Glycine protection against hypoxic injury in isolated rat proximal tubules: the role of proteases


Abstract

Introduction

Hypoperfusion of the kidney results in ischaemia and is a major cause of acute renal failure. In recent years, several groups have tried to delineate the mechanisms involved in ischemic renal injury using experimental animal models and cell culture techniques. It has been demonstrated that the small amino acid glycine affords profound protection against in vitro cell injury. In isolated rat and rabbit proximal tubules glycine strongly reduced the damage caused by various injurious processes e.g. hypoxia, ouabain, metabolic inhibitors, iomycin, and phosphate depletion. In the isolated perfused kidney glycine diminished the deterioration of renal function. The mechanism by which glycine protects is not known, however. Apparently, protection is not related to glycine metabolism, maintenance of cellular ATP, potassium or calcium levels, alterations in intracellular pH, prevention of activation of phospholipases, or removal of free fatty acids.

In recent studies it has been suggested that proteolytic enzymes play a role in hypoxia-induced cell injury and, therefore, inhibition of these proteases could be part of the protective effect of glycine. In general, four major classes of proteolytic enzymes are recognized based on either the amino acid present at the active site (cysteine, serine, aspartate proteases) or the necessity of a metal ion as a cofactor (metalloproteases). The four major classes comprise virtually all proteases characterized up to date, and form very heterogeneous groups. Since all cellular proteins are potential substrates for proteolytic enzymes, an increase in protease activity could result in partially degraded cell structures. Thus far, however, data on altered proteolytic activities during hypoxic insults are limited. In anoxic rat hepatocytes an increase in the activity of a pH-dependent non-lysosomal proteolytic activity was reported by Bronk and Gores. In subsequent studies, it was demonstrated that glycine inhibited protease activity. In livers stored on ice, glycine inhibited metallo and aspartate protease activity, whereas in hepatocytes exposed to anoxia inhibition of a cal-
cium-dependent non-lysosomal protease activity was observed [16,17]. In rat proximal tubules, Edelstein et al. [18] recently demonstrated that hypoxia induced an increase in activity of a calcium-dependent cysteine protease. These authors also provided evidence that activation of this particular protease plays an important role in cell injury.

To study the mechanisms involved in hypoxic cell injury and glycine protection, we have investigated the effect of hypoxia on different classes of proteolytic enzymes in isolated rat proximal tubules. In addition, the effect of glycine on the activity of these enzymes was studied.

**Subjects and methods**

**Preparation of isolated proximal tubules**

Suspensions of renal proximal tubules were prepared by methods previously described [3,19]. Male Sprague-Dawley rats (approximately 200–250 g of body weight) were anaesthetized with sodium pentobarbitone (60 mg/kg body weight i.p.) and the kidneys were flushed with 40 ml of an ice-cold oxygenated (95%O\(_2\) / 5%CO\(_2\)) heparin solution (1% of a 5000 IE/ml heparin (Organon, Oss, The Netherlands) in buffer A, which contains (in mM): NaCl 112, NaHCO\(_3\) 20, KCl 5, CaCl\(_2\) 1.6, NaHPO\(_4\) 2.0, MgSO\(_4\) 1.2, glucose 5, HEPES 10, mannitol 10, l-glutamine 1, sodium butyrate 1, and sodium lactate 1). Buffer A is adjusted on ice to pH 7.15, leading to a pH of 7.4 at 37 °C. Tubules were centrifuged and resuspended in warm, oxygenated (95%O\(_2\) / 5%CO\(_2\)) buffer A containing typically 1.5–3.0 mg protein/ml. The flask was then capped with a rubber stopper and warmed to 37 °C. The supernatant was recovered, incubated for 15 min at 37 °C, and centrifuged after 15 min. The supernatant was returned to the Erlenmeyer and the pellet washed and incubated for 15 min at 37 °C. After 5 min the flasks were sealed.

**Proteolytic activity in situ**

Freshly isolated tubules were warmed to 37 °C, washed and resuspended in oxygenated buffer B at 37 °C containing the succinyl-peptidyl-AMC derivatives, which were added from a 1000-fold concentrated stock solution in DMSO. The following substrates were used, n-succinyl-Ala-Ala-Ala-AMC (10 μM) for serine proteases, benzoylarginylcarbonyl-Arg-Gly-Phenylalanyl-Phe-Phe-Pro-AMC (10 μM) for aspartate proteases, n-succinyl-Leu-Tyr-AMC (10 μM) for cysteine proteases, n-succinyl-Ala-Ala-Phe-AMC (40 μM) for metalloproteases, and n-succinyl-Leu-Leu-Val-Tyr-AMC (10 μM) for calpain [16,21]. When appropriate, glycine was added from a concentrated stock solution (400 mM) to a final concentration of 2 mM, a concentration which provides maximal protection [3]. In view of the high constitutive activity of metalloproteases, the substrate for this class of enzymes was added during the last 5 min of incubation. A control sample to determine background AMC fluorescence was taken at the beginning of the incubation. After 15 min samples were taken to measure intracellular potassium concentration and release of LDH into the medium (see below). To determine the amount of released AMC, protease activity was stopped with 200 μl 4 M perchloric acid (PCA, final concentration 0.36 M) in 2 ml of suspension. The sample was kept on ice for 60 min and neutralized with 600 μl 4 M K\(_2\)HPO\(_4\) (final concentration 0.86 M). After centrifugation (6 min, 1500 g, 4 °C) fluorescence was measured in the supernatant. Proteolytic activity was expressed as the amount of AMC released per period of time corrected for protein content. Separate experiments were performed to calculate the maximal contribution of proteolytic activity in situ to the total amount of AMC released. In these experiments tubules were incubated in the absence of substrates and centrifuged after 15 min. The supernatant was recovered, incubated for 15 min at 37 °C with calpain substrate and the release of AMC was measured. Protein content of the supernatant was determined and the maximal contribution to total calpain activity was calculated.

**Measurement of protease activity**

The activity of the different classes of proteases was assessed using specific cell-permeant protease substrates, which were succinyl-peptidyl-7-amino-4-methylcoumarin derivatives. Proteolytic hydrolysis of the peptidyl-7-amino bond causes the release of the highly fluorescent 7-Amino-4-Methylcoumarin (AMC) [15]. Fluorescence was monitored at 360 nm excitation and 440 nm emission (10 nm band width) in a Shimadzu RF-510 spectrophotofluorometer. Proteolytic activity was measured in two experimental setups, i.e. in situ, measuring the release of AMC from tubules incubated in the presence of the cell-permeant substrates, or in a cysitolic fraction, in which proteolytic enzymes were set free by digitonin treatment of the tubules. The detailed procedures are described below.
Measurements of protease activity in cytosolic fractions

Cytosolic fractions were obtained from permeabilized tubules as described by Edelstein et al. [18]. Aliquots of proximal tubules (6–9 ml) were placed in siliconized Erlenmeyer flasks (25 ml) and were incubated under normoxic or hypoxic conditions without the substrates. After 15 min the suspension centrifuged (10,000 g, 4 °C), the supernatant was discarded and the pellet was resuspended in calcium-free imidazole-HCl buffer (containing in mM: 63.2 imidazole, 10 dithiothreitol, 1 EDTA, 10 EGTA, pH 7.3). This suspension was then incubated with 10 μM digitonin (from a 5 mM stock solution in ethanol) in a shaking waterbath at 37 °C for 5 min. After centrifugation (2 min, 1500 g, 4 °C) protease activity was assayed in the supernatant, using the succinyl-peptidyl-AMC derivatives. The calcium-dependent activity of cysteine and calpain proteases was calculated as the difference between AMC release in the presence and in the absence of 5 mM Ca²⁺. Serine and aspartate protease activity were always measured in the presence of 5 mM Ca²⁺. Briefly, 250 μl supernatant was pre-incubated with 250 μl imidazole-HCl buffer with or without 10 mM CaCl₂ for 10 min at 37 °C in a shaking waterbath. After 10 min 5 or 10 μl substrate was added (final concentrations 226 μM, 163 μM, 100 μM and 100 μM for the cysteine, calpain, serine and aspartate proteases respectively [18]). Imidazole-HCl buffer with or without 5 mM CaCl₂ was added to a final volume of 1 ml. After 30 min the reaction was stopped by adding 100 μl 4 M PCA (final concentration 0.36 M) and the samples were incubated on ice. After 10 min 300 μl 4 M K₂HPO₄ (final concentration 0.86 M) was added to neutralize the PCA and samples were centrifuged (6 min, 1500 g, 4 °C). AMC release was measured as described above.

Effect of glycine on calpain activity

To study the direct effect of glycine on calpain activity, hydrolysis of the calpain substrate by the cytosolic fraction was measured in the presence of glycine. Glycine was added from a concentrated stock solution (400 mM in water) to a final concentration of 2 or 10 mM.

Measurements of LDH, potassium, and protein

Samples to determine LDH-release and intracellular K⁺ content were taken before washing the warmed tubules and after 15 min of incubation. LDH was measured as described previously [22]. Increased release of LDH indicates complete loss of cell membrane integrity and reflects irreversible injury. LDH activity in the supernatant is expressed as a percentage of total LDH activity. Intracellular K⁺ content was indicative for K⁺ loss due to ATP depletion. To determine the intracellular K⁺ content, samples were centrifuged in an Eppendorf centrifuge (Centrifuge 5415C; 15,600 g) through a layer of 700 μl of 1-bromomocadecane (Aldrich, Axel, the Netherlands) into 200 μl of sucrose/ficoll (270 mM sucrose, 4% Ficoll 400, Pharmacia, Woerden, the Netherlands) and further processed for flame photometry (Eppendorf FCM 6343) as previously described [19,23]. Intracellular K⁺ content was expressed as nanomoles per milligram protein. Protein was quantified either by the Lowry method [24] or by using the Bio-Rad Protein assay (Bio-Rad Laboratories, München, Germany).

Materials

The protease substrate benzoylformcarbonyl-Arg-Gly-Phe-Phe-Pro-AMC (Z-Arg-Gly-Phe-Phe-Pro-AMC) was purchased from Enzyme Systems Products (Livermore, CA, USA) and the other substrates were from Sigma (St Louis, Ohio, USA). HEPES was obtained from Research Organics Inc. (Cleveland, Ohio, USA). All other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (St Louis, Ohio, USA) except when stated otherwise.

Statistical analysis

Multiple group comparisons were performed using analysis of variance, with post-tests according to Newman–Keuls (Statview 4.01, Superanov v. 1.11, Abacus Concepts, Inc., Berkeley, CA). Values are given as means± standard error. In case of a non-parametric distribution Wilcoxon’s rank test was used with Bonferoni correction, and values were given as medians with range. A P value less than 0.05 was considered the level of statistical significance.

Results

Effects of hypoxia and glycine on cell injury

During normoxic incubation of freshly-isolated rat proximal tubules a slight increase in LDH release occurred within 15 min (Figure 1A) and intracellular K⁺ content remained elevated (353±8 and 291±17 nmol/mg protein after 0 and 15 min incubation). Hypoxia caused major cell injury after 15 min as indicated by the release of LDH (Figure 1A) and the decrease in intracellular K⁺ content (Figure 1B).

Effects of hypoxia and glycine on protease activity

In situ measurements

The activities of the four known classes of proteases (serine, aspartate, cysteine, and metalloproteases) and of the calcium-dependent cysteine protease calpain were assessed using an in situ assay. All proteases exhibited a measurable activity during normoxia, as shown by the release of AMC from the various substrates (Table 1). Hypoxia caused a marked increase in the activity of serine, aspartate, and calpain proteases (Table 1, Figure 2), while the activities of cysteine and metalloproteases were not affected (Table 1). The addition of 2 mM glycine during the hypoxic incubation reduced the hypoxia-induced calpain activity (Figure 2), but glycine had no significant effect on the hypoxia-induced serine and aspartate protease activities (Table 1). Calpain activity at normoxia was not inhibited by glycine (data not shown). The AMC derivatives did not affect the parameters used to estimate cell injury and the ability of glycine to protect against hypoxia-induced injury (data not shown).

The increased calpain activity measured during hypoxia could have resulted from extracellular substrate
Fig. 2. Calpain activity of rat proximal tubules in suspension incubated for 15 min under: normoxic (open bar), hypoxic (hatched bar), and hypoxic conditions in the presence of 2 mM glycine (closed bar). The specific calpain substrate n-succinyl-Leu-Leu-Val-Tyr-AMC (10 μM) was added during normoxic and hypoxic incubations. Activity was expressed as the amount of AMC released from the substrate per time and per milligram protein. Values are presented as means ± SE. *P < 0.05 vs normoxia, **P < 0.05 vs hypoxia.

% for normoxic and hypoxic suspensions, respectively (not significant), and this can therefore not explain the observed attenuation of hypoxia-induced calpain activity.

Measurements on cytosolic fractions

Tubules were pelleted after 15 min of normoxic or hypoxic incubation and subsequently permeabilized with digitonin (see Subjects and methods). The digitonin-released fraction was assayed for protease activity using the same substrates as used for the in situ measurements. Metalloproteases were not included, since no hypoxia-induced increase in activity was found in the in situ measurements. Fractions recovered from hypoxic tubules exhibited a significantly higher activity in calpain, cysteine, serine, and aspartate proteases than those from the normoxic tubules (Table 2). The addition of glycine during the hypoxic incubation prevented these hypoxia-induced increases in protease activity (Table 2). Both calpain and cysteine protease activities were measured in the presence and in the absence of Ca<sup>2+</sup>. The hypoxia-induced activation as well as the suppressing effect of glycine were comparable in the presence or absence of Ca<sup>2+</sup> (Table 2). The calcium-dependent hydrolysis of the calpain substrate was calculated and is shown in Figure 3. Digitonin-released cytosolic fractions of hypoxic tubules show significantly higher Ca<sup>2+</sup>-dependent protease activities than their normoxic controls, and the hypoxia-induced increase is completely prevented by the presence of glycine during the hypoxic incubation (Figure 3).

To study the direct effect of glycine on calpain activity, we added glycine to the cytosolic fraction assay. Glycine (2 mM) had no effect on the hydrolysis of the calpain substrate measured in the presence of Ca<sup>2+</sup> (Figure 4). Also, a higher concentration of glycine (10 mM) had no significant effect.

Table 1. Activity of proteolytic enzymes in suspensions of rat proximal tubules incubated under normoxic and hypoxic conditions

<table>
<thead>
<tr>
<th>Class of protease</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Hypoxia + 2 mM glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate (pmolAMC/mg protein/15 min)</td>
<td>126 ± 17</td>
<td>252 ± 16*</td>
<td>225 ± 22*</td>
</tr>
<tr>
<td>Cysteine (pmolAMC/mg protein/15 min)</td>
<td>129 ± 26</td>
<td>100 ± 13</td>
<td>103 ± 11</td>
</tr>
<tr>
<td>Serine (pmolAMC/mg protein/15 min)</td>
<td>108 ± 12</td>
<td>196 ± 18*</td>
<td>166 ± 14*</td>
</tr>
<tr>
<td>Metallo (pmolAMC/mg protein/5 min)</td>
<td>4229 ± 150</td>
<td>4680 ± 215</td>
<td>3798 ± 177*</td>
</tr>
</tbody>
</table>

Activity of proteases was assessed by adding specific substrates to the suspension of freshly isolated rat proximal tubules (see Subjects and methods). Values are given as means ± SE in pmol AMC/mg protein/15 min or in pmol/mg protein/5 min. For all experiments: n ≥ 4, *P < 0.05 vs normoxia, **P < 0.05 vs hypoxia.

hydrolysis by proteases that have leaked from damaged tubules into the medium. We have therefore measured calpain activity in incubation medium harvested from a tubular suspension after normoxic or hypoxic incubation (see Subjects and methods). This procedure allowed us to calculate the maximal contribution of leaked proteases to the total substrate hydrolysis. Maximal contribution was 13.2 ± 2.9% and 15.0 ± 0.3% for normoxic and hypoxic suspensions, respectively (not significant), and this can therefore not explain the observed attenuation of hypoxia-induced calpain activity.
Table 2. Activity of classes of proteolytic enzymes in fractions obtained by digitonin-permeabilization of rat proximal tubules

<table>
<thead>
<tr>
<th>Class of protease</th>
<th>Calcium</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Hypoxia + 2 mM glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain</td>
<td>−</td>
<td>29 ± 2</td>
<td>60 ± 3*</td>
<td>30 ± 6*</td>
</tr>
<tr>
<td>Calpain</td>
<td>+</td>
<td>49 ± 4</td>
<td>112 ± 3*</td>
<td>55 ± 10*</td>
</tr>
<tr>
<td>Cysteine</td>
<td>−</td>
<td>1.9 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Cysteine</td>
<td>+</td>
<td>4.0 ± 0.3</td>
<td>7.9 ± 0.3</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>Aspartate</td>
<td>−</td>
<td>80 (60–122)</td>
<td>91 (79–177)</td>
<td>78 (52–131)</td>
</tr>
<tr>
<td>Serine</td>
<td>+</td>
<td>2.7 (2.2–8.2)</td>
<td>5.7 (3.3–9.0)</td>
<td>3.0 (2.2–6.5)</td>
</tr>
</tbody>
</table>

Activity of proteases was assessed by adding specific substrates to digitonin-releasable fractions from rat proximal tubules (see subjects and methods) in the presence (+) or absence (−) of 5 mM calcium. Values are expressed in nmol AMC/mg protein/30 min given as means ± SE or medians (range). For all experiments: n ≥ 4, *P < 0.05 vs normoxia, **P < 0.05 vs hypoxia.

In the above-mentioned experiments measurements were done after 15 min of incubation, a time point at which hypoxia had caused major cell injury. To further delineate the relationship between calpain activation, hypoxia, and glycine’s protection, we have performed additional experiments in which calpain activity and LDH release were measured at 7.5 min. At this time point no significant cell injury was noted (LDH release 11.5 ± 0.7%, 12.5 ± 1.1% and 11.4 ± 1.4% for normoxia, hypoxia and hypoxia in the presence of glycine, respectively, NS). However, also no significant difference in calpain activity was observed (Figure 5).

Discussion

Our experiments demonstrate that hypoxia increases the activity of several classes of proteolytic enzymes. It is of interest that in using two different protocols to assess protease activity, we obtained partially different results. It is possible that by each method a different subpopulation of proteases is preferentially measured, thus providing complementary information on activation of proteolytic enzymes in hypoxia. The first method includes the addition of specific substrates to
normoxic or hypoxic suspensions of proximal tubules. These substrates will be cleaved by their respective proteases after entering the cell and the fluorescent AMC is subsequently released. During hypoxia we observed an increased activity of serine and aspartate proteases, whereas the activity of cysteine and metalloproteases remained unchanged. The activity of calpain, a Ca$^{2+}$-dependent cysteine protease was also investigated and the calpain substrate showed enhanced degradation in hypoxia. A more precise appraisal of calpain activity would include an estimation of the Ca$^{2+}$-dependent substrate hydrolysis, but this is not feasible in the in situ assay. Previous studies showed that addition of the Ca$^{2+}$-chelator EGTA or the use of nominal Ca$^{2+}$-free media amplified hypoxic cell injury in proximal tubules [19]. Glycine significantly attenuated the hypoxia-induced increase in hydrolysis of the calpain substrate, but was without effect on hydrolysis of the other substrates.

One could argue that release of proteolytic enzymes from injured tubules into the medium could have substantially influenced the results. Therefore, we performed experiments to assess the proteolytic activity in the medium after normoxic and hypoxic incubations. The release of proteases in the medium could account for only 10–15% of total calpain activity. Most importantly, no difference was observed between media collected from normoxic and hypoxic incubations. This observation, as well as our experiments with serine and aspartate proteases, indicate that the increased activity observed during hypoxia is not the result of cell lysis and subsequent release of proteases into the medium. In the latter case, glycine, which completely prevents cell lysis, should also have attenuated hypoxia-induced serine and aspartate protease activity, but this was not the case.

It is obvious that the in situ assessment of protease activity, in which a substrate is added to intact tubules, has some disadvantages. The contribution of small amounts of protease activity leaked into the medium to the total protease activity precludes the detection of small changes in enzyme activities. Moreover, it is not feasible to realize a complete and homogeneous distribution of the substrate to the different cellular compartments. We have, therefore, used a second strategy to measure protease activity. Cytosolic fractions of digitonin-permeabilized tubules after hypoxic or normoxic incubation were assessed for protease activity under well-defined experimental conditions [18]. Utilizing this procedure, the Ca$^{2+}$-dependency of the degradation of the calpain substrate could be studied as well. In this situation, hypoxia again led to an increased calpain activity. Hypoxia also increased the activities of the other classes of proteases which were measured (cysteine, serine and aspartate proteases). Glycine present during the hypoxic incubation markedly attenuated all increased protease activities in the cytosolic fractions. These results may seem somewhat conflicting when compared to the results of the in situ assays. One must bear in mind, however, that the two methods are quite distinct and that they yield different kinds of information. In tubular suspensions, overall protease activity is assessed, to which cytosolic as well as membrane-bound proteases and proteases located in cellular organelles may contribute. Furthermore, activity may vary according to the local availability of the substrates. In the cytosolic fraction obtained after digitonin permeabilisation, activity of a subset of soluble proteases is measured under well-defined conditions. Furthermore, it should be appreciated that the overall activity of an entire group of proteases is determined and thus no information can be obtained about specific proteases involved in cell injury. An increased activity of a particular protease may easily go undetected if this increase falls within the variation of the activity of the class of proteases where it belongs to. This is illustrated by the observation that cysteine protease activity is not affected in hypoxia, whereas the activity of calpain, a member of the class of cysteine proteases, is markedly increased.

With respect to calpain the results of the in situ assay and the assay using cytosolic fractions were similar. Hypoxia consistently increased calpain activity. Calpains are calcium-dependent neutral cysteine proteases, forming a family consisting of at least six distinct members [25], and they can be divided in two groups on the basis of their expression pattern: either ubiquitous or tissue-specific. Ubiquitous calpains exist as inactive cysteine proteases (procalpain), which, in the presence of elevated intracellular free Ca$^{2+}$, translocate to the cell membrane. At the cell membrane calcium-dependent autocatalytic activation of procalpain to calpain takes place [26]. Our observations are in line with these properties. Hypoxia induced considerable LDH-release into the medium and stimulated hydrolysis of the calpain substrate, however, no additional calpain was released into the medium (calpain activity in normoxic and hypoxic medium were equal). This indicates that calpain remained bound to an intracellular site after being activated. The activated calpain could be released after treatment with the detergent digitonin.

Much is known about the structural and enzymological properties of the ubiquitous isozymes μ- and m-calpain, but information about their physiological function is limited [25]. It has been suggested that calpain might be important in cell differentiation [25,27], long-term memory [26], regulation of cell adhesion [25] and in signalling pathways [25]. Many physiologically important proteins are candidate substrates for calpain, such as cytoskeletal proteins [27–30], protein kinase C, protein kinase A, phospholipases, protein phosphatases [28], and the plasma membrane Ca$^{2+}$-ATPase [31]. Therefore activation of calpain during hypoxia is likely to have severe consequences for cell function and could initiate a cascade of reactions resulting in cell death. Indeed, several studies have demonstrated that calpain is activated during oxygen deprivation in various non-renal tissues and a correlation between calpain activity and cell injury was shown [15,17,28–30,32]. Furthermore, Edelstein et al. [18] have recently demonstrated an
increased activity of μ-calpain in hypoxic rat proximal tubules, which could be correlated with cell injury. Our study is in close agreement with these findings. Admittedly our experimental data do not allow us to draw definitive conclusions on the exact timing of the activation of calpain in relation to the occurrence of cell injury. Ideally, one should be able to measure intracellular Ca$^{2+}$, calpain activity and cell injury simultaneously. However, this is not feasible in isolated tubules in suspension. In our additional experiments, measuring calpain activity and LDH-release at an earlier time point (7.5 min instead of 15 min), no significant cell injury or calpain activation was observed. After this time point, cell injury and calpain activation became apparent. However, the rapid development of cell injury and the day to day variability which characterize the rat tubule preparation precluded us from dissecting these processes.

The cytoprotectant glycine prevented calpain activity at 15 min, but not at 7.5 min. Theoretically, there are plausible explanations for the suppressive effect of glycine on calpain activation. The small subunit of calpain contains a glycine-rich strand, which is important for membrane association [33]. Glycine could interfere with this interaction, preventing activation of the inactive proenzyme. Secondly, glycine could interact with factors that modulate calpain activity, such as the endogenous calpain inhibitor calpastatin or activators. A direct inhibitory effect of 2 and 10 mM glycine on calpain activation was excluded. Nichols et al. [17] measured the enzyme activity of purified μ- and m-calpain in the presence of different concentrations of glycine. These authors observed an inhibitory dose-response curve, with inhibition starting at 5 mM and being maximal at 10 mM glycine. This suggests that there may be species and tissue differences, since Nichols et al. used purified μ-calpain from human erythrocytes and m-calpain from bovine heart tissue, whereas we used a cytosolic fraction recovered from rat proximal tubules permeabilized with digitonin.

However, although glycine prevented the increase of calpain activity after 15 min of hypoxia, one cannot conclude from our data that inhibition of calpain activity contributes to the protective effects of glycine, since we were unable to demonstrate an increase of calpain activity and an effect of glycine at an earlier time point, i.e. before cell injury occurs. Therefore, it is quite possible that the increased activity of calpain after 15 min of hypoxia is largely explained by the increased calcium influx that occurs when cells become lethally damaged. The attenuation of calpain activation by glycine then merely reflects the ability of glycine to prevent lethal membrane injury and its attendant calcium influx.

With respect to the other proteases it is evident that the results of the in situ assay and the assay using cytosolic fractions were different. When measured in situ, in tubules in suspension, glycine did not attenuate the hypoxia-induced increase of serine and aspartate protease activity. In contrast, glycine attenuated the increased protease activities measured in the cytosolic fractions. It is possible that hypoxia modifies the release of proteins by digitonin, thus biasing the assay, and that glycine influences this behaviour. However, when digitonin-induced protein release of tubules incubated for 7.5 min under different conditions was measured, no difference was found (12.1±3.3%, 11.1±2.8% and 10.9±2.5% of total protein for normoxia, hypoxia, and hypoxia in the presence of glycine respectively, NS). Glycine therefore does not affect the protein release by digitonin treatment. However, theoretically, calculations of the specific activity in the in vitro assay could be influenced by loss of cytosolic proteins, and thus bias the conclusions. We feel that the in situ measurements suggest that glycine has no major effect on the activation of non-calpain proteases.

Studies addressing the role of other proteases in cell injury are very limited. Hepatocytes exposed to cold ischaemia demonstrated increased cytosolic activity of metallo- and aspartate proteases, but not serine and cysteine proteases [16]. Nichols et al. added a substrate specific for metalloproteases to liver cells incubated at anoxic conditions and did not observe an increased hydrolysis of this substrate [17]. Differences in methods as well as tissues may account for the differences in results between these and our studies.

In conclusion, hypoxia increases the cytosolic activity of several classes of proteolytic enzymes in rat proximal tubules. It is unclear whether changes in protease activity precede or merely reflect cell injury. Although glycine attenuated the increase in activity of several classes of proteases, in particular of calpain, it is unlikely that such inhibition of protease activity contributes importantly to the protective effects of glycine. To gain more insight in the role of proteolytic enzymes in cell injury, further studies should be aimed at measuring intracellular ions, protease activity and cell injury simultaneously, and at identifying and characterizing specific proteolytic enzymes and their cellular substrates.

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