Improved cytosolic free calcium mobilization and superoxide production in bicarbonate-based peritoneal dialysis solution

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Abstract

Background. Intraperitoneal phagocytes play an important role in local host defence to prevent CAPD peritonitis. The intracellular calcium $\left[ \text{Ca}^{2+} \right]_{i}$ is thought to be involved in the regulation of various cell functions. This study therefore investigates the effect of lactate-based dialysis solution (LBDS) and bicarbonate-based dialysis solution (BBDS) on cytosolic free calcium mobilization and superoxide production (SP) as important steps in signal transduction and bacterial killing.

Methods. We studied changes in $\left[ \text{Ca}^{2+} \right]_{i}$ and SP following stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP) in polymorphonuclear neutrophils (PMNs) incubated in either LBDS-pH 5.2, LBDS adjusted to pH 7.4, 1:10 diluted spent and fresh LBDS or BBDS-pH 7.4 with different glucose concentrations, comparing the data with cells treated with Hanks buffer (HBSS) pH 7.4 as control. To elucidate the effect of glucose and lactate PMNs were additionally incubated in HBSS-pH 7.4, containing glucose (HBSS-Glu-pH 7.4) or lactate (HBSS-Lact-pH 7.4) in the same concentrations as contained in CAPD solutions and tested as above. PMNs were isolated from healthy blood donors and incubated with dialysis solution 10 min prior to stimulation with fMLP.

Results. $\left[ \text{Ca}^{2+} \right]_{i}$ mobilization and SP were completely inhibited in PMNs incubated in LBDS pH 5.2, pH adjustment of LBDS to 7.4 and 1:10 dilution of spent and fresh LBDS corrected some of the suppression of the calcium influx and superoxide production. BBDS pH 7.4, however, preserved physiological cell function significantly better at low (1.5 and 2.3%) glucose concentrations.

Conclusion. In comparison to conventional lactate-based dialysis solution, pH adjusted and 1:10 diluted LBDS, bicarbonate-based dialysis solution is more biocompatible since it preserves significantly better neutrophil cell functions.

Key words: biocompatibility; CAPD; intracellular calcium; signal transduction; superoxide production

Introduction

The major complication of CAPD treatment is the incidence of infectious peritonitis. Since phagocytes play an important role in the peritoneal defence against micro-organisms, the proper function of these cells is crucial. Polymorphonuclear neutrophils (PMNs) are phagocytes that can be stimulated by a variety of chemoattractants (i.e. fMLP, C5a complement, IL-8) to activate complex signal transduction cascades. Phospholipase C (PLC) mediates hydrolysis of the phospholipid phosphatidylinositol-4,5-biphosphate (PIP₂), leading to the formation of inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ triggers the release of intracellular calcium $\left[ \text{Ca}^{2+} \right]_{i}$ and DAG activates the protein kinase C (PKC) [1,2]. Changes in the cytosolic free calcium concentration $\left[ \text{Ca}^{2+} \right]_{i}$, have evolved as a key intracellular messenger system to couple external stimuli to a variety of complex cellular responses [3]. In neutrophils, changes in $\left[ \text{Ca}^{2+} \right]_{i}$ follow stimulation of several receptors and are important for the production of superoxide anions [4]. This so-called respiratory burst is a key function required for killing bacteria, the biochemical basis of this process is unique to phagocytes [5]. NADPH generated in the hexose monophosphate shunt is oxidized by the activated NADPH-oxidase which transfers two electrons to each of the two molecules of oxygen, which are consequently reduced to the free radicals, the superoxide anions.

It has been suggested that commercially available lactate-based dialysis solutions with low pH of 5.2 may inhibit a variety of calcium-dependent phagocyte cell functions [6-8]. We recently showed an immediate and complete inhibition of C5a-complement-induced actin polymerization, chemotaxis and phagocytosis in cells incubated in various concentrations of lactate-based dialysis solutions (LBDS) with a pH of 5.2 [9]. As the acidic pH seems to be a major reason for this functional
impairment, it has been hypothesized that increasing the pH might improve phagocyte cell function.

To test this hypothesis we used a new bicarbonate-based dialysis solution (BBDS) with a pH of 7.4. We investigated the effect of the commercially available LBDS-pH 5.2, the pH adjusted solution LBDS-pH 7.4, and BBDS-pH 7.4 on fMLP-stimulated release of [Ca\(^{2+}\)], and superoxide production [SP] in PMNs from healthy donors. Previous studies [10] have shown that a residual volume of about 200 ml with a physiological pH remains after exchange of the dialysate fluid. In the case of 2000 ml fresh dialysate solution this corresponds to a ratio of 1:10 spent to fresh dialysate. Because of the buffering capacity of this residual peritoneal fluid, spent dialysate after a dwell period of 4 h was collected and diluted 1:10 in fresh dialysate to mimic the in-vivo situation. [Ca\(^{2+}\)] and SP in cells incubated in this 1:10 diluted spent and fresh LBDS was additionally studied.

To elucidate the effect of glucose and lactate, PMNs were incubated in HBSS-pH 7.4, containing glucose (HBSS-Glu-pH 7.4) or lactate (HBSS-Lact-pH 7.4) in the same concentrations as contained in CAPD solutions and tested as above.

**Subjects and methods**

**Materials**

N-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a complement, fura-2/AM, BSA, d-glucose and cytochrome C were obtained from Sigma (Deisendorf; Germany); Lactate-based dialysis solution (CAPD 2 = 1.5% anhydrous dextrose, CAPD 4 = 2.3% anhydrous dextrose, and CAPD 3 = 4.25% anhydrous dextrose), containing 35 mmol lactate, and bicarbonate-based dialysis solution (Bic 20, Bic 40 and Bic 30, corresponding to 1.5%, 2.3% and 4.25% glucose), containing 34.0 mM HCO\(_3\), were supplied by the Fresenius AG (Bad Homburg, Germany). Both the lactate- and the bicarbonate-based dialysis solutions contain the following constituents in mM: Na\(^+\): 134.0, Ca\(^{2+}\): 1.75, Mg\(^{2+}\): 0.5, Cl\(^-\): 103.5 (104.5 in bicarbonate-based dialysis solution). Hanks buffer without Ca\(^{2+}\), Mg\(^{2+}\), and Phenol Red was purchased by Biochrom KG, Berlin, Germany.

**Isolation of neutrophils**

Whole blood obtained from healthy donors was gently mixed with dextran (6% Hetastarch in 0.9% sodium chloride; Du Pont Pharmaceutical, Wilmington, DE, USA) and allowed to settle for 30 min. Cells were washed and resuspended in HBSS buffer. The supernatant was separated over an isotonic Percoll gradient (55% and 74%) by centrifugation at 900 g for 40 min. The cell layer at the interface of the first gradient step contained more than 95% neutrophils of which over 95% were viable as measured by trypan blue exclusion.

**pH Adjustment**

pH adjustment of the LBDS was accomplished by titrating the respective solutions with small amounts of concentrated sodium hydroxide. pH measurement was repeated prior to the experiments.

**Fura-2 measurement of cytosolic free [Ca\(^{2+}\)]**

Intracellular free calcium [Ca\(^{2+}\)] was determined with fura-2 according to the method described by Malgaroli et al. [11]. Cells were loaded by incubation with 1.5 μg/ml fura-2/AM for 30 min at 37°C in HBSS, washed and resuspended in BBDS supplemented with 0.5% bovine serum albumin. 1.5 x 10\(^6\) cells were incubated in the dialysate or in HBSS (control) in cuvettes for 10 min. The cuvettes were thermostated at 37°C and equipped with a magnetic stirring device. [Ca\(^{2+}\)] was analysed in a luminescence spectrometer (Perkin-Elmer LS-50). Excitation light was set with two excitation monochromators and a dual mirror chopping mechanism that allowed a rapid (10-Hz) alternating excitation of fura-2/AM at two wavelengths. Excitation wavelengths were set at 339 nm (peak excitation) and 359 nm (isobestic point) and the emission wavelengths at 509 nm. To ensure that the isobestic point was valid, a scanning either in the calcium-free or the calcium-bound form of the fura-2 dye prior to incubation in this 10 diluted spent and fresh LBDS was performed.

The experiment was started by observing the base signal for 20 s; 1 μM fMLP was then pipetted into the cell suspension. After each experiment a calibration was performed. To obtain maximum and minimum fluorescence signals, cell lysis was performed with TritonX (final concentration 0.1%), followed by the addition of EGTA 4 mM without calcium and Tris buffer 15 mM, pH 8.8, suggesting a free calcium activity of 10^\(^{-6.4}\) M. [Ca\(^{2+}\)] was calculated as described by Grynkiewicz et al. [12]. Each experiment was performed 10 times. Data are expressed as percentage of levels measured in control cells incubated in HBSS (containing 5.6 mM glucose, no albumin).

**Superoxide production**

Superoxide generation was gauged by quantifying the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C as described by Ing et al. [13]. As described above, PMNs were incubated for 10 min at 37°C in the tested solution; 100 nM fMLP were added, mixed, and incubated for another 10 min. After that, samples were centrifuged at 12000 g for 1 min and the absorbance of the supernatant was determined at 550 nm in a spectrophotometer. The superoxide generated was calculated in terms of the amount of ferricytochrome C (in nanomoles) reduced to ferrous furochrome C as described by Ing et al. [13]. Each experiment was performed 10 times. Data are expressed as percentage of levels measured in control cells incubated in HBSS.

**Intracellular calcium measurements**

[Ca\(^{2+}\)] response was measured in fura-2 loaded cells after stimulation with 1 μM fMLP. The fMLP-induced activation of cells incubated in control buffer induced a rapid increase in peak levels of intracellular calcium

**Statistical analysis**

Results are expressed as mean±SEM. Data were analysed using paired/unpaired t test, or Wilcoxon rank test as appropriate. Differences were considered to be significant for P<0.05.
up to 540 ± 32 nM for 1 µM fMLP, and was dependent on the fMLP-concentration used (Table 1). After addition of the fMLP there was a rapid increase in peak level of [Ca²⁺], response which declined to basal levels within 240 s. In the nominal absence of extracellular calcium—by adding 4 mM EGTA—the fMLP-induced [Ca²⁺], response was still present, suggesting calcium release from intramitochondrial stores (data not shown) [14].

[Ca²⁺], increase in cells incubated in LBDS-pH 5.2 and stimulated with 1 µM fMLP was absent at all glucose concentrations. Trypan blue exclusion (about 95% viable cells) confirmed that the inhibition of calcium release was not due to loss of viability of the LBDS-incubated cells. In comparison to the pH 5.2 solution, increasing the pH of the LBDS to 7.4 led to a significant (P < 0.001) improvement of the fMLP-induced mobilization of intracellular calcium to 76 ± 3.4, 67 ± 3.5 ± 3.3 and 49 ± 2.2% for the 1.5, 2.3 and 4.25% glucose concentration solutions respectively (Figure 1). The difference between the high (4.25%) and the low (1.5%) glucose concentration was also significant (P < 0.05).

Table 1. Effect of different concentrations of fMLP on the [Ca²⁺], response in fura-2 loaded PMNs

<table>
<thead>
<tr>
<th>fMLP (µM)</th>
<th>nM [Ca²⁺]</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µM</td>
<td>293.3 ± 17.9</td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>381.5 ± 33.8</td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td>540.0 ± 32.1</td>
<td></td>
</tr>
<tr>
<td>100 nM</td>
<td>506.7 ± 43.3</td>
<td></td>
</tr>
<tr>
<td>10 nM</td>
<td>374.7 ± 49</td>
<td></td>
</tr>
<tr>
<td>1 nM</td>
<td>44.5 ± 3.5</td>
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</tbody>
</table>

n = 10 ± SEM.

fMLP-stimulated [Ca²⁺], release in BBDS-incubated cells was 92.4 ± 3.2, 83.0 ± 2.5 and 57.8 ± 3.5% for the 1.5, 2.3 and 4.25% glucose concentration solutions in comparison to the cells incubated in control buffer (HBSS). The increase in cytoplasmic calcium for the low-glucose concentrations, did not differ significantly from the mobilization of intracellular calcium in cells treated with the control buffer (Figure 1). At all glucose concentrations BBDS-pH 7.4 incubated cells performed better (P < 0.05) than LBDS-pH 7.4-treated cells (Figure 1).

As shown in Figure 1 [Ca²⁺], mobilization in cells incubated in 1:10 diluted spent and fresh LBDS was not significantly different from [Ca²⁺], mobilization measured in pH 7.4-adjusted LBDS, 75.4 ± 6.8, 71.4 ± 3.9 and 54.3 ± 3.7 for 1.5, 2.3 and 4.25% glucose concentration respectively. After dilution of 1:10 spent and fresh dialysate the initial pH of 5.2 increased up to 6.8.

HBSS pH 5.2 blunted calcium mobilization up to 33 ± 2.4%, HBSS with glucose in 1.5, 2.3, and 4.25% concentration led to 86.0 ± 4.8, 68.6 ± 2.9 and 59.3 ± 3.6% and HBSS with 35 mmol/l lactate to 76.0 ± 2.4% [Ca²⁺], mobilization (Table 2).

**Superoxide production (SP)**

The fMLP-induced activation of cells incubated in control buffer (HBSS) induced an increase in superoxide anions up to 35 ± 7.3 nM/10⁶ cells.

SP in cells incubated in LBDS-pH 5.2 and stimulated with 100 nM fMLP was completely prevented (Figure 2). Trypan blue exclusion confirmed that the inhibition of SP was not due to loss of viability of the LBDS incubated cells. Increasing the pH of the LBDS to 7.4 improved the fMLP-induced production of superoxide anions significantly (65 ± 2.04, 51.4 ± 2.5 and 13.3 ± 1.5% for the 1.5, 2.3 and 4.25% glucose solutions respectively; P < 0.001). The difference between the high- (4.25%) and the low- (1.5%) glucose concentrations was significant (P < 0.05). fMLP-stimulated SP in BBDS-incubated cells was 98.1 ± 5.6, 83.7 ± 3.1 and 31.87 ± 5.4% for the 1.5, 2.3 and 4.25% glucose concentration (Figure 2). As shown in Figure 2 SP in cells incubated in 1:10 diluted spent and fresh dialysate solution was significantly higher (81.7 ± 4.5, 70.7 ± 3.5 and 23.0 ± 1.2% for cells incubated in the

Table 2. Effect of HBSS-pH 5.2, HBSS with different glucose concentrations (1.5, 2.3, 4.25%) and HBSS with 35 mmol/lactate on the intracellular [Ca²⁺], response and SP production

<table>
<thead>
<tr>
<th>[Ca²⁺],</th>
<th>SP</th>
</tr>
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<tbody>
<tr>
<td>HBSS pH 7.4</td>
<td>100%</td>
</tr>
<tr>
<td>HBSS pH 5.2</td>
<td>33.0 ± 2.4</td>
</tr>
<tr>
<td>HBSS-Glu-1.5%</td>
<td>86.0 ± 4.8</td>
</tr>
<tr>
<td>HBSS-Glu-2.3%</td>
<td>68.6 ± 2.9</td>
</tr>
<tr>
<td>HBSS-Glu-4.25%</td>
<td>59.3 ± 3.6</td>
</tr>
<tr>
<td>HBSS-lactate-35 mmol</td>
<td>76.0 ± 2.4</td>
</tr>
</tbody>
</table>

n = 10 ± SEM.
77.0 ± 2.5, 57.0 ± 1.4 and 24.0 ± 2.4%, and in HBSS with 35 mmol/l lactate 77.0 ± 4.0% (Table 2).

Discussion

Uraemic patients have an impaired host defence, which may contribute to a high rate of infectious complications. Therapeutic intervention in the treatment of uraemia should aim at improving or at least not worsening this state of vulnerability. Since it is well known that commercially available LBDS-pH 5.2 may have inhibitory effects on phagocytes acting as the first line of defence against micro-organisms, it is important to clarify the effects of various dialysis solutions. We recently showed a complete and persistent paralysis of C5a-induced actin polymerization in PMNs after incubation in fresh LBDS-pH 5.2, which was pH-dependent [9]. Thus a physiological pH seems to be important to reduce the cytotoxicity of a dialysis solution. In the present study we tested whether bicarbonate-based dialysis solution—by virtue of its physiological pH—is superior to conventional, commercially available LBDS. As a method of testing biocompatibility we focused on measuring change in cytoplasmic calcium (\([Ca^{2+}]\)) and superoxide production. A change in \([Ca^{2+}]\), occurs within seconds after stimulation with chemoattractants and is one basic step in signal transduction. Superoxide production has a key function required for bacterial killing.

Several investigators have suggested that bicarbonate-containing solutions are superior to LBDS in terms of different cell functions [15–17]. To compare the effect of dialysis solutions on early signal transduction mechanisms, especially mobilization of intracellular calcium and superoxide production, we incubated cells in LBDS-pH 5.2, BBDS-pH 7.4, the pH adjusted LBDS-pH 7.4, or 1:10 diluted spent and fresh LBDS prior to stimulation with fMLP. The adjustment of pH in this set of experiments and the 1:10 dilution may be relevant because in vivo the pH rapidly increases after intraperitoneal instillation of the LBDS due to the buffering capacity of residual peritoneal fluid [10]. To elucidate the effect of glucose and lactate, we additionally investigated \([Ca^{2+}]\), and SP in PMNs incubated in HBSS-pH 7.4 containing glucose (HBSS-Glu-pH 7.4) or lactate (HBSS-Lact-pH 7.4) in the same concentrations as contained in CAPD solutions and tested as above described.

The complete inhibition of calcium mobilization and superoxide production in cells treated with LBDS-pH 5.2 seen in our study may suggest a strong inhibition of neutrophil functions. This is in accordance with studies showing a dramatic inhibition of chemotaxis, phagocytosis and superoxide production by PMNs incubated in LBDS [9,18–20]. Topley et al. [20] showed that ATP content of human peritoneal mesothelial cells were significantly impaired after a 15-min. exposure to LBDS pH 5.2. Interestingly, they observed a transient recovery of ATP content in a recovery medium, but a subsequent relapse was seen after 240 min, suggesting a chronic injury.

In our experiments pH adjustment of the LBDS solutions could improve but not restore the \([Ca^{2+}]\), response and superoxide production. So the extracellular pH seems not to be the only reason for the impaired cell functions due to the LBDS dialysates. This could be confirmed by the fact that \([Ca^{2+}]\), response in HBSS-pH 5.2 in comparison to LBDS-pH 5.2 was not completely suppressed. Kaupke et al. [21] could show, that pH adjustment does not abrogate intracellular acidosis by lactate-based dialysis solutions, which is a reason for the inhibitory effect of LBDS despite extracellular pH adjustment. There is evidence that additionally the glucose concentrations and lactate have inhibitory effects on \([Ca^{2+}]\), response and superoxide production. In accordance with findings by Mene et al. [22] showing that higher glucose concentrations inhibit cytosolic calcium signalling in cultured rat mesangial cells, our data indicate that the increase in glucose significantly inhibits the cytosolic calcium mobilization as well as superoxide production in polymorphonuclear neutrophils. In each of the tested solutions, LBDS-pH 7.4, BBDS and 1:10 diluted spent and fresh dialysate, there was a glucose-concentration-dependent loss of \([Ca^{2+}]\), response of about 10% comparing the 1.5% and 2.3% glucose and about 20% comparing the 2.3% and 4.25% glucose solutions. In cells incubated in LBDS-pH 7.4. BBDS-pH 7.4-incubated cells performed better (\(P<0.05\)) than LBDS-pH 7.4-treated cells (Figure 2), and also better than cells in 1:10 diluted solutions at all glucose concentrations (Figure 2).

SP of cells incubated in HBSS with 1.5, 2.3 and 4.25% glucose solutions) than in cells incubated in LBDS-pH 7.4. BBDS-pH 7.4-incubated cells performed better (\(P<0.05\)) then LBDS-pH 7.4-treated cells (Figure 2), and also better than cells in 1:10 diluted solutions at all glucose concentrations (Figure 2).

**Fig. 2.** Effect of LBDS-pH 5.2, LBDS-pH 7.4, BBDS-pH 7.4, and 1:10 diluted fresh and spent LBDS (1:10) with different glucose concentrations (1.5, 2.3 and 4.25%) on the superoxide production prior to stimulation with 100 nM fMLP in polymorphonuclear neutrophils. Data are expressed as percentage of control cells incubated in Hanks’ buffer, considered as 100%. Data are mean ± SEM of 10 experiments. (*\(P<0.05\), LBDS-pH 7.4 vs BBDS for all three glucose concentrations, **\(P<0.001\), LBDS-pH 5.2 vs all other solutions).
Cytosolic free calcium mobilization in bicarbonate PD solution

References


Received for publication: 24.9.96
Accepted in revised form: 15.1.97