Intracellular acidification enhances neutrophil phagocytosis in chronic haemodialysis patients: possible role of CD11b/CD18

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Abstract

Background. We have demonstrated that uraemic neutrophils that exhibit a low intracellular pH (pHi) display enhanced phagocytosis. However, the underlying cellular mechanism is unclear.

Methods. We used neutrophils from three groups of haemodialysis (HD) patients before dialysis (Groups A, B and C) and also from age- and sex-matched healthy individuals to determine pHi, phagocytosis and expression of CD11b, CD18, CD14 and toll-like receptors (TLR)-2 and TLR-4. The patients were categorized based on three consecutive monthly pre-dialysis plasma bicarbonate concentrations (PbCO3) and pH values; Groups A, B and C had a constant pre-dialysis PbCO3 of ≤21, 21–26 and ≥26 mmol/L (mEq/L), respectively. We also studied the effects induced by the correction of metabolic acidosis and monoclonal antibodies (mAbs) against CD11b/CD18 on neutrophils in Group A. Furthermore, we investigated the effect of intracellular acidification on uraemic neutrophils ex vivo.

Results. We observed that the neutrophils in Group A exhibited significantly increased phagocytosis and expression of CD11b/CD18 compared with those in Groups B and C. Additionally, our ex vivo studies demonstrated that the mAbs against CD11b/CD18 partially blocked the enhancement of neutrophil phagocytosis in Group A. Moreover, the pH of uraemic neutrophils is inversely correlated with pHi and expression of CD11b/CD18.

Conclusions. HD patients with a low PbCO3 exhibited low neutrophil pHi that in turn increased the expression of CD11b/CD18 compared with neutrophils with a normal or high pHi. This increased expression of CD11b/CD18 on the uraemic neutrophils may contribute to the pHi-mediated phagocytosis.

Keywords: CD11b/CD18; haemodialysis; intracellular pH; neutrophil; phagocytosis

Introduction

Uraemic acidosis is usually mild, but if prolonged this form of metabolic acidosis may jeopardize patients’ health. This includes negative nitrogen balance [1], increased protein decomposition [2], bone lesions [3], hypercalciuria [4], enhanced parathyroid hormone secretion [5], hyperphosphataemia, reduced effect of 1α-hydroxylase [6], impaired functioning of the cardiovascular system, hormonal disturbances [7], insulin resistance [8], gluconeogenesis cycle disorders [9], and altered triglyceride metabolism [10]. Our previous data [11] were the first to demonstrate that HD patients with a low plasma bicarbonate concentration (PbCO3) exhibited low neutrophil intracellular pH (pHi). This intracellular acidification may contribute to the enhanced phagocytosis of these neutrophils as compared with their having a normal or higher pH. However, cellular mechanisms responsible for this phenomenon are unclear.

The CD11b/CD18 (Mac-1, CR3) is the most prevalent integrin expressed by monocytes and neutrophils [12,13]. The binding interactions between CD11b/CD18 and its counter-receptors or ligands are important for leukocyte adhesion and trans-endothelial migration, induction of inflammatory mediators and phagocytosis [14]. It is now widely accepted that CD14 together with lipopolysaccharide (LPS)-binding protein presents LPS to its high affinity transducer toll-like receptor (TLR)-4, thereby precipitating the molecular adjustments and cytokine profiles associated with
bacterial-induced systemic inflammation \cite{15, 16}. However, the effect of pH on the expression of CD11b/CD18, CD14, TLR-2 and TLR-4 and the possible role of these adhesion molecules in pH-mediated phagocytosis of uraemic neutrophils remains unclear. For this purpose, we used neutrophils from three groups of HD patients who had different pre-dialysis $P_{HCO_3}$ and pH values and also from age- and sex-matched healthy individuals. Their pH, phagocytosis and expression of CD11b, CD18, CD14, and TLR-2 and TLR-4 were investigated. We also investigated the effect induced by the correction of metabolic acidosis on neutrophils from the HD patients with low pre-dialysis $P_{HCO_3}$, and pH values (Group A). Further, we investigated the roles of CD11b and CD18 expressed on the neutrophils from Group A in the regulation of phagocytosis: this was established by determining the rate of phagocytosis after pre-treating these neutrophils with neutralizing monoclonal antibodies (mAbs) against CD11b, CD18, and CD11b/CD18 (combination of mAbs against CD11b and CD18). Furthermore, we investigated the effect of intracellular acidification on the regulation of phagocytosis and expression of CD11b, CD18, CD14, and TLR-2 and TLR-4 in uraemic neutrophils \textit{ex vivo}.

\textbf{Subjects and methods}

\textit{In vivo study design}

This study was multi-centred (3 HD centres), involving the three groups of HD: Groups A, B and C had a constant predialysis $P_{HCO_3}$ of $\leq 21$, 21–26 and $\geq 26$ mmol/L and pH $\leq 7.38$, 7.38–7.42 and $\geq 7.42$, respectively. The study also comprised age- and sex-matched healthy subjects (Group CN) with measurements of the neutrophil’s pH, phagocytosis and expression of CD11b, CD18, CD14, and TLR-2 and TLR-4. To evaluate the effect of metabolic acidosis correction on neutrophils, we investigated neutrophil function in acidicotic HD patients (Group A). Further, we investigated the roles of CD11b and CD18 expressed on the neutrophils from Group A in the regulation of phagocytosis: this was established by determining the rate of phagocytosis after pre-treating these neutrophils with neutralizing monoclonal antibodies (mAbs) against CD11b and CD18 (combination of mAbs against CD11b and CD18). Furthermore, we investigated the effect of intracellular acidification on the regulation of phagocytosis and expression of CD11b, CD18, CD14, and TLR-2 and TLR-4 in uraemic neutrophils \textit{ex vivo}.

\textit{Ex vivo study design}

Neutrophils from each HD patient were assayed for neutrophil pH, phagocytosis and expression of CD11b, CD18, CD14, TLR-2 and TLR-4 after incubating at different pH-adjusted (7.4, 6.5, 6.0, 5.5) culture media for 1 h \cite{i.e. each HD patient would have 4 (7.4, 6.5, 6.0, 5.5) groups of data}. We selected bicarbonate-buffered RPMI 1640 supplemented with 1% fetal calf serum (FCS) \cite{FCS containing $\leq 5$ pg 100 mL of LPS as recommended by the manufacturer (HyClone, South Logan, UT, USA)}; this medium was previously adjusted to the desired pH values (7.4, 6.5, 6.0 and 5.5). Aliquots of $10^8$/mL were cultured at 37°C in humidified 5% CO$_2$ (for cells suspended in the medium at pH 7.4) or 7% CO$_2$ (for cells suspended in media at lower pH values) incubators to maintain the media at the desired pH value. The pH of the culture media did not change significantly during the 1-h incubation (data not shown).

\textit{Patient selection}

After obtaining the approval of the Local Medical Ethics Committee, 122 anuric patients who had undergone maintenance HD (62 men and 60 women) were recruited for the study prior to which an informed consent was obtained. The mean age was 51.6 years (range 40–74 years). The mean dialysis duration was 37.7 months (range 8–52 months). The underlying primary renal diseases were glomerulopathy ($n = 57$), polycystic kidney disease ($n = 10$), tubulointerstitial nephritis ($n = 28$), nephrosclerosis ($n = 18$) and unknown ($n = 9$). In addition, all individuals had serum C-reactive protein (CRP) levels below 0.6 mg/dL. None of the patients suffered from diabetes mellitus, clinical infection, heart failure, liver cirrhosis, ketoacidosis and lactic acidosis. Further, none of the individuals were on calcium channel blockers, oral or intravenous active vitamin D, or iron. The HD patients in Groups A, B and C were screened for inclusion criteria in order to have similar serum ferritin (200–250 µg/L), transferrin saturation (TSAT) (20–50%), haemoglobin (10–11 g/dL), intact parathyroid hormone (PTH) (150–300 ng/L), length of HD (36–48 month) and K/V (1.2–1.5) (markers for adequacy of HD efficiency) during the experimental time period (monthly intervals for 3 months). All patient laboratory values presented in this study were within inclusion limits. As shown in Table 1, there were no statistically significant differences in serum ferritin, TSAT, haemoglobin, i-PTH, length of HD or K/V in HD patients among Groups A, B and C. The dose of erythropoietin for maintenance depends on the clinical indications.

\textbf{Haemodialysis}

HD was performed using a Fresenius Polysulfone dialyzer (F8 HPS–F10 HPS) containing a 1.8–2.4 m$^2$ polysulfone membrane and a dialysate flow rate of 500 mL/min. The dialysate fluid composition was as follows: sodium 140 mmol/L, potassium 2 mmol/L, calcium 1.75 mmol/L (7.01 mg/dL), HCO$_3$ = 35 mmol/L, acetate 4 mmol/L and glucose 5.5 mmol/L (99.1 mg/dL). The treatment duration was 4 h and the blood flow rates were around 200–250 mL/min. Anticoagulation was achieved using a predetermined loading dose and constant infusion of heparin. Net fluid

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removal was determined on an individual basis according to the patient’s clinical need.

**Biochemical analysis**

Albumin levels were measured using the Beckman Synchrom CX-9 Delta Chemistry Analyzer (GMI, MN, MN1, USA) according to the manufacturer's instructions. Serum iron and total iron-binding capacity were determined using spectrophotometry and detected by Integra 700 (Roche Diagnostics, Basel, Switzerland). Ferritin was measured using a polyclonal goat anti-ferritin antibody labelled with acridinium ester and detected by ACS:180 (R) SE Autoanalyzer System (Bayer, Tarrytown, NY, USA). Intact PTH was determined by means of biotinylated polyclonal goat anti-human PTH antibody compounded with acridinium ester-labelled polyclonal goat anti-human PTH antibody in phosphate buffer with goat IgG detection performed using the ADVIA Centaur Immunoassay system (Bayer, Tarrytown, NY, USA). Plasma CRP concentration was determined by the rate nephelometry (Beckman Instruments, Inc., Galway, Ireland). Plasma IL-6 concentration was measured by ELISA (R&D Systems Inc., Minneapolis, MN, USA).

**Neutrophil isolation and preparation**

Heparinized blood was procured before dialysis from the AV fistula or graft of HD patients in Groups A, B and C and also from age- and sex-matched healthy individuals who had taken no medication for at least 10 days prior to sampling. Neutrophils were isolated by sequential sedimentation in 6% dextran in 0.9% sodium chloride for 40 min at 22°C. Centrifugation was performed using Ficoll-Paque at 1500 rpm for 30 min to pellet granulocytes and the remaining erythrocytes, and centrifugation of the resuspended pellet was performed using an 81% isotonic Percoll gradient at 1750 rpm for 15 min to pellet the erythrocytes. The neutrophils from a diffuse layer at the interface were harvested, washed, resuspended in a medium and counted.

**Measurement of pH i**

Neutrophils were isolated from different conditions as described above. The pH i was measured using carboxy-SNARF-1-AM as described previously [17]. Neutrophils, at a concentration of 5 × 10^6/mL in the indicated pH-adjusted medium, were loaded with 10 μM carboxy-SNARF-1-AM for 1 h at 37°C, washed in phosphate buffer solution (PBS) and resuspended in the same buffer at 5 × 10^6 in 100 μL. Assays were performed by flow cytometry, with excitation at 488 nm and emission analysis at FL2 and FL3. Ten thousand events were collected. The pH i was estimated from the ratio of the emission intensities at two different wavelengths and standardized by comparison with the fluorescence intensity ratios of cells whose pH i values were fixed by incubation with nigericin (10 μM) in high-potassium buffers, as described previously [17].

**Neutrophil phagocytosis**

The phagocytosis capacity of bacteria was assayed by means of a commercially available kit (PHAGOTEST; ORPEGEN Pharma, Heidelberg, Germany). The neutrophils were isolated as described above and resuspended in 50% RPMI 1640 and 50% autologous plasma in v/v (previously pH adjusted and pH 7.4 was selected for the in vivo study). Subsequently, the opsonized FITC-labelled Escherichia coli was added and incubated for
Phagocytosis and CD11b/CD18

30 min in humidified 5% (was selected for in vivo study) or 7% CO2 incubators. The following procedure was performed according to the manufacturer’s instructions.

**Assay of the expression of CD11b, CD18, CD14 and TLR-2 and TLR-4 on neutrophils from HD patients and healthy individuals**

Neutrophils (10⁶ cells/mL) were isolated as described above and were incubated with 10 µg/mL anti-CD14 (MY4), anti-CD11b mAb (clone44), anti-CD18 mAb (IB4), anti-TLR2 (mAb 2392) and 4 mAb (HTA 125) or isotype control IgG (IgG1, IgG2a or IgG2b) (BD Biosciences, Pharmingen, San Diego, CA, USA) at 4°C for 15 min in PBS containing normal goat serum, 0.1% BSA. Cells were then washed with PBS containing 1% BSA, 0.01% NaN₃ and further incubated with 1 µg/mL FITC-conjugated goat anti-mouse IgG. The expressions of CD14, CD11b, CD18, TLR-2 and TLR-4 were analysed by flow cytometry through FCS/SSC gating [18,19] and expressed as mean fluorescence (difference in the fluorescence between with specific mAb and isotype control IgG).

**Statistical analyses**

All results are expressed as mean ± standard error of the mean (SEM). The difference among groups was analysed by one-way analysis of variance (ANOVA) followed by Scheffe’s test for multiple comparisons. Pearson’s correlation coefficient was used to determine the relationship between the neutrophil pHᵢ and function. A P value of <0.05 was considered statistically significant.

**Results**

**The expression of CD11b, CD18, CD14, TLR-2 and TLR-4 on neutrophils in HD patients and healthy individuals**

The expression of these adhesion molecules on neutrophils was investigated using mAbs against these integrins and quantified by flow cytometry using FITC-conjugated secondary antibodies. As shown in Figure 1A, flow cytometrical histogram profiles revealed that neutrophils from Groups A (A), B (B), C (C), A⁺ (A⁺) and CN (CN) expressed different levels of CD11b (1) and CD18 (2). The statistical analysis, as indicated in Figure 1B, of mean fluorescence intensities of CD11b and CD18 on neutrophils in Groups A, B, C and A⁺ was significantly higher than that on neutrophils in Group CN. In addition, neutrophils in Group A expressed significantly higher levels of CD11b and CD18 than those in Groups B, C and A⁺. Further, there was no significant difference in the expression of CD11b and CD18 on neutrophils in the HD patients among Groups B, C and A⁺. Nevertheless, as shown in Figure 2, there was no significant difference in the expression of CD14, TLR-2 and TLR-4 on the neutrophils in five study groups. Accordingly, our data suggest that neutrophils in uraemic patients expressed higher levels of CD11b and CD18 but similar levels of CD14, TLR-2 and TLR-4, when compared with the healthy individuals. In addition, the neutrophils in HD patients with a low pHᵢ expressed higher levels of CD11b and CD18, but similar levels of CD14, TLR-2 and TLR-4 than the individuals with normal or high pHᵢ. Furthermore, these alterations on neutrophils in Group A could be restored after the correction of metabolic acidosis over a period of 1 month.

**Effect of pHᵢ on the expression of CD11b, CD18, CD14 and TLR-2 and TLR-4 in uraemic neutrophils**

After demonstrating that the neutrophils of HD patients in Group A expressed significantly higher CD11b/CD18 than those from Groups B, C and A⁺, we planned to explore this phenomenon further by incubating uraemic neutrophils in...
The levels of the expression of CD14 and TLR-2 and TLR-4 on the neutrophils collected from Groups CN (CN; \(n = 28\)), A (A; \(n = 34\)), B (B; \(n = 51\)), C (C; \(n = 37\)) and A+ (A+; \(n = 34\)). As indicated in A, these cells (106 cells/mL) were incubated with anti-CD 14 mAb (1), anti-TLR-4 mAb (2) or isotype control IgG2a. As indicated in B, these cells (106 cells/mL) were incubated with anti-TLR-2 mAb (1) or isotype control IgG2b. After washing, the cells were further incubated with FITC-conjugated goat anti-mouse IgG, and the expressions of CD14, TLR-4 and -2 on neutrophils were analysed with flow cytometry. Background (BG) was assessed by using neutrophils that were not labelled with mAbs or mouse isotype control IgG but labelled with FITC-conjugated goat anti-mouse IgG. As illustrated in C, statistical results showed the expression (mean ± SEM) of CD14, TLR-4 and -2 on neutrophils in five experimental groups.

### Table 2. Uraemic neutrophil intracellular pH after 1 h incubation at various medium pH values

<table>
<thead>
<tr>
<th>Medium pH</th>
<th>Intracellular pH</th>
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<tbody>
<tr>
<td>7.4</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>6.5</td>
<td>6.2 ± 0.3*</td>
</tr>
<tr>
<td>6.0</td>
<td>6.0 ± 0.2*</td>
</tr>
<tr>
<td>5.5</td>
<td>5.8 ± 0.3*</td>
</tr>
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Neutrophils loaded with carboxy-SNARF-1-AM were suspended at 106/mL in the pH-adjusted medium, and pHi was determined after 1 h incubation. Shown are the means ± SEM of 21, each was in duplicate, experiments. *P < 0.05 versus similar medium at pH 7.4; **P < 0.001 versus similar medium at pH 7.4.

media with different extracellular pH (pHe) and investigated the expression of these adhesion molecules on the ureaemic neutrophils by using flow cytometry. As shown in Figure 3 and Table 2, the expression of CD11b/CD18 was significantly increased on the ureaemic neutrophils incubated in media with low pHe compared to those with high pHe. In addition, the expression of CD11b/CD18 on ureaemic neutrophils inversely correlated with pHi. However, there was no correlation between pHi and the level of CD14, TLR-2 and TLR-4 (data not shown). Accordingly, in compliance with the in vivo data, our ex vivo data suggested that CD11b/CD18 expression on ureaemic neutrophils was pHi related.

### Neutrophil phagocytosis of patients in Groups A, B, C and A+ and of healthy individuals

Uptake of FITC-labelled bacteria by neutrophils was tested by using Gram-negative species. Figure 4A showed the histograms of neutrophil phagocytosis in healthy individuals (CN) and patients in Groups A (A) [with or without pre-treatment of mAbs against CD11b, CD18 and both (combination of mAbs against CD11b and CD18)], B (B), (C) and A+ (A+). Group A neutrophils had significantly enhanced phagocytosis, which was similar to our previous data, compared to those in Groups B and C. The enhancement of phagocytosis in Group A was also reduced by correcting metabolic acidosis over a period of 1 month. In addition, mAbs against CD11b, CD18 and CD11b/CD18 significantly decreased neutrophil phagocytosis in Group A from ~78% to ~58%. The statistical results shown in Figure 4B reveal that neutrophil phagocytosis was significantly increased in the case of Group A patients compared to Groups CN, B, C, A+ and Group A patients pre-treated with mAbs against CD11b, CD18 and CD11b/CD18. However, there were no significant differences for neutrophil phagocytosis among HD patients in Groups B, C, A+, Group A pre-treated with mAbs against CD11b, CD18 and CD11b/CD18, and healthy subjects in Group CN. Accordingly, the enhanced neutrophil phagocytosis for patients in Group A could be significantly inhibited by mAbs against CD11b, CD18 and CD11b/CD18.

### Discussion

Our previous data [11] had demonstrated that ureaemic patients on maintenance haemodialysis can be classified into...
three categories based on their $P_{\text{HCO}_3}$ and pH values and that these three groups of patients have different pHi and neutrophil function. To the best of our knowledge, this is the first work to clarifying that individuals with a lower $P_{\text{HCO}_3}$ had a lower pHi, which may in turn contribute to the enhanced phagocytosis for uraemic neutrophils through the CD11b/CD18 signalling pathways.

The basal expressions of CD11b/CD18 on uraemic neutrophils from HD patients have been demonstrated to be higher than those for age- and sex-matched healthy individuals [20]. In addition, these two adhesion molecules were expressed at higher levels than those in the basal states during and after HD when using bio-incompatible dialyzers [21,22]. Hypercarbic acidosis with 20% CO$_2$ increased normal and not uraemic neutrophil CD18 expression and enhanced neutrophil adhesion despite a decrease in ICAM-1 expression on endothelial cells in acidic microenvironments such as ischaemia/inflammation states [23]. On the other hand, Takeshita and co-workers [24] concluded that hypercarbic acidosis did not alter the expression of CD11b or CD18 for neutrophils when compared with the situation under normocapnic conditions. However, the culture media used in the latter study did not contain bicarbonate. Furthermore, normal neutrophil chemotaxis was demonstrated to be regulated by pH [25]. Accordingly, CD11b/CD18 expression and/or CD11b/CD18-mediated functions (adhesion, migration, etc.) for normal neutrophils could be regulated by extra- and intracellular acidosis, and bicarbonate-containing media appear to be one of the essential factors in the alteration of neutrophil function. In this study, in compliance with previous works, we demonstrated that uraemic neutrophils had a higher CD11b/CD18 expression as compared with the normal ones. In addition, uraemic neutrophils from HD patients with a lower pHi (Group A$^+$) but also the expression of CD11b/CD18. Moreover, similar results were also found in the ex vivo study. We found that for uraemic neutrophils, the expression of CD11b/CD18, but not CD14/TLR-2/4, correlated inversely with their pHi.

Experiments using blocking antibodies revealed that CD14 and TLR2 but not TLR4 play a major role in lipoteichoic acid (a component of Gram-positive bacteria) [26] and bacterial lipoprotein [27]-mediated inhibition of spontaneous apoptosis for neutrophils. However, none of the previous studies have mentioned the effects of extra- and/or intracellular acidosis on the regulation of the expression for CD14, TLR2 and TLR-4 on and/or of functions of uraemic neutrophils. Ex vivo and in vivo studies for this work reported that the changes in pHi did not alter the expression levels of CD14, TLR-2 and TLR-4 on uraemic neutrophils. In addition, there was no difference in the expressions of CD14, TLR-2 and TLR-4 on neutrophils between HD patients and healthy individuals. However, the similar expression levels of CD14, TLR-2 and TLR-4 on neutrophils do not necessarily imply similar functional modulation. Further studies on the same are necessary. Accordingly, our data suggested that pHi was one of the effectors regulating the expression of CD11b/CD18 on uraemic neutrophils.

However, the increased expression of CD11b/18 for uraemic neutrophils having a lower pHi did not necessarily account for alterations in the function of uraemic neutrophils. Surprisingly, only few studies have focused on the cellular regulation of phagocytosis for uraemic neutrophils. In a non-immune host, the bacteria must be opsonized with the C3b or C3bi fragments to enable identification and subsequently the engulfing by neutrophils. The recognition is mediated by the binding of C3b and C3bi to specific complement receptors (CRs) on the neutrophil surface, which include CD35 (CR1), and the integrins CD11b/CD18 and CD11c/CD18 [28]. The β2 integrin CD11b/CD18 has been found to be important both for the adhesion of neutrophils to different biological surfaces [29] and for phagocytosis [30]. To explore this further, we investigated neutrophil
phagocytosis ex vivo after blocking CD11b/CD18 by using mAbs against both CD11b, CD18. We found that phagocytosis for uraemic neutrophils with a lower pH could be significantly inhibited by mAbs against both CD11b, CD18. Our data suggest that intracellular acidification for uraemic neutrophils enhanced phagocytosis through the CD11b/18 signaling pathways.

In conclusion, we demonstrated that HD patients having a lower $P_{HCO_3}$ had lower pH and this intracellular acidification may contribute to the increased expression of CD11b/CD18 on neutrophils when compared with those having a normal or higher pH. In addition, the increased expression of CD11b/18 might contribute to the pH-mediated enhanced phagocytosis of uraemic neutrophils.

Conflict of interest statement. None declared.

References


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