Nephrol Dial Transplant (2013) 28: 421–429
doi: 10.1093/ndt/gfs454
Advance Access publication 9 December 2012

The uraemic toxin phenylacetic acid contributes to inflammation by priming polymorphonuclear leucocytes

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Abstract

Background. The activation of polymorphonuclear leucocytes (PMNLs) causes inflammation and as a result cardiovascular disease, which is a main risk factor for increased morbidity and mortality in patients with chronic kidney disease. Toxins accumulating in uraemic patients play a major role in modulating essential PMNL functions and apoptosis, the latter being crucial for a coordinated resolution of inflammation. One uraemic toxin is phenylacetic acid (PAA). We therefore investigated whether PAA contributes to the deranged immune response in uraemia by modulating PMNL activities.

Methods. PMNL oxidative burst, phagocytosis and surface expression of the activation markers CD11b and CD18 were measured by flow cytometry in whole blood from healthy subjects in the presence and absence of PAA. Spontaneous apoptosis of isolated PMNLs was assessed by evaluating morphological features under the fluorescence microscope and by measuring the DNA content by flow cytometry. PMNL chemotaxis was tested by the under-agarose method.

Results. PAA significantly enhanced the stimulation of PMNL oxidative burst by *Escherichia coli*, phagocytosis of *E. coli* by PMNLs and the expression of CD11b and CD18 at the PMNL surface. PAA significantly decreased PMNL apoptosis resulting in an increased percentage of viable cells. PAA affected neither the oxidative burst stimulated by phorbol-12-myristate-13-acetate nor PMNL chemotaxis.

Conclusions. PAA increases the activation of various PMNL functions and the expression of surface activation markers, while it attenuates PMNL apoptotic cell death. Therefore, PAA may contribute to the inflammatory state and consequently to increased cardiovascular risk in uraemic patients.

Keywords: apoptosis; oxidative burst; phagocytosis; polymorphonuclear leucocytes; uraemic toxin

Introduction

Cardiovascular disease and infections are the main risk factors leading to increased morbidity and mortality in patients with chronic kidney disease (CKD) [1]. Both complications are directly or indirectly linked to a compromised immune defence. Disturbed functions of polymorphonuclear leucocytes (PMNLs) play a key role in the defective non-specific immune response in CKD [2]. PMNLs, cells of the first-line non-specific immune defence, migrate to the site of infection along a chemotactic gradient, ingest the invading microorganisms by phagocytosis and kill them with proteolytic enzymes and toxic oxygen radicals produced during the oxidative burst. Their normal response can be impaired giving rise to infectious diseases or can be pre-activated/primed leading to inflammation and consequently to cardiovascular disease. Whereas the coordinated removal via apoptosis of activated immune cells is crucial for the resolution of inflammation [3, 4], inappropriately high apoptotic rates lead to a diminished immune response.

Various compounds usually excreted by the kidneys are retained in uraemic patients. One uraemic toxin is phenylacetic acid (PAA). It was not detected by NMR spectroscopy in the plasma of healthy subjects but in the plasma of CKD patients [5]. PAA in patients with end-stage renal disease (ESRD) inhibits the expression of inducible nitric oxide synthase (iNOS) in mononuclear leucocytes [5]. Total plasma PAA level consists of a protein-bound fraction and an unbound biologically active fraction. In ESRD patients, total and free plasma concentrations of PAA were found to be 3.49 ± 0.33 and 2.5 ± 0.5 mM, respectively [5]. PAA is the major metabolite of the neurotransmitter phenylethylamine and therefore also studied in psychiatry. Of note, PAA has been detected in plasma samples of schizophrenic patients and also in plasma samples of patients with various neurological diseases [6, 7]. However, in both studies, no information about the kidney function of those patients was provided.

PAA impairs macrophage functions such as the intracellular killing of bacteria, as well as lipopolysaccharide (LPS)/interferon-γ-induced iNOS expression in a murine macrophage cell line [8].

Uraemic retention solutes have been classified by a European expert panel [9–12]. One potential uraemic

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toxin is PAA. Several uraemic retention solutes have already been studied and shown to affect essential PMNL functions [13–20]. In contrast, so far no study has addressed the potential effects of the uraemic toxin PAA on PMNLs. *In vitro* assays testing the biological effects of uraemic retention solutes represent a straightforward way to identify uraemic toxins and to choose candidates for further in-depth analysis [21]. Thus, we investigated whether PAA affects PMNL functions such as chemotaxis, phagocytosis, oxidative burst and the expression of surface adhesion markers, and also whether PAA affects spontaneous PMNL apoptosis using whole blood samples or isolated PMNLs from healthy subjects.

Materials and methods

Materials

Giemsa was from Merck (Darmstadt, Germany), Ficoll-Hypaque from GE Healthcare (Uppsala, Sweden) and Hank's buffered saline from Gibco (Paisley, UK). PAA and all other chemicals were from Sigma-Aldrich, St Louis, MO, unless otherwise indicated. The PAA stock solution was prepared as follows: PAA was dissolved in ethanol to obtain a 2 M PAA solution. The pH value of this stock solution was adjusted to 7.25 with NaOH dissolved in phosphate-buffered saline (PBS, pH 7.4; Gibco). The final assay concentration of ethanol was 0.5 v/v % and was used as control in all assays.

LPS assay

Previous unpublished experiments from our laboratory showed that PMNL surface expression of adhesion markers are increased in the presence of 1 EU/mL LPS. Therefore, we tested PAA for potential LPS contamination.

To check the LPS content of the samples, we used the Limulus Amebocyte Lysate assay QCL-1000™ (Lonza, Walkersville, MD) which utilizes a modified Limulus Amebocyte Lysate and a colour producing substrate to detect endotoxin. No endotoxin was detected in the samples.

Blood donors

There were 21 healthy male and 16 healthy female blood donors with average ages of 40.3 ± 2.2 and 40.6 ± 2.3 years, respectively. Informed consent was given by all donors. People taking medication or having infection or inflammation were excluded. The blood donors had no renal, psychiatric or neurological disease.

Oxidative burst

PMNL oxidative burst was measured in whole blood with the Bursttest® (Opregen Pharma, Heidelberg, Germany). Ten microlitres of PAA stock or control solution were added to 90 µL heparinized blood and incubated for 15 min at 37°C. After stimulation with opsonized *Escherichia coli* (Opregen Pharma) at a final concentration of 1.7×10^8 cells/mL, with phorbol-12-myristate-13-acetate (PMA; Opregen Pharma) at a final concentration of 1.35×10^{-6} M or with formyl-methyl-leucyl-phenylalanine (fMLP; Opregen Pharma) at a final concentration of 8.3×10^{-7} M, the incubation was continued for another 10 min and PMNLs were exposed to the fluorogenic substrate dihydrorhodamine 123 for exactly 10 min at 37°C. Erythrocytes were lysed for 20 min at room temperature with lysis buffer (Opregen Pharma). PMNLs were identified by their forward/sideward scatter properties. The conversion of dihydrorhodamine 123 to fluorescent rhodamine 123 was quantified by flow cytometry (Epics XL-MCL; Coulter, Hialeah, FL) and measured as mean fluorescence intensity (MFI).

Phagocytosis

PMNL phagocytosis was measured using the Phagotest® (Opregen Pharma). To 90 µL heparinized whole blood, 10 µL PAA stock or control solution was added and incubated for 15 min at 37°C. After addition of fluorescein isothiocyanate (FITC)-labelled opsonized *E. coli* (Opregen Pharma), the incubation was continued for exactly 10 min at 37°C. *Escherichia coli* uptake was stopped by putting the samples on ice. Adding quenching solution (Opregen Pharma) allowed the discrimination between attachment and internalization of bacteria by quenching the FITC fluorescence of surface-bound bacteria leaving the fluorescence of internalized particles unaltered. Erythrocytes were lysed for 20 min at room temperature. The percentage of PMNL that had been taken up and the amount of ingested *E. coli* per PMNL measured as MFI in the absence and presence of PAA was determined by flow cytometry (Epics XL-MCL).

Expression of CD11b and CD18 on PMNL surface

To 90 µL whole blood anti-coagulated in 10 mL Lithium Heparin Vacutainer tubes (Vacuette®, Greiner bio-one, Kremsmünster, Austria), 10 µL of a PAA stock or control solution was added and incubated at 37°C for 60 min. Fluorescently labelled antibodies (PC5-anti-CD11b, ECD-anti-PE-anti-CD18; Coulter International Corp.) were added and the samples incubated for 10 min at room temperature. After lysis of erythrocytes using the automatic sample processing ImmunoPrep Reagent System (Beckman Coulter, Inc.) and Multi-Q-Prep (Coulter International Corp.), the samples were immediately analysed. The flow cytometry was performed on an Epics XL-MCL (Coulter International Corp.). PMNLs were gated using the forward/sideward scatter characteristics.

Isolation of PMNLs

As previously described [17], PMNLs were isolated from heparinized blood of healthy volunteers by discontinuous Ficoll–Hypaque density gradient centrifugation and hypotonic lysis of erythrocytes and finally suspended in PBS. The viability of the PMNLs obtained by this protocol was >95% as determined under the fluorescence microscope.

Apoptosis

Incubations. PMNLs isolated under sterile conditions were incubated at 6×10^6 cells/mL at 37°C for 20 h in PBS supplemented with 100 U/mL penicillin–streptomycin (GibcoBRL).

Morphological features. After mixing the cell suspension with the fluorescent DNA-binding dyes ethidium bromide (GibcoBRL) and acridine orange (Merck) at a final concentration of 5 µg/mL each, PMNLs were examined under the fluorescence microscope. Acridine orange binds to DNA and appears green. PMNLs with a damaged plasma membrane take up ethidium bromide staining DNA orange to a stronger extent. DNA in non-apoptotic cells is structured within the nucleus, while DNA in apoptotic cells is condensed. Therefore, viable non-apoptotic (green, structured nucleus), apoptotic (green, condensed nucleus) and secondary necrotic (orange, condensed nucleus) cells can be observed.

Analysis of the DNA content by flow cytometry. As a result of DNA cleavage by an activated nuclease, apoptotic cells have a lower DNA content. PMNLs (1.2×10^6/200 µL) were centrifuged at 360*g* for 10 min and washed with PBS. To the pellet, 250 µL ice-cold 70% ethanol was added. After 60 min incubation on ice, centrifugation and washing with PBS, PMNLs were resuspended in 200 µL PBS containing 250 µg/mL RNAse (type I-A; Sigma, St Louis, MO) and 50 µg/mL propidium iodide (Sigma). Samples were kept on ice in the dark until flow cytometric analysis.

Data presentation. Our data are presented as the percentage of viable PMNLs. Apoptotic PMNLs are in a stage between viability and secondary necrosis. Under *in vivo* conditions, apoptotic PMNLs would be readily phagocytozed. Therefore, viable PMNLs are most important for the interpretation of our results.

Activity of intracellular kinases

Incubations. PMNLs (3×10^6 in 100 µL) were incubated in the presence of PAA or control at 37°C for 5 min. Twenty microlitres of PBS or stimulus were added and incubated for 5 min. For PMA, the final concentration was 1.35 µM and for *E. coli* (from the Bursttest® kit) 1.2 to 2.4×10^8 cells/mL.

Preparation of extracts. PMNLs were suspended in 125 µL lysis buffer [20 mM Tris, pH7.5; 150 mM NaCl; 1 mM ethylene diamine tetraacetic acid; 1 mM ethylene glycol tetraacetic acid, 1%(v/v) Triton-X100; 2.5 mM sodium pyrophosphate; 1 mM Na_3VO_4; 1 µg/mL leupeptin; 1 mM phenyl-methyl sulphonyl fluoride]. After addition of protease inhibitor cocktail (6.25 µL), cells were incubated on ice for 5 min and sonicated

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four times for 5 s each. PMNL extracts were centrifuged (4°C, 10 s, 10 000*g*), and the supernatant was used for western blotting.

Western blotting. A half volume of 3× sample buffer [187 mM Tris, pH6.8; 6% (v/v) sodium dodecyl sulphate (SDS); 0.03% (w/v) bromphenol blue; 7.5% (v/v) β-mercapto ethanol] was added to the lysate. Proteins were then separated by SDS–polyacrylamide gel electrophoresis (GE Healthcare) and electrotransfered to a nitrocellulose membrane. Polyclonal rabbit anti-p38 mitogen-activated protein kinase (MAPK), anti-phospho-p38 MAPK, anti-p44/42 kinase and anti-phospho-p44/42 kinase (Cell Signaling Technology Inc., Beverly, MA) were used as primary antibodies and detected by a horseradish peroxidase-labelled goat-anti-mouse antibody and the enhanced chemiluminescence detection system (GE Healthcare). The intensity of the bands was quantified as MFI with the Personal Densitometer SI (Molecular Dynamics) and the image master 1D software (Amersham Biosciences).

Enzyme-linked immunosorbent assay. Endogenous phosphorylated and total c-Jun N-terminal kinase (JNK) in PMNL lysates were detected by an enzyme-linked immunosorbent assay (ELISA; Cell Signaling Technology, Inc.). Microtitre plates were precoated with a mouse monoclonal antibody specific for phosphorylated and total JNK, respectively. JNK in the lysates was bound to the immobilized antibody. After washing away unbound substances, a rabbit antibody specific for phosphorylated or total JNK conjugated to horseradish peroxidase was added. Unbound reagent was washed away and the substrate tetramethylbenzidine was added. After stopping the colour development with sulphuric acid, the OD was quantified with a microplate reader (Anthos Reader HT 3; Anthos Labtec Instruments; Wals-Siezenheim; Austria) at 450 nm.

Chemotaxis

PMNL chemotaxis was determined by the under-agarose method [17]. Formyl-methyl-leucyl-phenylalanine fMLP dissolved in PBS at a final concentration of 4×10^{-7} M was used as chemoattractant. Five × 10^5 PMNLs were resuspended in 10 μL control or PAA solution. After incubation for 2 h at 37°C, the cells were fixed with methanol and paraformaldehyde and stained with Giemsa (Merck). The distance migrated under the agarose was measured under the microscope.

Statistical analysis

The Wilcoxon matched-pair signed-rank test was used to analyse data from at least six independent experiments. When less than six independent experiments were performed, data were analysed by paired two-tailed *t*-test. Data are presented as mean values ± standard error of the mean (SEM).

Results

PAA enhances the stimulation of PMNL oxidative burst by E. coli

The effect of PAA on the oxidative burst was tested in whole blood obtained from healthy subjects. PAA is not detectable in serum samples of healthy subjects. Therefore, different PAA concentrations were added to whole blood samples obtained from healthy persons [5]. PAA alone did not affect the basal production of reactive oxygen species (Table 1). Pre-incubation of whole blood with PAA significantly increased the subsequent stimulation of PMNL oxidative burst induced by *E. coli* relative to the basal levels (Figure 1). Already a final level of 2.5 mM, the biologically active concentration of PAA measured in ESRD patients [22], leads to this priming effect. A further increase in PAA levels (5 or 10 mM) resulted in an additional dose-dependent increase in oxidative burst stimulation by *E. coli*. When PMA was used as a stimulant, no priming by PAA was observed within the concentration range tested (data not shown). fMLP did not stimulate the PMNL oxidative burst, neither in the absence nor in the presence of PAA (data not shown).

Table 1. Effect of PAA on the basal activity of PMNL oxidative burst expressed as MFI and the percentage (values in the absence of PAA set as 100%)

	PAA (mM)			
	0	2.5	5	10
MFI	0.57 ± 0.04	0.54 ± 0.04	0.54 ± 0.03	0.54 ± 0.02
Percentage	100 ± 0	96 ± 2	95 ± 2	96 ± 3

n = 9; mean values ± SEM.

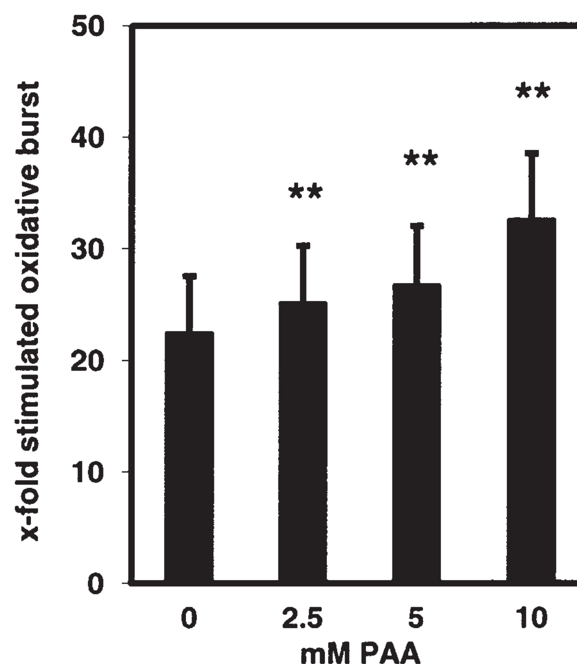

Fig. 1. PAA enhances the stimulation of PMNL oxidative burst by *E. coli*. PAA was added to whole blood from healthy persons to final concentrations of 2.5, 5 and 10 mM. The degree of stimulation relative to the basal levels (absence of any stimulus) calculated from the MFIs is shown. Nine independent experiments were performed. **P < 0.01 versus 0 mM PAA, mean values ± SEM.

PAA increases the phagocytosis of E. coli by PMNLs

We measured the percentage of PMNL taking up *E. coli* bacteria by phagocytosis and the amount of *E. coli* taken up per PMNL expressed as MFI. 95.1 ± 0.7% PMNLs took up *E. coli* and there was no significant change when PAA was added (data not shown). However, the number of *E. coli* taken up per PMNL was increased in the presence of 2.5 mM PAA (Figure 2). A further increase in PAA concentration did not lead to a higher *E. coli* uptake.

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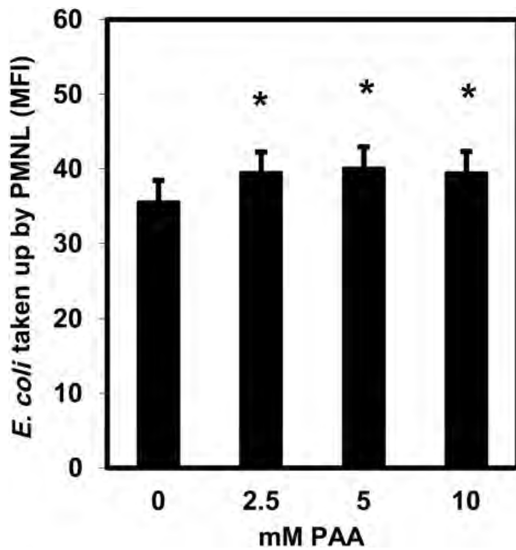

Fig. 2. PAA increases the phagocytosis of *E. coli* by PMNLs. PAA was added to whole blood from healthy persons to final concentrations of 2.5, 5 and 10 mM. The uptake of FITC-labelled opsonized *E. coli* by PMNLs is shown as MFI. Seven independent experiments were performed. *P < 0.05 versus 0 mM PAA, mean values ± SEM.

PAA increases the expression of CD11b and CD18 on PMNLs

Addition of PAA to whole blood significantly increased the expression of the activation markers CD11b (Figure 3A) and CD18 (Figure 3B) on the surface of PMNLs.

PAA increases PMNL viability by reducing spontaneous apoptosis

Already a final PAA concentration of 1.25 mM, half of the biologically active concentration observed in ESRD patients, reduced spontaneous PMNL apoptosis and as a result increased the viability of PMNLs as determined by evaluating morphological features (Figure 4A) and by measuring the DNA content (Figure 4B). This suggests that this effect may occur already in early stages of CKD with a small rise in serum PAA concentration.

PAA does not affect the activation of p38 MAPK, p44/42 MAPK and JNK

We assessed the activation of the intracellular kinases p38 MAPK and p44/42 MAPK by measuring their degree of phosphorylation via western blotting with specific antibodies. *Escherichia coli* and PMA significantly induced the activation of p38 MAPK (Figure 5A and B) and of p44/42 MAPK (Figure 6A and B). Pre-incubation of PMNLs with 2.5 mM PAA did not lead to a change in stimulation by *E. coli* or PMA. The total amount of p38 MAPK (Figure 5) and of p44/42 MAPK (Figure 6) was not affected by *E. coli*, PMA or PAA.

In contrast to PMA, *E. coli* did not induce an activation of JNK in PMNLs (data not shown). Incubation of the

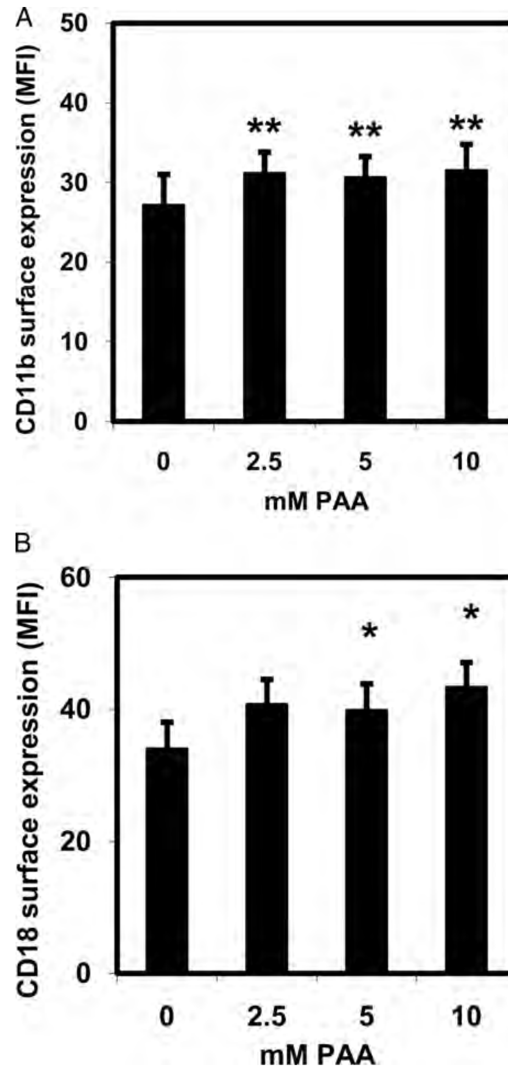

Fig. 3. PAA increases the expression of CD11b and CD18 on PMNLs. PAA was added to whole blood from healthy persons to final concentrations of 2.5, 5 and 10 mM. The expression of CD11b (**A**) and CD18 (**B**) on the PMNL surface is shown as MFI. Seven independent experiments were performed. *P < 0.05 and **P < 0.01 versus 0 mM PAA, mean values ± SEM.

cells in the presence of PAA also had no effect on the phosphorylation status of JNK protein (data not shown).

PAA does not affect PMNL migration

At final concentrations of up to 10 mM, PAA had no effect on random PMNL movement (chemokinesis) and directed migration towards fMLP (chemotaxis; data not shown).

Discussion

PAA, a partially protein-bound low-molecular weight substance, accumulates in the blood of patients with kidney failure [5, 23, 24]. We tested whether PAA affects essential functions of PMNLs and thereby contributes to the deranged immune defence in CKD patients. PAA

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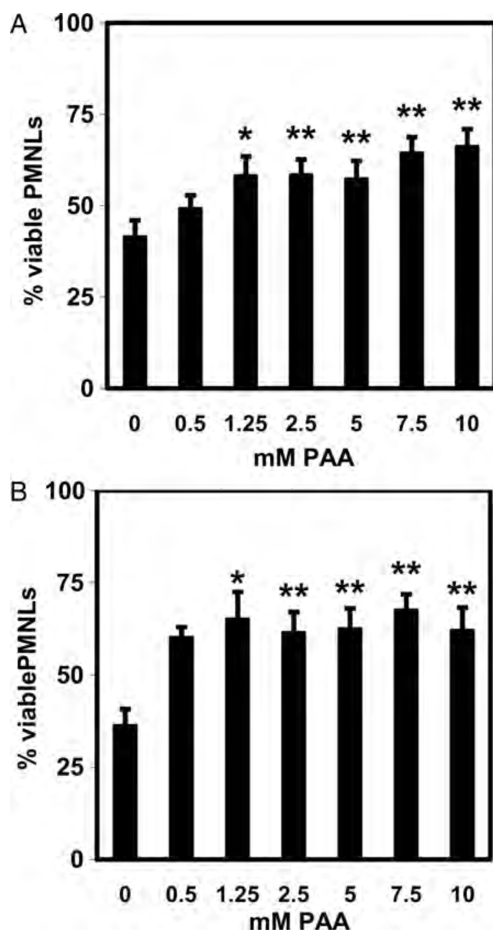

Fig. 4. PAA increases PMNL viability by reducing spontaneous apoptosis. PMNLs isolated from healthy subjects were incubated for 20 h in the absence of PAA (0 mM) or at final PAA concentrations of 0.5, 1.25, 2.5, 5, 7.5 and 10 mM. The percentage of viable PMNLs was assessed by measuring morphological features (**A**) and the DNA-content (**B**). Six to 8 independent experiments were performed. $^*P < 0.05$ and $^{**}P < 0.01$ versus 0 mM PAA, mean values ± SEM.

enhanced the activation of the oxidative burst, phagocytosis of bacteria and surface expression of β2-integrins. PAA also increased the viability of those primed PMNLs and therefore PAA may contribute to the inflammatory state in CKD and as a consequence to cardiovascular complications. One could argue that the observed effects, even though statistically significant, are only relatively small and of little biological relevance. However, the concerted action of several uraemic toxins leads to a more pronounced effect on essential functions than caused by the increased concentration of a single compound.

Patients with higher evidence of leucocyte activation have increased mortality rates [25] and *in vivo* cell preactivation is a risk factor for cardiovascular diseases [26]. In CKD patients, immunodeficiency coexists with the activation of immune cells contributing to chronic inflammation [27, 28]. The disturbed non-specific immune system in CKD is mainly a consequence of deranged PMNL functions [29–31]. Uraemic toxins, substances that accumulate in CKD patients play a pivotal role in disturbing PMNL activities [17–20, 32, 33].

The newly identified uraemic toxin PAA inhibits iNOS of mononuclear leucocytes [5]. PAA was not detectable in the plasma of healthy people [5]. Of note, PAA, the major metabolite of the neuromodulator phenylethylamine, has been previously detected in schizophrenic patients and subjects with neurological diseases [6, 7]. Plasma levels of total PAA in ESRD patients are 3.49 ± 0.33 mM. As a result of protein binding, the free, biologically active concentration is 2.44 mM [5]. During haemodialysis, only one-third of PAA is removed [23]. PAA is also not sufficiently eliminated by haemofiltration because of protein binding [34] and as a result of fast refilling of the vascular compartment from the extravascular space [24]. In the present study, we tested the *in vitro* effect of PAA on PMNL functions at concentrations previously shown to impair macrophage functions [8].

The priming of leucocytes controls host defence responses leading to a continuum of activation states [35]. During priming, the functional response to a stimulus is amplified by previous exposure to a priming agent. An inappropriate extent of priming contributes to inflammation. PMNL priming, a key mediator of low-grade inflammation and oxidative stress in CKD patients, is observed already before the onset of renal replacement therapies [36]. We showed that PAA primes the stimulation of PMNL oxidative burst by *E. coli* and therefore may contribute to inflammation in CKD. Pre-treatment with PAA not only evoked an increased PMNL oxidative burst after stimulation by *E. coli* but also increased the uptake of *E. coli* by phagocytosis. In contrast, the phagocytic activity of the murine macrophage cell line was not significantly affected by PAA [8].

Adhesion of circulating PMNLs via β2-integrin expression to activated vascular endothelial cells is a first step in the pathogenesis of inflammatory conditions leading to vascular damage [37, 38] and an increased risk of atherosclerosis [39]. PMNLs of CKD patients show a high CD11b expression [40] contributing to the pre-activation of the immune system [41]. PMNLs of patients with mild CKD already have already an altered PMNL integrin pattern reflecting a state of PMNL activation related to the high incidence of cardiovascular events in CKD [42]. Our results show that PAA increases the expression of the activation markers CD11b and CD18 on PMNLs and may therefore account for the inflammatory state of CKD patients.

While PMNLs contribute to an efficient defence against infections, any discharge of PMNL cytotoxic products into the extracellular space leads to a prolonged inflammation and tissue destruction [43]. Therefore, the coordinated removal of PMNLs via apoptosis is important for the resolution of inflammation [44]. We previously identified and characterized free Ig-light chains [16] and hydroxy-hippuric acid [45] as PMNL apoptosis-attenuating uraemic toxins [18]. In the present study, we found that also PAA significantly reduces spontaneous PMNL apoptosis (Figure 4). Increasing the viability of primed PMNLs may represent another effect of PAA contributing to inflammation in CKD.

Coxon *et al*. [46] previously showed that pro-apoptotic effects of CD11b/CD18 activation may be mediated by

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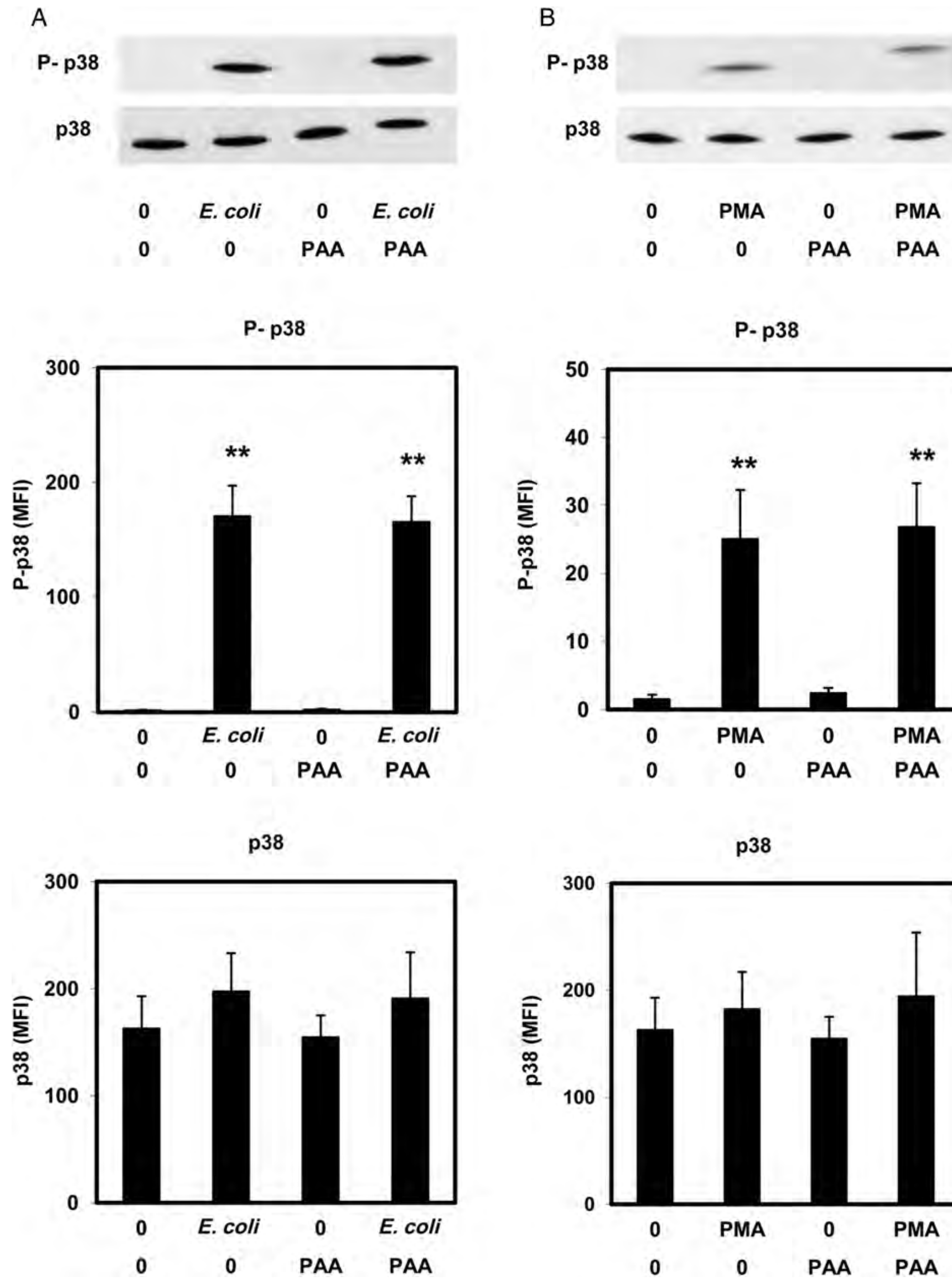

Fig. 5. PAA does not affect the activation of p38 MAPK. PMNLs from healthy subjects were incubated in the presence of 2.5 mM PAA or buffer alone and in the absence or presence of *E. coli* (**A**) or PMA (**B**). The amount of total p38 MAPK (p38) and of phosphorylated p38 MAPK (P-p38) is shown as MFI. Representative western blots are displayed. Four independent experiments were performed. $*P < 0.05$ versus unstimulated cells in the absence of PAA, mean values ± SEM.

oxidants in PMNLs. Our data indicate that increased CD11b/CD18 expression and enhanced stimulation of the oxidative burst by *E. coli* goes along with reduced PMNL apoptosis rate in the presence of PAA. There are however several differences in the experimental setup. Coxon *et al*. [46] analysed the basal activity of the oxidative burst of PMNLs from CD11b/CD18-deficient mice. We used an *in vitro* assay to test the stimulation of the oxidative burst by *E. coli*. In contrast to our experiments, Coxon *et al*. used PMNLs transmigrated to the peritoneal cavity. PMNLs transmigrated through endothelial monolayers have modulated functions and a different tendency to undergo apoptosis [47]. Furthermore, there is no difference in apoptosis rates between normal and NADPH oxidase-deficient PMNLs, arguing against a role of oxygen radicals in PMNL apoptosis [48].

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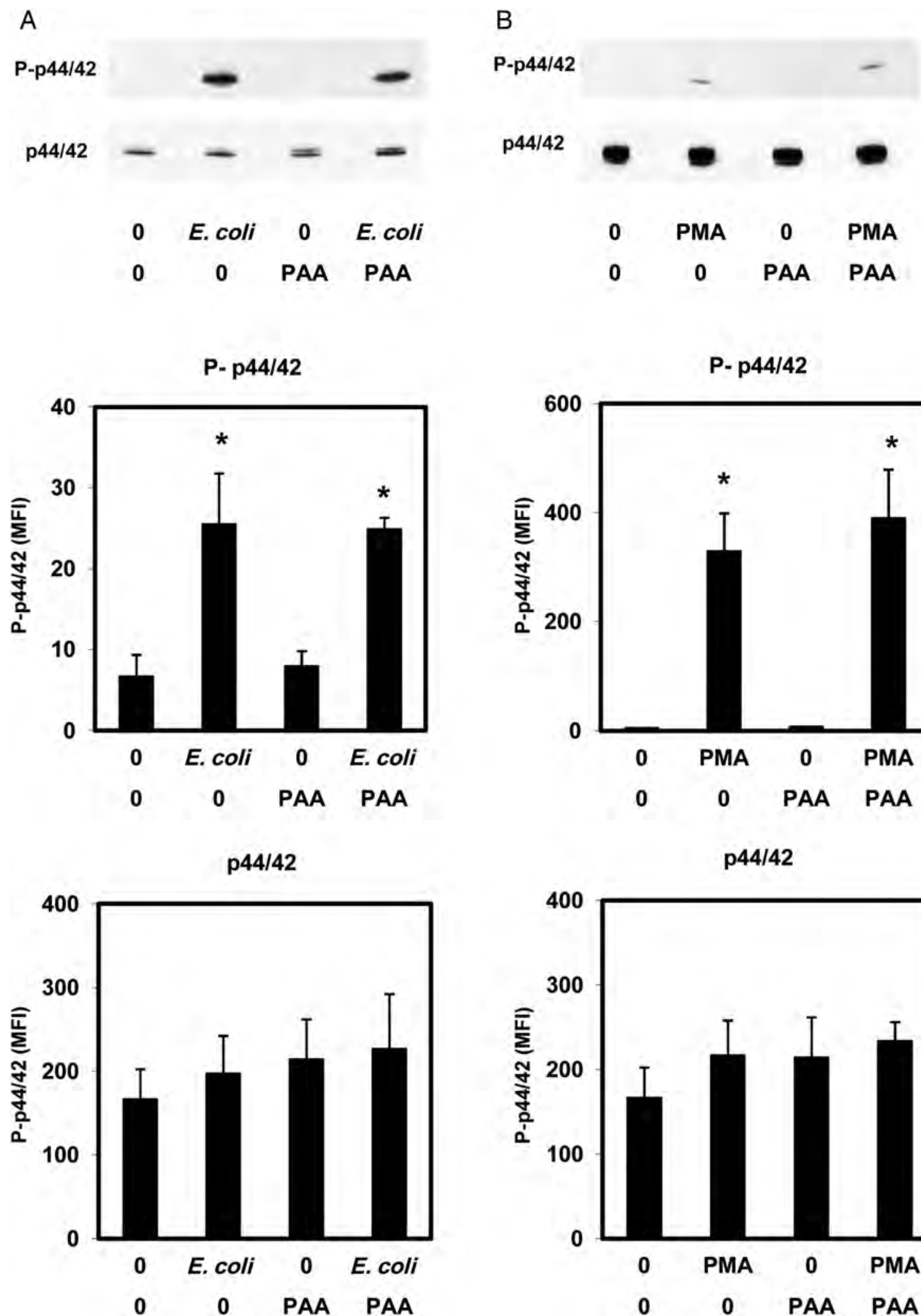

Fig. 6. PAA does not affect the activation of p44/42 MAPK. PMNLs from healthy subjects were incubated in the presence of 2.5 mM PAA or buffer alone and in the absence or presence of *E. coli* (**A**) or PMA (**B**). The amount of total p44/42 MAPK (p44/42) and of phosphorylated p44/42 MAPK (P-p44/42) is shown as MFI. Representative western blots are displayed. Four independent experiments were performed. *P < 0.05 and **P < 0.01 versus unstimulated cells in the absence of PAA, mean values ± SEM.

MAPK pathways participate in signal transduction in many leucocyte effector functions. In the murine macrophage cell line RAW 264.7, p44/42 MAPK and JNK activation was blocked by PAA, whereas p38 MAPK activation was not affected [8]. We found that in human PMNLs, the activation of p38 MAPK (Figure 5) and p44/42 MAPK (Figure 6) by *E. coli* or PMA was not altered by PAA, suggesting that the corresponding pathways are not affected by PAA. The activation of PMNL oxidative burst by PMA, a direct activator of protein kinase C, was not influenced by PAA, indicating that the target of PAA action is upstream or independent of protein kinase C.

In line with reports showing that JNK plays a minor role in PMNLs, JNK was not activated by *E. coli*. JNK activation is unnecessary to induce cell migration in human PMNLs [49], differentiation of HL-60 cells toward the PMNL phenotype results in a loss of JNK activation

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[50] and LPS activates JNK only in an adherent PMNL system but not in suspended PMNLs [51].

Whereas PAA has been identified as an inhibitor of iNOS in a murine macrophage cell line [5], *in vitro*-stimulated macrophages from normal donors do not express iNOS [22]. On the other hand, macrophages from infected or inflamed patients do express iNOS. Similarly, iNOS has only been detected in PMNLs from urine of patients with urinary tract infections [52]. Accordingly, we did not detect iNOS expression in unstimulated or activated PMNLs (unpublished observation) because we used human PMNLs from healthy, uninfected donors.

Except for the activation of the oxidative burst, we did not observe a dose dependency at PAA concentrations above 2.5 mM, implying that the pathways mediating the effects on phagocytosis, CD11b/CD18 surface expression and apoptosis are fully activated at this PAA concentration. This does not exclude the possibility that other substances act in a synergistic manner with PAA via different pathways.

In conclusion, the uraemic toxin PAA increases the activation of the PMNL oxidative burst, phagocytosis and surface expression of the activation markers CD11b and CD18, while it attenuates PMNL apoptotic cell death. As a consequence, PAA gives rise to the prolonged survival of primed PMNLs and thereby contributes to the inflammatory state observed in patients with CKD, leading to an increased risk for cardiovascular diseases.

Acknowledgements. This study was made possible by an unrestricted grant from Baxter Healthcare GmbH. The authors acknowledge the European Uraemic Toxin (EUTox) Work Group, a group of European researchers involved in studies and reviews related to uraemic toxicity. EUTox was created under the auspices of the European Society for Artificial Organs and is composed by 20 research groups throughout Europe. The authors thank Dr Anthony Winiski for critically revising the manuscript.

Conflict of interest statement. None declared.

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Received for publication: 15.12.2011; Accepted in revised form: 6.8.2012

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