Changes in release of N-acetyl-β-D-glucosaminidase, γ-glutamyl transpeptidase and leucine aminopeptidase from renal tubular cells under anaerobic conditions

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Abstract

Although the urinary excretion of NAG and γ-GTP has been employed as an index of renal injury, the mechanism of release of these enzymes remains unclear. In the case of renal ischemic injury, we recently reported that u-NAG and u-γ-GTP were not correlated well with the degree of renal damage especially in the late phase of ischemic injury [1]. In order to elucidate the relationship between release of these enzymes and renal ischemic injury, we devised an in vitro model of anoxic injuries to renal tubular cells and measured the time-course release of NAG (a lysosomal enzyme), γ-GTP and LAP (brush border enzymes) at 37°C under five different aerobic and anaerobic conditions (95% O2, 20% O2, 10% O2, 3% O2 and 0% O2). The ATP and lactate levels in the system were also measured to estimate the metabolic state of the tubular cells. In the 3% O2, 10% O2 and 20% O2 groups, NAG, γ-GTP and LAP were released into the media at a similar rate to that in the 95% O2 group, and anaerobic damage to the renal tubular cells was not observed. In the 0% O2 group, the NAG in the medium increased from 60 to 180 min at a significantly higher rate than that of the 95% O2 group, and a linear relationship was observed between NAG concentration and incubation time (r=0.73), although NAG did not increase significantly at 30 min. The release of γ-GTP and LAP was significantly increased in the 0% O2 group even at 30 min as compared to that in the 95% O2 group, but did not increase significantly after 90 min as compared to that at 60 min in contrast to the release of NAG. Although all three enzymes were released from in vitro renal tubular cells under anaerobic conditions, the release of NAG was found to correlate most precisely with the degree of renal anaerobic damage of the renal tubular cells.

Introduction

In order to assess renal proximal tubular injury, the urinary concentrations of the lysosomal enzyme, NAG, and brush border enzymes, γ-GTP and LAP, are widely employed as indices in clinical examinations [2]. Especially in the case of hypertensive nephropathy, diabetes nephropathy and drug-induced renal disorders, u-NAG has been shown to correlate better with the degree of renal damage than the two brush border enzymes [3, 4]. Furthermore, u-NAG is now extensively utilized to evaluate the level of general renal injury [5]. Nevertheless, the mechanism and changes in urinary levels of NAG, γ-GTP and LAP induced by renal ischemic damage often resulting in renal failure, remain to be elucidated. In a previous clinical study performed during elective operations, we found that the u-NAG levels reflected the degree of renal ischemic disorders [6, 7]. Furthermore, when renal function tests (Ccr, FENa and CH2O) were compared with renal injury indices, such as u-NAG and u-γ-GTP, after different periods of in vivo warm ischemia in the rabbit kidney [8], the urinary excretion of the two enzymes was increased at 60 min after complete renal artery occlusion. Following 90 min of ischemia, however, u-NAG and u-γ-GTP were not significantly increased as compared to the levels
at 60 min, although renal function as represented by Ccr was markedly decreased [1]. These findings might derive from a decreased release of NAG and γ-GTP from the renal proximal tubules or a decreased washing out rate of NAG and γ-GTP into the urine, and suggest that u-NAG and u-γ-GTP do not always provide adequate indices of renal ischemic damage. In the present experiments, we measured the concentrations of NAG, γ-GTP and LAP released from in vitro renal proximal tubular cells under different anaerobic conditions at different incubation periods in order to evaluate the correlation between the release of these enzymes and the anaerobic damage at the cellular level. Based on the results obtained, together with several other vital indices, we discuss whether the most useful index for assessing renal ischemic damage is u-NAG or some other parameter.

Methods

Preparation of suspension of renal tubular fragments: Renal tubular fragments, mainly comprising renal proximal tubular cells, were prepared from rabbit renal cortex by the method of Balaban et al. with slight modifications, which was developed to obtain a functionally intact renal tubular suspension applicable for the analysis of aerobic metabolism [9]. The practical procedures and buffers used were as described in the report of Endou et al. [10]. Male Japanese white rabbits weighing 2.8-3.0 kg were anesthetized with 5% pentobarbital sodium (50 mg/kg). The right and left kidneys were mobilized and perfused via the renal artery with 20 ml of ice-cold Hanks' solution (NaCl, 137; KCl, 5.0; CaCl₂, 1.38; MgCl₂, 1.0; NaH₂PO₄, 1.0; NaHCO₃, 24 mM) by repeated suspension and centrifugation at 25 g for 5 min at 4°C to remove the dispersed cells, cell debris and red blood cells remaining in the supernatant. Light microscopic observation indicated that rather homogeneous tubular fragments were prepared by these filtration and washing procedures. The renal tubular fragments in the pellets were finally suspended by shaking with 7 ml of Krebs-Hensleit solution and then used for the experiments. In the report of Balaban et al., renal tubular fragments were finally purified by centrifugation with Ficoll to remove a large number of nonvital cells [9]. In our experiments, however, more than 95% of the cells were vital and were not stained by trypan blue. We therefore omitted the step of centrifugation with Ficoll.

Five different anaerobic conditions: Five kinds of aerobic and anaerobic conditions were employed, as follows: 1) 95% O₂/5% CO₂ (control group, N=11), 2) 20% O₂/5% CO₂/75% N₂ (20% O₂ group, N=4), 3) 10% O₂/5% CO₂/85% N₂ (10% O₂ group, N=4), 4) 3% O₂/5% CO₂/92% N₂ (3% O₂ group, N=4), and 5) 0% O₂/5% CO₂/95% N₂ (0% O₂ group, N=17). Twenty ml flasks containing 3.5 ml of Krebs-Hensleit solution were bubbled with each gas at a flow rate of 6 L/min for 30, 60, 90 and 120 s, respectively, and the pH, pCO₂ and pO₂ of each solution were measured with a blood gas analyzer (Corning 178). Under these conditions, the pO₂ was constant but the pCO₂ and pH were changed by flushing with these gases for over 90 s. Therefore, 90 s flushing with each gas at a flow rate of 6 L/min was used for the experiments.

Incubation system: To 0.5 ml of the suspension of renal tubular fragments, 3.5 ml of Krebs-Hensleit solution were bubbled with each gas at a flow rate of 6 L/min for 30, 60, 90 and 120 s, respectively, and the pH, pCO₂ and pO₂ of each solution were measured with a blood gas analyzer (Corning 178). Under these conditions, the pO₂ was constant but the pCO₂ and pH were changed by flushing with these gases for over 90 s. Therefore, 90 s flushing with each gas at a flow rate of 6 L/min was used for the experiments.
then incubated at 37°C for 0, 30, 60, 90, 120, and 180 min. After incubation, each resulting suspension was centrifuged at 1100 g for 10 min at 4°C. The supernatants were collected, and the activities of the three enzymes (NAG, γ-GTP and LAP) and the concentration of lactate released into the media were measured as described below. Renal tubular fragments in the pellets were homogenized with a Polytron mixer for 30 s in 0.6 M perchloric acid solution for deproteinization. After centrifugation at 1100 g for 10 min, the resulting supernatant was used for determination of the ATP contents [13]. The protein contents of the renal tubular fragments were measured by the method of Lowry after complete homogenization of the original tubular fragment suspension aliquoted at 0 min. Although we usually prepared the tissue suspensions starting from 0.2 g wet tissue/7 ml of Krebs-Hensleit solution, the data were corrected on the basis of the protein concentration. Thus, all experimental data are shown as NAG/P, γ-GTP/P, LAP/P, lactate/P and ATP/P, where P is the protein concentration. The total enzyme activities of NAG, γ-GTP and LAP in the renal tubular fragments at 0 min were also measured after complete homogenization of the original tubular suspension to estimate the percentage of released enzymes. The viability of the cells exposed to each gas composition and at each incubation time was examined by the trypan blue test [14], and the pO2, pCO2 and pH of the incubation medium were also monitored during the incubation period [12].

Measurements of NAG, γ-GTP, LAP, ATP and lactate: a) The assay of NAG was performed by the method for NAG activity estimation with sodi-m-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide employing a commercially available kit (Shionogi, Osaka, Japan). b) The activity of γ-GTP was assayed by using γ-glutamyl-p-N-hydroxethylaminoanilide as a substrate (Wako, Osaka, Japan). The reaction product was determined by measuring the absorbance at 660 nm. c) The LAP activity was assayed by using l-leucyl-p-N,N-disulphopropylaminoanilide as a substrate (Kyowa, Tokyo, Japan). d) The concentration of l-lactate was estimated from the oxidative reaction of l-lactate to pyruvate with lactate dehydrogenase. The substrates and enzymes were purchased from Boehringer (Mannheim, Germany). The increase of NADH in this reaction was determined by measuring the absorbance at 340 nm. e) The concentration of ATP was assayed as follows. ATP and glycerate 1-phosphate were first converted to glyceraldehyde 1,3-diphosphate enzymatically. The resulting glyceraldehyde 1,3-diphosphate consumed NADH when it was further converted into glycerol 1,3-diphosphate. The amount of NADH consumed was estimated by measuring the absorbance at 340 nm. The reagents used were purchased from Boehringer as a kit.

Statistical analysis: Statistical analysis was performed by one-way analysis of variance, the Dunnett t-test and Scheffe F-test.

Results

Renal tubular cell conditions during the incubation periods:

First, the viability and metabolic states of the renal tubular cells in the control group exposed to 95% O2/5% CO2 were examined throughout the incubation periods (Fig. 1). In the control group,
the ATP contents in the tubular fragments increased gradually, but no such rise was observed in the other incubation groups. The lactate released into the media increased linearly from 0 to 180 min, especially from 0 to 30 min (p<0.01). However, no significant changes were observed from 30 to 90 min. The CO2 product (CO2 released from the tubular fragments) in the media also increased up to 60 mmHg at 120 min. As a result of aerobic metabolism, accumulation of CO2 product lowered the pH of the media from 7.41 to 7.21 at 120 min. On the other hand, the tubular cells in the fragments were hardly stained by trypan blue by the end of incubation.

The ATP contents of the tubular fragments after different incubation periods varied according to the O2 composition of the incubation atmosphere (Fig. 2). As mentioned above, the ATP contents of the fragments increased in the control group and did not change significantly in the 20% O2 group. In the 10% O2 group, the cellular ATP level decreased significantly after 60 min (P<0.01). Under conditions of less than 3% O2, the ATP contents in the fragments fell significantly even at 30 min incubation (P<0.01).

The lactate concentration in the media was determined in the present study, since renal tubular fragments were used for measurement of the ATP content, and the lactate released into the media was stable during incubation and storage. As shown in Fig. 3, the lactate concentration in the media increased almost linearly in every incubation group. The rate of lactate release from the tubular cells was at a minimum in the control group exposed to 95% O2, and the 20% and 10% O2 groups revealed comparable rates of lactate release. In the 3% O2 group, the lactate concentration in the incubation media increased rapidly from 30 to 120 min (P<0.01), and its value was about 6 times higher than that in the control group at 120 min. However, in the 0% O2 group, the lactate release rate decreased nearly to the levels in the 10% and 20% O2 groups.

In the 0% O2 group, the CO2 product did not increase even after 120 min incubation. The ATP in the renal tubular fragments decreased significantly from 30 min incubation and was consumed completely after 120 min incubation (P<0.01). Furthermore, the tubular cells were...
clearly stained by trypan blue after 90 min incubation.

Time-course changes in concentrations of three enzymes during incubation:

In the control group, NAG, γ-GTP and LAP were released into the media, and their concentrations increased significantly between 0 and 30 min (P<0.01), as shown in Fig. 1. The concentrations of these enzymes did not increase significantly after 60 min.

The release of NAG, γ-GTP and LAP from renal tubular suspensions into the media under the five different O2 concentrations is illustrated in Figs. 4-6. For these three enzymes, the amounts of enzymes released into the media were not significantly different among the 95% O2, 20% O2, 10% O2, and 3% O2 groups at any incubation time. In the case of the 0% O2 group, however, the amounts of γ-GTP and LAP in the media were higher than those under the other incubation conditions at each incubation time, and NAG was higher than that after 60 min incubation. In particular, in the case of NAG, the value of NAG/P increased significantly between 30 and 90 min (P<0.01) and between 60 and 90 min (P<0.05), and there was a linear relationship between NAG/P and incubation time (r=0.73), as shown in Fig. 4. Furthermore, the values of NAG/P in the 0% O2 group were significantly higher than those of the other incubation groups after 60 min (P<0.01).

The concentrations of released γ-GTP and LAP in the media in the 0% O2 group increased significantly at incubation times of 30-120 min as compared to those under the other four conditions (P<0.01) (Figs. 5 and 6). γ-GTP/P and LAP/P increased significantly between 0 and 60 min (P<0.05). After 60 min, the concentrations of these two enzymes remained at nearly the same levels, increasing slightly between 120 and 180 min. The differences in γ-GTP/P and LAP/P between 60 and 180 min were not statistically significant, in contrast to that of NAG/P.

The ratio of the released enzyme activity to the total enzyme activity in the renal tubular suspension system is shown in Fig. 7. The three enzymes were completely stable during the incubation, and the total amount of each was constant within the
Fig. 6. Release of LAP under five aerobic and anaerobic conditions. Data are shown as LAP/P. Values are expressed as the mean \( \pm \) S.E. * \( P < 0.01 \), compared to the 95% O2 group.

Fig. 7. Ratio of released enzymes to total enzyme contents during incubation in the 0% O2 group. The ratio is expressed as the released enzyme activity/total enzyme activity in the renal tubular fragments at 0 min. Values are expressed as the mean \( \pm \) S.E.

Discussion

In the present study, renal tubular cells suspended as fragments in the media needed to survive in a good condition for up to 180 min of incubation in order to evaluate the anaerobic damage. Therefore, we first investigated the viability and metabolic states of the renal tubular cells under all the experimental conditions employed, using three indices including the cellular ATP contents, lactate concentration in the media and trypan blue staining of the tubular cells. In the 95% O2 group, the ATP contents in the renal tubular cells increased gradually, and the lactate release into the media was the lowest among the five incubation groups (Figs. 2 and 3). Although the accumulation of CO2 product generated by aerobic metabolism lowered the pH of the media from 7.40 to 7.21 at 120 min incubation, Kleinman et al. [15] have shown that renal cell function can remain normal even when the extracellular pH is as low as pH 7.02. Furthermore, the tubular cells were not stained by trypan blue even at the end of incubation. The metabolic state of the renal tubular cells in the 95% O2 group was therefore concluded to be at least sufficiently good as to maintain cellular function [16, 17].

During the time-course, the ATP contents in the renal tubular fragments were found to shift towards 0 as the O2 composition in the incubation system decreased [13]. Concerning the lactate concentration in the media, the highest lactate release was observed in the 3% O2 group and the level was rather inhibited in the 0% O2 group. With the trypan blue assay system, most of the tubular cells in the 0% O2 group were stained at the end of the incubation. These findings indicate that the incubation system of the 0% O2 group was sufficient to induce anaerobic damage to the tubular cells. Therefore, in the following discussion, we compare limits of experimental error. Thus, the data shown in Fig. 7 represent the rate of enzyme release from the cells into the media. The ratio of released NAG to total enzyme increased linearly and significantly from 60 to 180 min as compared to that in the 95% O2 control group. On the other hand, the ratio of released \( \gamma \)-GTP and LAP increased significantly from 0 to 60 min, but increased only gradually after 60 min.
the data for the 0% O₂ group with those for the 95% O₂ group.

As shown in Fig. 4, the NAG/P value of the 0% O₂ group increased significantly from 60 to 180 min. The NAG concentration in the media at 180 min was about 14 times higher than that at 0 min, while the NAG/P of the 95% O₂ group increased gradually and was about 4 times higher at the end of the incubation than that at 0 min. On the other hand, the γ-GTP/P and LAP/P values already increased significantly between 0 and 60 min, but remained at a comparable level after 60 min (Figs. 5 and 6). The ratios of released γ-GTP and LAP to the total cellular enzymes were 2 and 8 times higher than that of NAG at 60 min, but were 1.3 and 3 times higher than that of NAG at 180 min, respectively. These results indicate that γ-GTP and LAP respond to anaerobic damage of the renal tubular cells from a relatively early stage. NAG release from the tubular cells had a slow onset, and there was no difference in NAG concentration between the 0% and 95% O₂ groups at 30 min. However, the NAG in the media increased linearly as the exposure time of the tubular fragments to the 0% O₂ environment was extended from 30 to 180 min.

Among the three enzymes, the lysosomal enzyme NAG was found to correlate better with the anoxic injuries than the other two enzymes. The discordance noted between NAG and γ-GTP or LAP in response to anaerobic damage was probably related to differences in their localization in the cell. Morphological changes of the proximal tubules in the rat kidney induced by transient ischemia have been examined in in vivo systems [18-21]. At 30-60 min of ischemia, the microvilli in the brush border were swollen and distended tortuously, and the membranes of the microvilli occasionally formed myelin figures in the lumen at 120 min. On the other hand, Mergner et al. [22] reported that 60 min of ischemia did not cause serious morphological changes to the lysosomes and that slight enlargement and swelling of the lysosomes were observed with clearing of the matrix. At 120 min of ischemia, lysosomal hydrolases including NAG spread in parallel to degradation of the cellular organelles and a portion of the lysosomal enzymes leaked out of the tubular cells [23]. Since NAG was stable and the total amount of NAG in the system was constant during the incubation period, the concentration of NAG in the media probably corresponded to the degree of morphological change and destruction of the lysosomal membrane. On the other hand, γ-GTP and LAP are localized in the brush border and are thought to be released from tubular cells by shorter exposure to anaerobic conditions. The present data, together with the previous observations described above, suggest the possibility that release of lysosomal enzyme NAG into the media may reflect the destruction of the membrane structure in the renal tubular cells, which probably leads to cell death. The ratio of NAG released from the cells may thus correlate better with the degree of anoxic injury of the proximal tubular cells than the corresponding values for the brush border enzymes, γ-GT and LAP.

In our previous in vivo ischemic model of complete renal artery occlusion, Ccr and C_H₂O decreased in proportion to the ischemic period, and they were clearly recognized as valid indices for evaluating renal function [1]. In that system, the levels of u-NAG increased significantly from 30 to 60 min of occlusion time, although they did not change significantly with longer occlusion. Thus, u-NAG was considered to be an appropriate index only for the early stage of renal tubular cell damage. As mentioned above, however, NAG is thought to represent a better index for evaluating the tubular cell damage in the in vitro assay system used in the present study, since the NAG release is apparently correlated with the anaerobic damage to the tubular cells up to 180 min. Such a discrepancy between in vitro and in vivo assay systems may reflect obstruction of the secondary excretion step of NAG after it has leaked out of the tubular cells. These findings suggest that in clinical cases, in order to evaluate the renal ischemic injury, u-NAG should be monitored in relation to the renal function indices, Ccr, C_H₂O and FENa.

In the 20% O₂, 10% O₂, and 3% O₂ groups, anaerobic metabolism tended to be predominant in accordance with decrease of the oxygen concentration. However, the NAG, γ-GTP and LAP levels increased gradually in a similar manner to those of the 95% O₂ group. On the other hand, the time-courses of ATP/P and lactate/P during
the experiments were markedly different under each incubation condition. Especially in the case of the 3% O₂ group, lactate/P increased linearly and significantly from an incubation time of 30 min, while the concentrations of all the three enzymes in the 3% O₂ group were comparable to those of the 95% O₂ group. These findings suggest that the metabolic change induced by a low oxygen environment is not directly synchronized with the damage to the renal tubular cells, i.e. destruction of the cell membrane organization takes place after the occurrence of metabolic changes in the cell. Although the significant increase in lactate release in the 3% O₂ group may be due to accelerated glycolysis in order to supply NAD⁺ under the anaerobic conditions of the 3% O₂ group, the lactate concentration in the 0% O₂ group was less than one third of that in the 3% O₂ group and was no less than that in the 95% O₂ group [19, 20]. This could be explained by the fact that activities of enzymes such as hexokinase which function in glycolysis are inhibited by the completely anaerobic conditions of 0% O₂ used in the present study. These differences observed between the 0% O₂ and 3% O₂ groups are also thought to support the dissociation of metabolic change and cellular damage [24].

Conclusion

Under completely anaerobic conditions of an in vitro renal tubular suspension system, the lysosomal enzyme NAG leaks out into the media almost linearly with incubation time. The brush border enzymes, γ-GTP and LAP, are also released from the tubular cells even at an early stage of anoxic damage but do not increase significantly at extended exposure to anaerobic conditions. NAG is thought to represent a more useful index than γ-GTP and LAP for estimating the anoxic damage to the renal tubular cells in the in vitro system.

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Abbreviations: NAG, N-acetyl-β-D-glucosaminidase; γ-GTP, γ-glutamyl transpeptidase; LAP, leucine aminopeptidase; Ccr, creatinine clearance; FENa, fractional excretion of Na; C₁H₂O, free water clearance; u-, urinary excretion of.

The LAP activity was estimated using l-leucine-p-N,N-disulfopropanoamide as a substrate. Although this LAP activity, which is usually employed for clinical examinations, is derived from alanine aminopeptidase (arylamidase) [EC 3.4.11.2], we designate it as “LAP” in this paper according to conventional terminology.

References

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