The uraemic retention solute para-hydroxy-hippuric acid attenuates apoptosis of polymorphonuclear leukocytes from healthy subjects but not from haemodialysis patients

Gerald Cohen, Jana Raupachova, Thomas Wimmer, Robert Deicher and Walter H. Hörl

Division of Nephrology and Dialysis, Department of Medicine III, Medical University of Vienna, Austria

Abstract

Background. Disturbed polymorphonuclear leukocyte (PMNL) apoptosis contributes to the dysregulation of the non-specific immune system in uraemia. Intracellular Ca\(^{2+}\) modulates PMNL apoptotic cell death. We investigated the effect of para-hydroxy-hippuric acid (PHA), an erythrocyte plasma membrane Ca\(^{2+}\)-ATPase inhibitor accumulating in uraemic sera, and of cyclopiazonic acid (CPA), an inhibitor of the sarko/endoplasmatic Ca\(^{2+}\)-ATPase, on PMNL apoptosis.

Methods. Apoptosis of PMNLs from healthy subjects and from haemodialysis (HD) patients was assessed after incubation for 20 h by evaluating morphological features under the fluorescence microscope and by measuring the DNA content and caspase activities by flow cytometry. The intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) was determined by measurement of fura-2 fluorescence using the 340/380 nm dual wavelength excitation.

Results. Spontaneous apoptosis of PMNLs from healthy subjects and from HD patients did not differ. PHA significantly attenuated, while CPA increased, the apoptotic cell death of PMNLs from healthy subjects. The PHA effect was not observed with PMNLs from HD patients, irrespective of whether the blood was drawn before or after HD treatment. Baseline [Ca\(^{2+}\)]\(_i\) was increased in PMNLs obtained from HD patients before dialysis but reversed after dialysis. The PHA effects were not mediated via [Ca\(^{2+}\)]. The chemotactic peptide N-formyl-methionyl-L-leucyl-L-phenylalanine (fMLP) induced a [Ca\(^{2+}\)]\(_i\) increase and reduced PMNL survival. Extracellular Ca\(^{2+}\) did not affect CPA- and fMLP-induced apoptosis.

Conclusions. PHA, without affecting [Ca\(^{2+}\)], attenuates apoptosis of healthy but not of uraemic PMNLs. CPA and fMLP enhance PMNL apoptosis independently of Ca\(^{2+}\) influx.

Keywords: apoptosis; haemodialysis; intracellular calcium; polymorphonuclear leukocytes; uraemia

Introduction

Killing of invading microorganisms by polymorphonuclear leukocytes (PMNLs) is crucial for a successful immune response. Patients with end-stage renal disease (ESRD) have a higher risk of infection and consequently an increased incidence of morbidity and mortality. The dysregulation of the non-specific immune system in uraemia is mainly caused by disturbed PMNL functions [1–3]. This immuno-deficiency coexists with the activation of immuno-competent cells contributing to a chronic inflammatory state [4,5]. Therefore, the coordinated removal of apoptotic PMNLs is important for the resolution of inflammation [6,7]. Substances accumulating in the plasma of ESRD patients interfere with PMNL functions and apoptosis. A number of those uraemic toxins have been purified and characterized by our group [8–12].

Para-hydroxy-hippuric acid (PHA), an inhibitor of the erythrocyte plasma membrane Ca\(^{2+}\)-ATPase [13], accumulates in sera of ESRD patients and can be reduced by 53% by dialysis treatment [13]. PHA is not detectable in sera of healthy subjects.

Ca\(^{2+}\)-ATPases restore Ca\(^{2+}\) homeostasis after cell activation by keeping the Ca\(^{2+}\) concentration in the cytosol ([Ca\(^{2+}\)]\(_c\)) low. The plasma membrane Ca\(^{2+}\)-ATPase pumps Ca\(^{2+}\) out of the cell; the endomembrane Ca\(^{2+}\)-ATPase sequesters Ca\(^{2+}\) into intracellular stores [14,15]. Therefore, inhibition of Ca\(^{2+}\)-ATPases results in increased [Ca\(^{2+}\)]. Increases in [Ca\(^{2+}\)] modulate various PMNL functions [16] such as oxidative burst and degranulation as well as PMNL apoptosis [17].

In this study, we investigated the effect of PHA on spontaneous apoptosis of PMNLs. Whereas previous studies used cells from healthy subjects [9–12,18–21], we performed our experiments with PMNLs isolated from healthy donors and from uraemic patients undergoing regular haemodialysis (HD) treatment. We also tested...
the effect of cyclopiazonic acid (CPA), an inhibitor of the sarko/endoplasmatic Ca\textsuperscript{2+}-ATPase, and of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), a chemotactic peptide that evokes a sudden increase in [Ca\textsuperscript{2+}].

**Subjects and methods**

**Materials**

PHA was purchased from Bachem AG (Bubendorf, Switzerland), CPA from Alexis Biochemicals (Lausen, Switzerland), fMLP from Sigma-Aldrich (St. Louis, MO, USA), ethyleneglycol-bis(2-aminoethylether)\textsuperscript{N,N'}-tetraacetic acid (EGTA) from Serva Feinbiochemica (Heidelberg, Germany) and fura-acetoxymethyl ester (fura-AM) from Molecular Probes (Eugene, OR, USA). PHA and CPA were dissolved in methanol. The final methanol concentration was 0.1 and 0.2% (v/v), respectively.

**Patients**

A written informed consent was obtained from nine patients undergoing regular HD treatment without any sign of infection and not receiving medications affecting their plasma calcium levels. They were on dialysis for 71.3 ± 32.2 months [mean value ± standard error of the mean (SEM); range 20.5–340.3 months]. The Kt/V was 1.31 ± 0.05. Six patients were dialysed with polysulphone and three with nitrocellulose triacetate membranes. Four patients had ESRD of unknown origin; the others had chronic glomerulonephritis, ESRD after acute renal failure induced by a contrast medium, and diabetic, hypertensive and analgesics nephropathy each.

**Spontaneous PMNL apoptosis**

**PMNL isolation.** PMNLs were isolated from heparinized blood using discontinuous Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and hypotonic lysis of erythrocytes [12]. The cell viability was >95% as determined by assessing exclusion of ethidium bromide (Gibco-BRL Life Technologies, Gaithersburg, MD, USA).

**Incubations.** PMNLs isolated under sterile conditions were incubated at 37°C for 20 h in phosphate-buffered saline (PBS, pH 7.4; Gibco, Paisley, Scotland, UK) containing Ca\textsuperscript{2+}, or in PBS containing 0.1 mM EGTA without Ca\textsuperscript{2+} at 6 × 10\textsuperscript{6} cells/ml. All samples contained 100 U/ml penicillin–streptomycin (Gibco-BRL).

PMNLs isolated from healthy adults were incubated in the presence of PHA concentrations resembling serum concentrations in ESRD patients pre- (100 μM) and post-dialysis (50 μM) based on mean uraemic concentrations (C\textsubscript{u}) given by Jankowski et al. [13]. As highest concentration, we used the theoretical maximal uraemic concentration C\textsubscript{max} = 160 μM based on mean (C\textsubscript{u}) + 2SD as previously suggested [22]. The lowest concentration tested (15 μM) is the minimal effective concentration inhibiting the plasma membrane Ca\textsuperscript{2+}-ATPase [13].

**Morphological features.** PMNLs were mixed with the fluorescent DNA-binding dyes acridine orange (Merck, Darmstadt, Germany) and ethidium bromide (Gibco-BRL) at a final concentration of 5 μg/ml each and examined under the fluorescence microscope as previously described [11,12].

**DNA content.** Apoptotic cells have a lower DNA content that was analysed by flow cytometry. PMNLs (1.2 × 10\textsuperscript{6}/200 μl) were centrifuged at 360 g for 20 min and washed twice with PBS. After 60 min of incubation in 250 μl ice-cold 70% ethanol on ice, the PMNLs were centrifuged, washed once with PBS and resuspended in 200 μl PBS containing 250 μg/ml RNase (type I-A; Sigma-Aldrich) and 50 μg/ml propidium iodide (Sigma-Aldrich). The samples were analysed after 15 min at room temperature in the dark.

**Data presentation.** Apoptotic PMNLs are in a stage between viability and secondary necrosis. The values for viable PMNLs are most important for the interpretation of our results, because apoptotic PMNLs are readily phagocytosed under in vivo conditions. Therefore, our data are presented as the PMNL viability factor. The PMNLs in buffer only or in the presence of the solvent in which the substance under investigation was dissolved are set as 1.00. Consequently, data obtained with substances dissolved in different solvents or obtained in different sets of experiments can be presented in one figure. This is possible because the percentage of viable PMNLs from healthy donors did not significantly differ from the values for cells from HD patients obtained before or after a HD session (‘Results’ section, Figure 1).

**Caspase activities.** The caspase-3 activity was determined by flow cytometry using the CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (BioVision, Mountain View, CA, USA). Caspase-8 and -9 activities were measured with the CaspGLOW™ Red Active Caspase-8 and -9 Staining Kit, respectively (BioVision), according to the instructions of the supplier.

**Intracellular calcium concentrations**

[Ca\textsuperscript{2+}]\textsubscript{i} was determined by measurement of fura-2 fluorescence in a luminescence spectrometer LS50B (Perkin Elmer, Langen, Germany). To 10\textsuperscript{6} PMNLs in 4 ml Hank’s buffer (HBSS, GibcoBRL), 4 μl fura-2AM was added, incubated at 37°C for 30 min in the dark and centrifuged at 300 g for 10 min at 4°C. From this point on the PMNLs were kept on ice. The cells were taken up in 1 ml Hank’s buffer. We obtained 0.5 × 10\textsuperscript{6} PMNLs per ml were obtained by addition of Hank’s buffer. The fluorescence was measured at 505 nm using the 340/360 nm dual wavelength excitation spectrofluorometry as described by Grynkiewicz et al. [23].

**Statistics**

The results are given as mean value ± SEM and were evaluated by paired analysis using the Wilcoxon test or by the Student t-test.
Results

Spontaneous apoptosis of PMNLs from healthy donors and haemodialysis patients

The percentage of viable PMNLs as determined by morphological criteria was 29 ± 2% for cells isolated from healthy donors (Figure 1a). In agreement with the literature [3,24], the values for viable PMNLs from HD patients were in the same range (30 ± 2% for pre-HD and 32 ± 1% for post-HD). By determining the DNA content (Figure 1b), we confirmed the finding that PMNLs obtained pre- and post-dialysis did not behave differently from PMNLs from normal subjects before PHA has been added.

Effect of p-hydroxy-hippuric acid on spontaneous apoptosis of PMNLs from healthy donors

PHA attenuated PMNL apoptosis and thereby increased the viability of PMNLs in a concentration-dependent manner as assessed by morphological features (Figure 2). By determining the DNA content, the PMNL viability factor in the presence of 160 μM PHA was 1.22 ± 0.10 when compared to the control set as 1.00 (n = 13; P < 0.005). In accordance with the increased viability of the PMNLs, the activities of caspases 3, 8 and 9 were significantly diminished by 160 μM PHA (Figure 3).

Influence of uraemia on the PHA and CPA effect

Blood cells of ESRD patients are continuously exposed to the uraemic milieu and do not necessarily react in the same way as cells obtained from healthy subjects. We compared the influence of PHA on apoptosis of PMNLs from healthy subjects and from HD patients before and after HD treatment. The viability of healthy PMNLs was significantly increased by 160 μM PHA, or determined significantly increased the viability of healthy PMNLs as determined by morphological criteria (Figure 4a) and by assessing the DNA content (Figure 4b), and it diminished the caspase-3 activity (Figure 4C). PMNLs from uraemic patients did not show any significant changes, irrespective of whether if the blood was drawn before or after HD treatment. Considering the same degree of apoptosis in the absence of PHA (Figure 1a and 1b), these data show that cells from uraemic patients on regular HD treatment behave differently from those obtained from normal subjects and that those differences are not reversed by haemodialysis. On the other hand, 5 μM
PHA attenuates apoptosis of PMNL from healthy subjects but not from HD patients.

Fig. 4. Influence of p-hydroxy-hippuric acid (PHA, striped bars) at a final concentration of 160 µM on spontaneous apoptosis of polymorphonuclear leukocytes (PMNLs) from healthy subjects (HS) and from uraemic patients before (PRE) and after (POST) haemodialysis treatment as determined by morphological features (a), DNA content (b) and caspase-3 activity (c). PMNLs in the absence of PHA acted as control (black bars) and were set as 1.00. For HS, PRE and POST n = 14, 9 and 9 for (a), n = 13, 9 and 9 for (b) and n = 8 for (c). Mean values ± SEM. *P < 0.05 versus controls.

CPA decreased the viability of PMNLs from healthy subjects and from HD patients (Figure 5).

**Influence of PHA and CPA on intracellular calcium**

As expected, addition of CPA led to a gradual sustained increase in [Ca²⁺], (Figure 6). However, PHA did not affect [Ca²⁺], (Figure 6), even during incubations up to 3 h (data not shown). Therefore, the effect of PHA on PMNL apoptosis is not mediated by changes in [Ca²⁺], of PMNLs under the conditions used. We obtained similar results for CPA (Figure 7a) and for PHA (Figure 7b) when we used PMNLs from uraemic patients before or after HD treatment. [Ca²⁺], was higher in PMNLs from uraemic patients before HD treatment as compared to cells from healthy subjects (Figure 7c). This effect cannot be attributed to PHA because PHA had no effect on [Ca²⁺]. HD treatment reduced [Ca²⁺], to the normal range (Figure 7c).

**Influence of CPA and fMLP in the presence and absence of extracellular calcium**

In parallel with CPA, we tested the effect of fMLP which causes a rapid increase in [Ca²⁺]. The PMNL viability was reduced by fMLP to 0.70 ± 0.05 (n = 13; P < 0.0005), as determined by morphological criteria, and to 0.89 ± 0.03% (n = 13; p < 0.01) by assessing the DNA content.

As expected, fMLP led to a quicker and more pronounced increase in [Ca²⁺], than CPA in the presence (1.27 mM) of extracellular calcium (Figure 8). In the absence of extracellular calcium, the effect of both compounds was reduced.
2516 G. Cohen et al.

Fig. 7. Effect of cyclopiazonic acid (CPA; a) at a concentration of 5 µM and of p-hydroxy-hippuric acid (PHA; b) at a concentration of 160 µM on the intracellular calcium concentration ([Ca^{2+}]_i) of polymorphonuclear leukocytes (PMNLs) isolated from uremic patients before (PRE) and after (POST) haemodialysis treatment as compared to changes in [Ca^{2+}]_i without the addition of a substance. Mean value of eight patients. (c) Basal values of [Ca^{2+}]_i of PMNLs isolated from five healthy subjects (HS) and from uremic patients (n = 8) before (PRE) and after (POST) haemodialysis treatment. Mean values ± SEM. **P < 0.01 versus HS or POST.

because only the emptying of the intracellular calcium stores contributes to the raise in [Ca^{2+}]_i.

The viability of PMNLs from healthy subjects did not significantly differ in the absence and presence of extra-cellular calcium. The absence of extracellular Ca^{2+} did not affect CPA- and fMLP-induced apoptosis either (Figure 9).

Discussion

The coordinated removal of PMNLs via apoptosis is crucial for the coordinated resolution of inflammation. The maintenance of a balance between anti-apoptotic and pro-apoptotic factors is important to prevent a reduced immune function in the case of increased apoptotic cell death or a state of increased inflammation in the case of reduced PMNL apoptosis [8]. Furthermore, the microenvironment and the local
concentrations of PMNL modulating compounds have to be considered. The characterization of factors affecting PMNL apoptosis is a first step towards the understanding of this complex scenario. We previously described free Ig-light chains as apoptosis-attenuating [11] and glucose-modified proteins as apoptosis-enhancing factors [12]. In the present work, we describe PHA as a compound that is able to reduce apoptosis of PMNLs from healthy subjects but not from HD patients.

In the literature, there are conflicting results regarding the involvement of uraemic toxins [3,23,26] did not find differences in apoptosis of PMNLs from HD patients and healthy controls.

The uraemic retention solute PHA is an inhibitor of the erythrocyte plasma membrane Ca^{2+}-ATPase [13]. Because modulation of intracellular calcium levels is involved in the regulation of apoptosis, we investigated the influence of PHA on spontaneous PMNL apoptosis. PHA attenuated PMNL apoptotic cell death and diminished the activities of caspases 3, 8 and 9, indicating that the PHA interferes with the activation of the caspase cascade both from the cell membrane and the mitochondria. CPA, an inhibitor of the sarko/endoplasmatic Ca^{2+}-ATPase, and fMLP, a chemotactic peptide that evokes a sudden increase in [Ca^{2+}], increased spontaneous PMNL apoptosis.

Surprisingly, PHA, unlike CPA and fMLP, did not influence [Ca^{2+}]. Therefore, the attenuating effect of PHA on PMNL apoptosis is not mediated by changes in [Ca^{2+}]. This finding is compatible with the fact that only part of PHA may penetrate into the intracellular space to inhibit the Ca^{2+}-ATPase because of protein binding of PHA [27].

Extracellular acidosis depresses PMNL apoptosis [28]. However, the pH value of the test media in the presence of PHA at the concentrations used in our assays was <0.1 units more acidic than the control medium (data not shown). Furthermore, PMNL apoptosis did not change in buffers with pH values up to 0.2 units more acidic than the standard test medium (unpublished observation). Therefore, it is unlikely that the effect of PHA is caused by changes in pH. Moreover, acidification leading to depressed apoptosis was connected with changes in [Ca^{2+}], which was not observed after PHA addition in our assays.

So far, several in vitro experiments exploring the effect of potential uraemic toxins were performed with PMNLs from healthy subjects [9–12,18–21]. However, the reaction of blood cells of ESRD patients continuously exposed to elevated levels of serum factors may differ from the response of healthy cells, e.g. because of different surface receptor expression or activity [29]. It is known that cells from uraemic patients respond to many stimuli in a different way when compared to cells from normal individuals. Mononuclear cells from CKD patients show a significantly reduced cytokine production in response to lipopolysaccharide [30–32] and a reduced proliferative response to antigens in vitro [33]. PMNLs from uraemic patients produce less reactive oxygen species in response to stimuli such as PMA or fMLP. However, to our knowledge, the present paper is the first report on the impact of a uraemic retention solute on PMNLs from uraemic patients. PHA increased the viability of healthy PMNLs, whereas PMNLs from uraemic patients before and after HD treatment did not show any significant changes (Figure 4a–4c). This suggests that the continuous exposure to the uraemic milieu, especially to the complex mixture of uraemic retention solutes, may lead to the down-modulation of receptors/mediators of PHA action and/or the triggering of pathways, also activated by PHA, finally resulting in a desensitization of uraemic PMNLs. Therefore, results obtained with PMNLs from healthy subjects may reflect the response to an acute exposure of PMNLs to uraemic toxins, but cannot necessarily be extrapolated to the chronic effect of uraemic retention solutes. The fact that apoptosis of PMNLs obtained after HD treatment was not affected by PHA either suggests that the PHA effect is not shielded by a dialyzable factor in the uraemic plasma and points to an inherent property of uraemic PMNLs.

A sudden temporary increase in [Ca^{2+}], acts as an important second messenger in PMNLs [34,35] leading to functional responses and modulation of apoptotic cell death [17,36,37]. In agreement with the literature [35,38–40], we found increased basal levels of [Ca^{2+}], in PMNLs from haemodialysis patients (Figure 7). However, this cannot be attributed to PHA because PHA at the maximal reported uraemic concentration had no effect on [Ca^{2+}], in PMNLs obtained from healthy subjects or from uraemic patients before and after dialysis. A number of factors such as uraemic retention solutes (e.g. parathyroid hormone) or rHuEpo therapy [39] have been made responsible for this change. This increased basal [Ca^{2+}], is associated with a decreased reactivity upon stimulation [41,42]. Whereas HD treatment with bioincompatible membranes leads to an increase in [Ca^{2+}], the use of biocompatible membranes did not change [Ca^{2+}], [39,43]. In our study, we found that dialysis treatment reverted the increased [Ca^{2+}], (Figure 7C) suggesting a removal of factors responsible for the increased basal levels of [Ca^{2+}]. The fact that only the basal [Ca^{2+}], is different between the control and the pre-HD group (as opposed to a sudden temporary increase) is in agreement with our finding that the change in [Ca^{2+}], is not associated with any change viability in vivo (Figure 1).

CPA blocks the Ca^{2+} uptake into the intracellular stores followed by store-operated Ca^{2+} entry (SOCE) [44,45]. We showed that CPA significantly increased PMNL apoptosis. In agreement with our observation, Lucas and Diaz [17] found that thapsigargin, another inhibitor of the sarko/endoplasmatic Ca^{2+}-ATPase, produced a rapid and sustained rise in [Ca^{2+}], and activated an apoptotic endonuclease which caused PMNL DNA breakdown.

The effect of fMLP on PMNL apoptosis is controversial. Chemoattractants have been suggested to be unable to modulate PMNL survival despite the activation of phosphatidylinositol-3 kinase and elevation of [Ca^{2+}] [46]. Klein et al. [47] reported that fMLP had no effect on PMNL apoptosis, Kettritz et al. [36] showed that fMLP significantly increased PMNL apoptosis at 24 h and Hu et al. [37] found that fMLP inhibited PMNL apoptosis. In the present study, fMLP significantly increases PMNL apoptosis (Figure 8). Consistent with these results, we previously
showed that glucose-modified proteins activate PMNL functions [12] and increase the rise in $[\text{Ca}^{2+}]_i$ in PMNLs after fMLP stimulation (unpublished observation) while causing enhanced PMNL apoptosis.

The degree of PMNL apoptosis was not dependent on the presence of extracellular Ca$^{2+}$ (Figure 9), in contrast to Chacon-Cruz et al. [48] reporting that Ca$^{2+}$ free buffer delayed spontaneous apoptosis in human PMNLs. However, Chacon-Cruz et al. used a high EGTA concentration (10 mM) in the medium and thereby actively depleted internal Ca$^{2+}$ stores, whereas our experiment was designed to investigate if Ca$^{2+}$ influx influences PMNL survival. Figure 9 shows that CPA and fMLP induced apoptosis to the same extent in the absence and presence of extracellular Ca$^{2+}$. For both fMLP and CPA, the increase of $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca$^{2+}$ is caused by store emptying only, while the rise in $[\text{Ca}^{2+}]_i$ in the presence of Ca$^{2+}$ in the medium results from the combination of store emptying followed by SOCE. This suggests that Ca$^{2+}$ influx via SOCE channels did not participate in the regulation of PMNL apoptosis in our experiments.

In conclusion, the uremic retention solute PHA—without inducing changes in $[\text{Ca}^{2+}]_i$—attenuates in vitro the spontaneous apoptosis of healthy PMNLs but not of PMNLs from HD patients. CPA which elevates $[\text{Ca}^{2+}]_i$ directly by inhibiting intracellular store refilling, and fMLP, an agent which causes a rise in $[\text{Ca}^{2+}]_i$ via several signal transduction pathways, enhance PMNL apoptosis independently of SOCE.

**Conflicts of interest statement.** None declared.

**References**

17. Lucas M, Diaz P. Thapsigargin-induced calcium entry and apoptotic death of neutrophils are blocked by activation of protein kinase C. Pharmacology 2001; 63: 191–196
PHA attenuates apoptosis of PMNL from healthy subjects but not from HD patients


Received for publication: 11.9.07
Accepted in revised form: 1.2.08