Cellular acidification occurs during anoxia in cultured, but not in freshly isolated, rabbit proximal tubular cells

Abstract In a variety of cells it has been shown that acidosis is protective against anoxic injury. We have demonstrated previously that proximal tubule (PT) cells in primary culture were more resistant to anoxia-induced cell injury than were freshly isolated cells. Therefore, we asked the question of whether a difference in cellular acidification during anoxia could explain this difference in susceptibility to anoxia. To answer this question, intracellular pH (pH_i) was measured during anoxic incubation of PT cells in culture and those that were freshly isolated. PT cells were incubated in an anoxic chamber at 37°C after loading with 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein ace!oxymethyl ester (BCECF-AM) or fura-2 acetoxymethyl ester (fura-2-AM). pH_i and cytosolic free Ca^{2+} ([Ca^{2+}]_i) were measured by digital imaging fluorescence microscopy. During anoxia, pH_i in cultured PT cells decreased from 7.3 ± 0.1 to 6.8 ± 0.1, whereas pH_i in freshly isolated cells did not decrease significantly. In addition, the intrinsic buffering capacities (\beta_i) in cultured and freshly isolated PT cells were determined and turned out to be the same at a pH_i greater than or equal to 7.3. Below pH_i 7.3, \beta_i increased several fold in freshly isolated PT cells, and rose to significantly higher levels than in cultured PT cells. During 1 h of anoxia, cell viability of freshly isolated PT cells decreased significantly to 54% ± 2% (P < 0.05), while no loss in viability was observed in cultured PT cells. Clamping the pH_i during anoxia at 6.7 and 6.1 significantly increased cell viability in freshly isolated PT cells to 76% ± 5% and 72% ± 4%, respectively (P < 0.05). In contrast, prevention of acidification in cultured PT cells during anoxia did not lead to increased cell death. Therefore, the differences in susceptibility to anoxic injury between cultured and freshly isolated PT cells cannot be explained by cellular acidification in cultured cells, but must be sought elsewhere.

Key words Ischaemia • Intracellular pH • Proximal tubule • Primary culture

Introduction

ATP depletion and acidosis are prominent features during hypoxic or ischemic insults in many tissues, including the kidney [28]. In general, ATP depletion results in the disturbance of intracellular ion homeostasis which leads eventually to cell damage [15, 27]. Increased cytosolic Ca^{2+} ([Ca^{2+}]_i) has also been suggested to mediate injury during ATP depletion in several organ systems, including the kidney [7, 23, 27, 28], liver [4, 22], brain [2] and heart [14]. It has been shown that acidification of hypoxic tissue, resulting from glycolytic lactate production, ATP hydrolysis and CO_2 accumulation, can enhance the resistance to the damaging effects of O_2 deprivation in the kidney [7, 24, 26–29], cardiomyocytes [3, 16], and hepatocytes [10, 11, 13, 18]. However, the mechanism behind the protection offered by lowering the pH is unknown. In some cells, such as cardiomyocytes, acidosis has an energy-conserving effect caused by a lower energy demand [16]. In addition, intracellular pH (pH_i) plays a role in the preservation of ionic gradients across the plasma membrane during ATP depletion, by decreasing plasma membrane conductance pathways of, for example Ca^{2+}, or inhibition of Ca^{2+}-calmodulin-regulated processes [26]. Moreover, the mechanisms responsible for membrane and cell damage, such as
phospholipid and protein degradation by phospholipases and proteases, appear to be pH dependent, with maximal activity at or near physiological pH and minimal activity at acidic pH [13]. Protection by extracellular acidosis has been shown to be mediated by intracellular acidification in hepatocytes [6], but until now this has not been confirmed in renal cells [7, 24, 26–29].

Previous studies on anoxia-induced cell injury in proximal tubular (PT) cells revealed a striking difference in the sensitivity to anoxia between freshly isolated and cultured PT cells [20, 21]. The fact that cultured cells were more resistant to anoxia could be due to the presence of a protective factor such as, for example, intracellular acidosis. The objective of the present study was to measure pH and [Ca2+]i during anoxia in cultured and freshly isolated PT cells and to investigate whether cytosolic acidosis plays a role in protection against anoxia-induced cell injury in PT cells.

Materials and methods

Isolation of PT cells

Rabbit kidney PT cells were isolated by immunodissociation as described previously [20]. Briefly, kidneys were excised from New Zealand white rabbits (approx. 0.5 kg). A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 min on ice with monoclonal antibodies 85CS and 101F12, recognizing cell surface antigens specific for the PT. After three washes, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 min at 20°C. The dishes were carefully washed and adherent PT cells were collected and resuspended in a mixture of Dulbecco’s Modified Eagles medium (Invitrogen No. 1 466 14, Hampshire, UK) and Ham’s F12 medium (Gibco, No. 041 01765M, Paisley, UK) (1:1), supplemented with 5% (v/v) fetal calf serum (PCS) during the first 60 min, and 5% FCS at 37°C for de-esterification. After loading and de-esterification of the PT cells, they were washed twice in the experimental medium containing (in mM): 112 potassium gluconate, 28 KCl, 10 NaCl, 3.1 MgCl2, 0.01 CaCl2, 5 4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid (HEPES), 5 L-alanine, 10 D-glucose, pH 7.4; hereafter this medium is referred to as Kt medium. PT cells were exposed for 4 min to the same solution except that now 40 mM NH4Cl was substituted by 40 mM NH4Cl. Subsequently, the pH of the solution was adjusted to pH 6.8 with NH4OH, and the PT cells were incubated in the presence of 0.3 mM probenecid to inhibit fura-2 or BCECF leakage.

Fura-2 fluorescence was monitored using digital imaging equipment (MagiCal, Applied Imaging systems, Tyne and Wear, UK). The fura-2-loaded PT cells were alternately excited at 340 nm and 380 nm and the emitted light was captured at 510 nm with a CCD camera followed by digital imaging using TARDIS software (Applied Imaging International, Tyne and Wear, UK). The 340–380 nm capturing sequence was interrupted by 30-s periods, of which 15 s was used for and 15 s for excitation at 340 nm and 380 nm respectively, 0.02% (w/v) pluronic F127 (Molecular Probes), 4% (v/v) FCS and 3 mM probenecid (Sigma, St. Louis, Mo., USA). PT cells were loaded with 2.7- bis-(2-carboxyethyl)-5,6-carboxyfluorescin acetoxy methyl ester (BCECF-AM; Molecular Probes) by incubating the coverslips for 45 min at 37°C in Kt plus 5% FCS medium containing 2 μM BCECF-AM and 3 mM probenecid (Sigma). Thereafter PT cells were incubated for 30 min in Kt plus 5% FCS at 37°C for de-esterification. After loading and de-esterification, the PT cells were washed twice in the experimental medium and were used immediately. All experiments were performed in the presence of 0.3 mM probenecid to inhibit fura-2 or BCECF leakage.

Intrinsic buffering capacity of PT cells

The intrinsic buffering capacity (βf) was estimated using a method described by Boyarski et al. [5]. Briefly, all mechanisms for pHj regulation in the PT cells were inhibited by omitting Na+ and HCO3- from the perfusate [composition in mM: 140 N-methyl t-glucamine, chloride salt (NMGCJ), 5 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 5 L-alanine, 10 D-glucose, pH 7.4]. After 6 min, the PT cells were exposed for 4 min to the same solution except that now 40 mM NaHCO3 was substituted by 40 mM NH4Cl. Subsequently, the NH4Cl concentration was stepwise lowered to 20, 10, 5 and 0 mM at 4-min intervals. Each experiment was followed by a pHj calibration.
according to the procedure described above. \( \beta \) was calculated from the pH change induced by reducing the \( \text{NH}_4\text{Cl} \) concentration according to the formula \( \beta = \frac{\Delta B}{\Delta \text{pH}} = - \left( \frac{\Delta A}{\Delta \text{pH}} \right) \), where \( \Delta B \) is the amount (in mM) of strong base added to the solution, \( \Delta A \) is the amount of strong acid, and \( \Delta \text{pH} \) is the resultant change in pH [5].

Anoxic chamber experiment

Anoxic conditions were realized in an anoxic chamber as described previously [20]. Fura-2- or BCECF-loaded PT cells on coverslips were mounted in this anoxic chamber at 37°C. After filling the anoxic chamber with 100% \( \text{N}_2 \), gassed modified Krebs Henseleit Buffer [KHB; composition in mM: 138 NaCl, 5 KCl, 1 MgSO\(_4\), 2 CaCl\(_2\), 1 l-alanine, 5 l-lactate, 20 HEPES/TRIS and 360 mU/ml Oxyrase (Oxyrase, Ashland, Ohio, USA)], \([\text{Ca}^{2+}]\), and pH, were monitored for 45-60 min.

Estimation of cell viability

PT cells were mounted in the anoxic chamber under continuous monitoring of partial \( \text{O}_2 \) pressure using a Clark-type electrode [20]. After anoxic incubations, cell viability was estimated by means of lactate dehydrogenase (LDH) leakage for cultured cells [20] and by means of trypan blue exclusion of propidium iodide staining for freshly isolated cells [21]. For LDH activity measurements, medium and cells were collected separately. To this end, cells were scraped off the coverslip in 1 ml MilliQ water and the resulting cell suspension was sonicated for 30 s at 100 W to release all LDH. Both cell and medium samples were centrifugated for 5 min at 200 \( g \) and LDH content was measured as described previously [20]. For trypan blue and/or propidium iodide staining, coverslips with PT cells were incubated for 1-2 min in 0.08% (w/v) trypan blue or 5 \( \mu \)g/ml propidium iodide. The percentage of stained cells was determined by counting using light microscopy for trypan blue staining, or by using fluorescence microscopy for propidium iodide staining as described previously [21].

Materials

All chemicals were of the purest grade and obtained from Sigma unless otherwise indicated.

Statistical analysis

All reported data are expressed as means ± SEM. Statistical analysis was performed on ratio and viability values using analysis of variance (\( P < 0.05 \) being significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher [25].

Results

\([\text{Ca}^{2+}]\), and pH, during anoxia

\([\text{Ca}^{2+}]\), and pH, were monitored during substrate-free anoxia in cultured and freshly isolated PT cells. Figure 1A shows a typical example of cultured PT cells, where anoxia induced increases in \([\text{Ca}^{2+}]\) within 20 min, reaching a maximal level within 30 min. After reintroducing \( \text{O}_2 \) and glucose, i.e. reperfusion, \([\text{Ca}^{2+}]\), returned completely to basal levels, as reported previously [20]. In cultured PT cells, anoxia also induced a decrease in pH,. The ratio of fluorescence at 490 nm and 440 nm declined significantly from 3.11 ± 0.10 to 2.44 ± 0.11 (\( P < 0.05 \)) within the first 15 min of anoxia. This level was maintained throughout the entire anoxic period until reperfusion. At reperfusion, the BCECF ratio value increased significantly to 3.04 ± 0.11 (\( P < 0.05 \)). Figure 1B shows a typical experiment in which anoxia induced an increase in \([\text{Ca}^{2+}]\) in freshly isolated PT cells. Upon reperfusion \([\text{Ca}^{2+}]\), did not always return to initial levels, as described before (Table 1 and [21]). In contrast to cultured PT cells, pH, did not decrease significantly during 45 min of anoxia in freshly isolated PT cells. In order to provide real pH values, a calibration was performed in both cultured and freshly isolated PT cells. PT cells were incubated in a high-K\(^+\) medium containing 10 \( \mu \)M nigericin with a pH ranging from pH 6.0 to pH 7.9. Cultured PT cells were calibrated directly after the anoxic experiment. In some freshly isolated PT cells this was not feasible.
Table 1 The effect of intracellular acidosis on \([\text{Ca}^{2+}]_i\) in freshly isolated proximal tubular (PT) cells. Anoxic incubation of freshly isolated PT cells at normal and low pH medium. Initial \([\text{Ca}^{2+}]_i\) which is \([\text{Ca}^{2+}]_i\), 10 min after filling the chamber, anoxic \([\text{Ca}^{2+}]_i\) and \([\text{Ca}^{2+}]_i\), 10 min after reperfusion, are presented in nM. All data are mean values ± SEM of at least 5 experiments. (pHn = 7.4).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>([\text{Ca}^{2+}]_i), (nM)</th>
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<tr>
<td>pHn = 7.4</td>
<td>111 ± 16</td>
</tr>
<tr>
<td>pHn = 6.0</td>
<td>150 ± 1*</td>
</tr>
<tr>
<td>Initial</td>
<td>512 ± 33*</td>
</tr>
<tr>
<td>Anoxia</td>
<td>412 ± 5**</td>
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<tr>
<td>Reperfusion</td>
<td>213 ± 7*</td>
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*\(P < 0.05\) Anoxia and reperfusion values versus pre-anoxic values,
**\(P < 0.01\) for values at pHn = 6.0 versus pHn = 7.4

Fig. 3 pH of cultured (♦) and freshly isolated (○) PT cells measured 10 min after filling the anoxic chamber, (initial), during the steady-state reached after 20 min of anoxia (anoxic) and at the introduction of O2 and substrate (reperfusion). Each point represents the mean pHi ± SE = M with \(N \geq 8\) (*\(P < 0.05\) anoxic versus basal pHi).

Protection by cytosolic acidosis

Cell viability of cultured PT cells after 60 min of anoxic incubation was unaltered, as reported previously [20]. Cell viability of freshly isolated PT cells had decreased already during the preparation and preservation of the cells. After the cell isolation procedure, which takes almost 3 h, cell viability was, on average, 84% ± 1%. During the following 5 h of preservation in K+ medium, cell viability decreased further to 67% ± 3%. We have shown in a previous study that 1 h of anoxia decreased percentage cell viability independent of the time which had passed since the commencement of cell isolation [21]. Therefore, in all experiments, the viability of the control normoxic group was set at 100% [21]. When expressed in this way, 1 h of anoxic incubation of freshly isolated PT cells decreased cell viability to 54% ± 2%. To test whether intracellular acidosis protects against anoxic injury, pHi was clamped at low extracellular pH (pHn) during 60 min of anoxia. To this end, freshly isolated PT cells were incubated in substrate-free high-K+ medium of pH 6.6 or 6.0 containing 10 \(\mu\)M nigericin. Figure 4 shows a typical experiment of clamping the pHi at 6.0. During anoxic incubation, the BCECF ratio was stabilized at 1.23, which corresponds to a pHi of 6.0. On average, pHi was 6.7 ± 0.1 and 6.1 ± 0.1 during clamping in pHn 6.6 and 6.0, respectively. After 60 min of anoxic incubation at pHn 6.6 or...
Fig. 4 Typical pH trace of a pH clamp of freshly isolated PT cells during anoxic incubation in substrate-free high-K+ buffer of pH 6.0, containing 10 μM nigericin. During this pH clamp, the 490/440 nm ratio declines to 1.23 pH is presented as 490/440 nm ratios as well as real pH values.

Fig. 5 Cell viability of freshly isolated PT cells after 60 min of anoxic incubation in substrate-free Krebs-Henseleit buffer (KHB) of pH 7.4 (pH<sub>6</sub> = 7.4) or in substrate-free pH clamp buffer (pH<sub>6</sub> = 6.6 and pH<sub>4</sub> = 6.0). Columns represent mean viability values ± SEM with N > 19 (*P < 0.05 pH<sub>6</sub> = 6.6 and 6.0 versus pH<sub>6</sub> = 7.4).

6.0, in the presence of nigericin, cell viability was significantly higher than in the control situation (Fig. 5). Incubation of PT cells in a nigericin-free medium of pH 6.0 resulted in a pH<sub>6</sub> of 6.8 ± 0.1 and a cell viability of 77% ± 4%, which is not significantly different from the results of experiments in which nigericin was present (P > 0.1). This control excludes an obscuring toxic effect of nigericin in the protection by cellular acidosis. In addition, we tried to clamp the pH of cultured PT cells during anoxia at 7.3 to see whether this protocol induced cell injury. However, pH<sub>6</sub> measurements revealed that in cultured PT cells we were unable to clamp pH<sub>6</sub>, since the cells still acidified during anoxia. Even in the presence of valinomycin or amiloride pH<sub>6</sub> could not successfully be clamped at pH<sub>6</sub> 7.3. Finally, cultured PT cells were incubated in a medium of pH 8.0 resulting in a pH<sub>6</sub> of 7.3 ± 0.1. However, cell viability did not decrease during a 1-h anoxic incubation at a pH<sub>6</sub> of 8.0. We did observe, however, a slight tendency for cells to start to detach themselves from the collagen coating, which could be an early sign of cell injury.

In Table 1 the influence of cytosolic acidosis on [Ca<sup>2+</sup>]<sub>i</sub> is summarized for freshly isolated PT cells. It is shown that intracellular acidosis increased initial [Ca<sup>2+</sup>]<sub>i</sub> significantly, but, on the other hand, maximal anoxic [Ca<sup>2+</sup>]<sub>i</sub> was significantly reduced when compared to anoxia in the absence of acidosis. The mean [Ca<sup>2+</sup>]<sub>i</sub> after 10 min of reperfusion was significantly higher than the initial level, independent of the pH during anoxia.

Estimation of β<sub>i</sub>

A factor which could partly explain, in theory, the difference in pH<sub>6</sub> values in cultured and freshly isolated PT cells during anoxic incubations is the β<sub>i</sub> for both cell types; therefore, β<sub>i</sub> was estimated. Figure 6 shows β<sub>i</sub> values as a function of pH<sub>6</sub>. In the pH range 8.0 to 7.5, β<sub>i</sub> values were similar for cultured and freshly isolated PT cells. However, at lower pH<sub>6</sub>, β<sub>i</sub> values in freshly isolated PT cells started to increase and to deviate significantly from those measured in cultured cells. Nevertheless, the absence of cytosolic acidosis during anoxia in freshly isolated PT cells cannot be explained solely on the basis of a higher β<sub>i</sub> at lower pH<sub>6</sub>, but must be sought in the absence of glycolysis.

Discussion

The present study demonstrates that during anoxic incubation, intracellular acidosis develops in cultured PT cells, but does not occur in freshly isolated PT cells. Differences in cellular acidification must be due to differences in glycolytic capacities and cannot be explained by different β<sub>i</sub> between cultured and freshly isolated PT cells. In addition, we have demonstrated
that intracellular acidosis is protective against anoxia-induced cell injury, since acidosis was protective in freshly isolated PT cells.

Studies using isolated perfused rat kidney have shown that acidosis, caused by ATP hydrolysis and accumulation of CO₂, protected against hypoxic cell injury [24]. Until now, in vitro experiments have demonstrated only a protective effect of extracellular acidosis against hypoxia/anoxia-induced cell injury in the kidney [7, 24, 26-29], hepatocytes [15] and cardiomyocytes [3]. In these studies, however, it was not shown that protection by low pH is mediated via intracellular acidosis. Our study demonstrates that anoxia caused intracellular acidosis in cultured, but not in freshly isolated, PT cells. Cultured cells, in general, are more glycolytic than are freshly isolated cells and, therefore, endogenous substrates at the onset of anoxia will be metabolized, forming lactic acid and ATP. In addition, hydrolysis of ATP leads to an intracellular acid load and, thus, to cytosolic acidification [8]. In contrast, non-cultured PT cells have less glycolytic capacity and do not form lactic acid during anoxia [9]. Finally, we showed that freshly isolated PT cells have a higher 1̄ than do cultured PT cells, which could cause a slower development of acidosis, but cannot explain its absence. By subjecting freshly isolated PT cells to a pH clamp during anoxia, it could be shown that acidosis is also protective in these cells, obviously mediated by intracellular acidosis, since pH clamp experiments resulted in pH values of 6.7 and 6.1.

There are several mechanisms by which acidosis may protect against anoxia-induced cell death. First, hypoxia/anoxia-induced phospholipid degradation has been reported to be pH dependent [13]. Also ATP depletion, in chemical anoxia, resulted in a loss of phospholipid mass and increased permeability of the plasma membrane and cellular organelles [28]. Second, low pH has been shown to preserve metabolic energy in cardiomyocytes [14, 16]. In PTs, however, no increase in cellular ATP content was found during anoxia at in cultured, but not in freshly isolated, PT cells. Cultured cells, in general, are more glycolytic than are freshly isolated cells and, therefore, endogenous substrates at the onset of anoxia will be metabolized, forming lactic acid and ATP. In addition, hydrolysis of ATP leads to an intracellular acid load and, thus, to cytosolic acidification [8]. In contrast, non-cultured PT cells have less glycolytic capacity and do not form lactic acid during anoxia [9]. Finally, we showed that freshly isolated PT cells have a higher 1̄ than do cultured PT cells, which could cause a slower development of acidosis, but cannot explain its absence. By subjecting freshly isolated PT cells to a pH clamp during anoxia, it could be shown that acidosis is also protective in these cells, obviously mediated by intracellular acidosis, since pH clamp experiments resulted in pH values of 6.7 and 6.1.