α priming, but is not involved in exocytosis 31-33,50. Another MAPK, i.e. JNK, was studied for involvement in mPR3 expression as well. Also in other studies 51,52, JNK was not phosphorylated during stimulation with TNF-α. Here, we demonstrate that also during apoptosis induction by combined treatment with TNF and CHX or by UV-B irradiation, no phosphorylation of JNK can be observed (data not shown).

Phosphatidylinositol 3-kinase (PI 3-K) is a heterodimeric enzyme consisting of an 85 kDa regulatory and a 110 kDa catalytic subunit 53. It has been shown to be involved in exocytosis of granules as well 29,31,35,54. We observed that phosphatidylinositol 3-kinase (PI 3-K) is involved in the translocation of PR3 during TNF-α/CHX-accelerated apoptosis, but not in apoptosis accelerated by UV-B irradiation. Blocking PI3-K activity with the pharmacological inhibitor LY294002, which reversibly inhibits PI3-K via interactions with the ATP-binding site, resulted in an almost complete inhibition of mPR3 expression during TNF-α / CHX accelerated apoptosis. Apoptosis itself was not affected. No such effects were, however, observed during apoptosis by UV-B irradiation, implying a less pronounced role of PI3-K during UV-B induced apoptosis. Finally, we confirmed that translocation of PR3 during priming with TNF-α was independent on PI3-K activation, which is in agreement with a study of Kettritz et al 34,35. The discrepancy between PI3-K dependent mPr3 expression during TNF-α-accelerated apoptosis and PI3- K independent mPr3 expression during TNF-α-induced priming may be attributed to the differential mobilization of granules and vesicles during TNF-α priming or TNF-α-accelerated apoptosis. For comparison, upregulation of CD11b/CD18 via exocytosis of secretory vesicles by fMLP is independent of PI3-K, whereas exocytosis of specific azurophilic granules is PI3-K dependent 29. As such, translocation of PR3 during priming appears to be primarily dependent on exocytosis of secretory vesicles, whereas upregulation during TNF-α / CHX- accelerated apoptosis is more dependent on mobilization of specific granules and, to a lesser extent, of secretory vesicles.

In conclusion, both priming and apoptosis results in the expression of proteinase 3 on the cell membrane, and the same subpopulation of neutrophils in bimodal donors seems to be involved. In addition, mPR3 expression is transient after priming and induced expression of mPR3 can be blocked by pharmacological inhibitors interfering with p38 MAPK and, dependent on the type of stimulation, PI3-K activity. Furthermore, induced expression of mPR3 was independent of ERK and JNK activity. As autoantigen expression plays a dominant role in the pathophysiology of ANCA-associated vasculitis, pharmacological inhibition of p38 MAPK (or PI3-K) may be an effective therapeutic strategy in the management of ANCA-associated vasculitis.
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