TRANSLOCATION OF AMINO ACIDS AND GLUTATHIONE STUDIED WITH THE PERFUSED KIDNEY AND ISOLATED RENAL CELLS

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1. Introduction

The central role of the kidney in the metabolism of plasma glutathione is now well established [1,2], and recent studies have elucidated a series of oxidative and degradative reactions involved in renal metabolism of extracellular glutathione [3,4]. The kidney itself contains a high intracellular level of reduced GSH, present mainly in proximal tubular cells, which undergoes a continuous turnover at high rate. The steady state concentration of renal GSH is a function of its utilization and resynthesis, both of which are influenced by various physiological factors and amenable to experimental manipulation.

The involvement of the 'γ-glutamyl cycle' in amino acid translocation across the tubular epithelium is strongly suggested by [5]. Observations supporting this theory have been made in our laboratory, on the basis of experiments with isolated renal tubular cells [6], and is also presented in several reports of amino acid transport in yeast [7–9]. The physiological significance of this transport system for the uptake of amino acids from the glomerular filtrate has, however, not yet been strictly defined.

The function of γGT in the renal degradation of extracellular glutathione has, however, been proven [2,3], and a coupling between extra- and intracellular glutathione turnover in the kidney appears probable. Moreover, renal 'extraction' of plasma glutathione has been observed in rodents in vivo [10] and in experiments with recirculating perfusion of an isolated organ system [11]. With isolated rabbit kidneys the addition of glutathione to the perfusate has been found to counteract the loss of intracellular GSH otherwise seen during ischaemic perfusion, suggesting a supporting role of extracellular glutathione on maintenance of renal GSH concentration [11]. The nature of renal 'extraction' of extracellular glutathione, i.e., whether this process involves uptake of intact glutathione molecules or absorption of constituent amino acids after extracellular hydrolysis, has not yet been clarified.

Here, we have used the perfused rat kidney and freshly isolated renal epithelial cells to further characterize the turnover of intracellular glutathione in the kidney. Renal perfusion with a buffer devoid of precursor amino acids for GSH synthesis, but containing other amino acids able to act as γ-glutamyl acceptors in the transpeptidation reaction [12], led to a considerable loss of glutathione from the tissue. This effect became even more pronounced under conditions of inhibited GSH synthesis, and it could be abolished by ureter ligation. Supplementing the perfusate with glutathione efficiently counteracted renal GSH depletion, an effect which was scarcely influenced by inhibition of cellular GSH synthesis. In isolated renal cells, containing ~30% of their normal amount of glutathione, replenishment of intracellular GSH occurred rapidly, when the incubation medium contained GSH, GSSG or equimolar concentrations of the constituent amino acids. Amino acid-dependent GSH resynthesis rate could be lowered by 50–60% by inhibition of γGT with serine · borate, or of γ-glutamylcysteine synthetase with methionine sulfoximine. The rate of replenishment of intracellular GSH in presence of exogenous glutathione was

Abbreviations: GSH, reduced glutathione; GSSG, glutathione disulfide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; γGT, γ-glutamyltransferase. The term 'glutathione' is used without inference to the redox state; it thus includes GSH as well as GSSG

much less sensitive to methionine sulfoximine, but more sensitive to serine · borate.

Taken together, these observations support a role of renal glutathione in amino acid translocation as well as a supportive function of exogenous glutathione on renal intracellular GSH level. The latter effect appears to consist partly of γGT-mediated, direct uptake of GSH or GSSG and partly of the uptake of the constituent amino acids (released by γGT-initiated hydrolysis of extracellular GSH) which are subsequently utilized for GSH biosynthesis.

2. Materials and methods

2.1. *Animals and chemicals*

Male Sprague-Dawley rats (190–230 g) were fed water and pelleted rat food ad libitum. With the animals in ether anaesthesia surgical procedures for isolation of the kidneys were performed as in [13]. Renal tubular cells were isolated according to [14].

GSH, GSSG and L-methionine-D,L-sulfoximine were obtained from Sigma Chemical Co., St Louis, MO. All other reagents were of analytical grade and purchased from local commercial sources.

2.2. *Experimental procedure*

Isolated renal cells were incubated in rotating, round-bottom flasks at 37°C in a modified Krebs-Henseleit buffer (pH 7.4) supplemented with 25 mM Hepes, and continuously gassed with carbogen (95% O_2, 5% CO_2). Samples were removed at regular intervals, the cells were washed once with Krebs-Henseleit buffer (pH 7.4) and subsequently deproteinated with 5% HPO_3. Cells partly depleted of glutathione were obtained by perfusing the kidneys for 5 min with a calcium-free Hanks' solution containing 4 μM diethylmaleate, diluted in dimethyl sulfoxide (1:10), before adding collagenase to the perfusate [6].

Isolated kidneys were perfused in vitro, with modified Krebs-Henseleit buffer (pH 7.4) supplemented with 25 mM Hepes, in a thermostated glass chamber at 37°C at a pump pressure of 60 cm H_2O, and a flowrate of ~3 ml . kidney^{-1} . min^{-1}. The perfusate was recirculated and continuously gassed with carbogen. In the beginning of each experiment, both kidneys were perfused simultaneously with buffer. After 2 min, the right renal artery was ligated and the right kidney removed and placed in ice-cold 0.25 M sucrose. When indicated, additions were made to the perfusate, which was further recirculated through the remaining left kidney for 15 or 30 min. Finally, the kidney was flushed gently with 25 ml buffer to remove residual perfusate additions from the vascular bed.

The kidneys were homogenized in ice-cold 0.25 M sucrose, and the homogenate was diluted to 5 ml/g kidney wet wt.

2.3. *Assays*

GSH was assayed in deproteinated aliquots of cell incubates or kidney homogenates according to the spectrophotometric method in [15]. Protein was measured as in [16].

3. Results and discussion

3.1. *Amino acid-mediated decrease in renal GSH level*

Perfusion of isolated kidneys with a medium containing amino acids known to act as γ-glutamyl acceptors in the γGT-mediated transpeptidation reaction [12], led to a loss of cellular GSH (fig.1). The effect

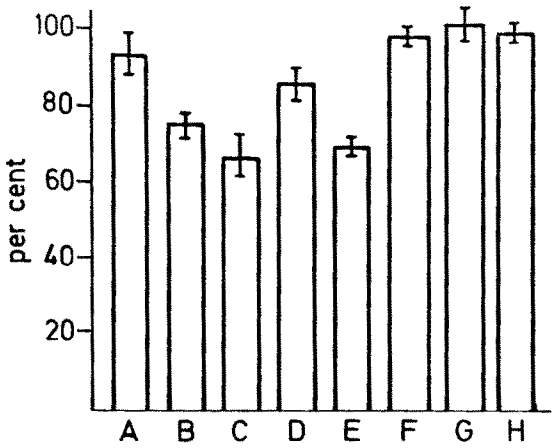

Fig.1. GSH content in homogenate of rat kidneys perfused in vitro with a modified Krebs-Henseleit buffer (pH 7.4) supplemented with Hepes, 25 mM and continuously gassed with carbogen (95% O_2, 5% CO_2). Both kidneys were perfused with this buffer for 2 min, then the right kidney was removed and the left kidney perfused for another 15 min with additions to the perfusate as indicated. Results are given as % ± SD of left renal GSH level compared to right renal GSH content from the same rat. Letters indicate additions to perfusate after removal of right kidney: (A) none; (B) glycylglycine, 0.2 mM; (C) glycylglycine, 1 mM; (D) methionine, 0.2 mM; (E) methionine, 1 mM; (F) methionine, 1 mM + serine · borate, 20 mM; (G) serine · borate, 20 mM; (H) methionine, 1 mM and ureter closed.

was concentration dependent and could be abolished by adding serine · borate to the perfusate or by ligating the ureter to cessate glomerular filtration.

A similar effect of methionine, glycylglycine and some other amino acids on renal GSH level has been observed in vivo [5], as well as in isolated kidney cells [6], and has been taken as an indication of the participation of the γ-glutamyl cycle in renal amino acid translocation. The present results provide additional support for this hypothesis and are also in accordance with histochemical [17], immunocytochemical [18] and functional [19] evidence for a strictly luminal localization of renal tubular γGT.

3.2. *Maintenance of GSH in isolated perfused kidneys*

A rapid turnover of renal GSH has been demonstrated in vivo [5] as well as with isolated kidney cells [6]. Fig.2 illustrates the effect of 30 min kidney perfusion on renal GSH level. Perfusion with Krebs-Henseleit buffer led to a 24±5% decrease, which became even more pronounced in the presence of the γ-glutamyl synthetase inhibitor, L-methionine-D,L-sulfoximine, indicating continuous resynthesis of GSH from endogenous precursors in the absence of this inhibitor. Addition of glutathione to the perfusate, either in reduced form (C, D) or in oxidized (disulfide) form (E, F), prevented this loss, and a slight increase in renal GSH content could be observed. Inhibition of GSH resynthesis by methionine sulfoximine affected the supportive effect of extracellular glutathione only slightly, suggesting that this effect was not solely due to perfusate glutathione acting as a reservoir for the constituent amino acids made available through γGT-initiated breakdown. Ligation of the ureter prevented GSH loss during perfusion, and also abolished the expected increase in renal glutathione in the presence of glutathione in the perfusate. Since ureter ligation leads to a rapid cessation of glomerular filtration, these observations suggest that the involved translocation process is located on the luminal side of the tubular epithelium.

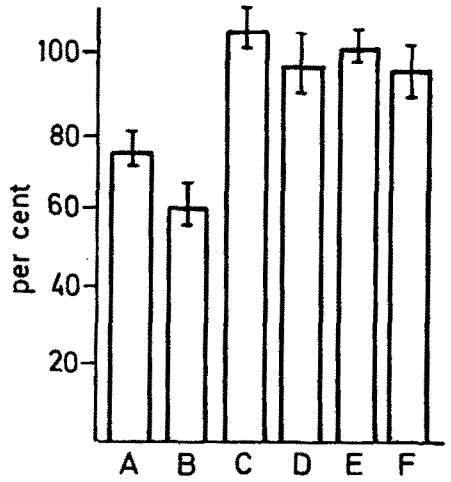

Fig.2. GSH content in homogenate of rat kidneys perfused in vitro under the same basic conditions as in fig.1. The right kidney was removed after 2 min perfusion with buffer, while the left kidney was perfused for another 30 min with additions to the perfusate as indicated. Results are given as % ± SD of left renal GSH level compared to right renal GSH content from the same rat. Letters indicate additions to perfusate after removal of right kidney: (A) none; (B) L-methionine-D,L-sulfoximine, 5 mM; (C) GSH, 1 mM; (D) GSH, 1 mM + L-methionine-D,L-sulfoximine, 5 mM; (E) GSSG, 0.5 mM; (F) GSSG, 0.5 mM + L-methionine-D,L-sulfoximine, 5 mM.

3.3. *Replenishment of GSH in tubular cells isolated from diethylmaleate-treated kidneys*

Prompted by our observations on GSH resynthesis in partly depleted renal cells [6] and by the present indication for a supportive effect of extracellular glutathione on GSH level in the perfused rat kidney, we proceeded to incubate cells isolated from diethylmaleate-perfused kidneys in media containing various combinations of compounds which could be expected to influence intracellular GSH concentration. Rates of cellular GSH replenishment, as measured during the first 10 min incubation period, are presented in table 1. Cells from diethylmaleate-treated kidneys contained ~30% of their normal glutathione, and in the absence of exogenous precursors they were able to resynthesize GSH at a low, but measurable rate. The presence of methionine sulfoximine in the medium abolished GSH replenishment under these conditions and led to a gradual further loss of cellular GSH (not shown) accompanied by a loss of cell viability as judged by a decrease in trypan blue exclusion frequency. In the presence of glutamate, glycine and cystine in the medium, GSH replenishment occurred at ~0.85 nmol . 10^6 cells^{-1} . min^{-1}. Inhibition of γGT by serine · borate lowered this rate by 60%, whereas methionine sulfoximine at the concentration used inhibited the amino acid-mediated GSH replenishment by some 50% (table 1).

Addition of GSH or GSSG to the incubation medium was more effective than addition of the pre-

Table 1
Replenishment of cellular GSH in isolated cells from rat kidneys pretreated with diethylmaleate to lower intracellular glutathione levels

Addition	GSH replenishment rate (nmol . 10^6 cells^{-1} . min^{-1})
None (15)	0.11 ± 0.05
GSH, 1 mM (4)	1.12 ± 0.30
GSH, 1 mM + serine · borate, 20 mM (3)	0.07 ± 0.03
GSH, 1 mM + L-methionine-D,L-sulfoximine, 5 mM (3)	0.82 ± 0.22
GSSG, 0.5 mM (4)	1.02 ± 0.34
GSSG, 0.5 mM + serine · borate, 20 mM (3)	0.08 ± 0.03
GSSG, 0.5 mM + L-methionine-D,L-sulfoximine, 5 mM (3)	0.78 ± 0.24
Glu, 1 mM + Gly, 1 mM + Cys–*S*–Cys, 0.2 mM (3)	0.84 ± 0.12
Glu, 1 mM + Gly, 1 mM + Cys–*S*–Cys, 0.2 mM + serine · borate, 20 mM (3)	0.32 ± 0.10
Glu, 1 mM + Gly, 1 mM + Cys–*S*–Cys, 0.2 mM + L-methionine-D,L-sulfoximine, 5 mM (3)	0.40 ± 0.14

At the beginning of the incubation cellular GSH was 8.9 ± 1.2 nmol/10^6 cells. Incubations were performed in a medium consisting of modified Krebs-Henseleit buffer (pH 7.4) supplemented with 25 mM Hepes and additions as indicated. Results are given as mean ± range; no. expt. listed in parentheses

cursor amino acids in stimulating GSH replenishment in partly depleted cells, and no difference seemed to exist between GSH and GSSG in this respect (table 1, fig.3). Serine · borate abolished the effect of exogenous glutathione completely, whereas methionine sulfoximine was a markedly less effective inhibitor of glutathione-mediated GSH replenishment as compared to its effect on the amino acid-stimulated GSH accumulation (table 1). Taken together with the kinetic data shown in fig.3, these observations support a double effect of extracellular glutathione on replenishment of intracellular GSH in the kidney:

(i) GSH is degraded extracellularly in a sequence of hydrolytic reactions mediated by γGT and cysteincylglycine dipeptidase [3,4] and the constituent amino acids are subsequently taken up and utilized for GSH resynthesis;

(ii) Extracellular glutathione is translocated directly without prior degradation to constituent amino acids.

γGT activity appears to be involved also in the latter translocation process, since the supportive effect of

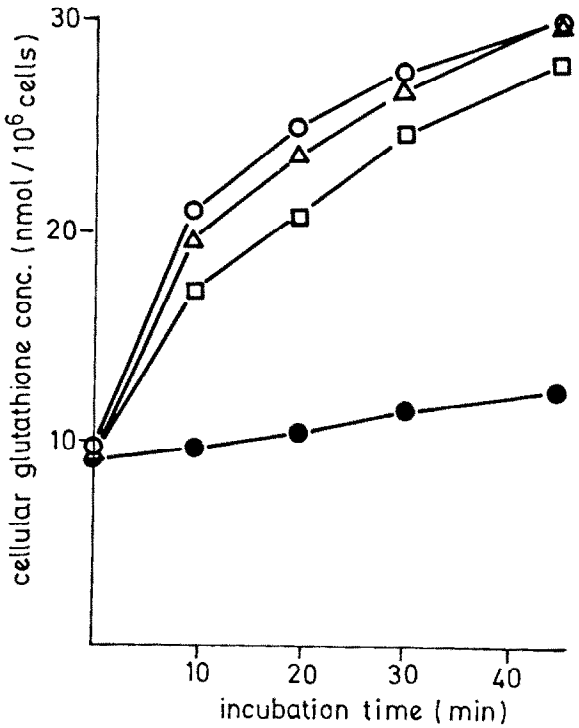

Fig.3. GSH concentration in cells isolated from diethylmaleate-pretreated kidneys. Incubations were performed at 37°C under carbogen atmosphere in a medium consisting of Krebs-Henseleit buffer (pH 7.4) supplemented with Hepes, 25 mM. Additions as indicated: (●——●) none; (△——△) GSSG, 0.5 mM; (○——○) GSH, 1.0 mM; (□——□) Glu, 1.0 mM, Gly, 1.0 mM + Cys–*S*–Cys, 0.2 mM. One experiment typical of 3.

extracellular glutathione on GSH reaccumulation in renal cells is abolished by serine · borate.

From these experiments it appears that extracellular glutathione may play a role in the maintenance of optimal intracellular GSH level in the kidney, partly by supplying precursor amino acids for intracellular GSH biosynthesis and partly by direct uptake into renal cells. In both cases translocation most probably occurs across the brush border membrane facing the tubular lumen. However, direct uptake of glutathione may be of significance only under extreme conditions, e.g., [10,11] and our own perfusion experiments with glutathione levels in the perfusate 200–300-times above the physiological concentration. Under physiological conditions the concentrations of glutathione in plasma and glomerular filtrate are only in the μM range [10,20]. Filtered glutathione is very rapidly hydrolysed in the tubular lumen [19] and thus will be accessible for reuptake in the form of its constituent amino acids. The rate of uptake of glutathione as such will be dependent on its relative affinity for the γ-glutamyl acceptor site of the enzyme, as well as on the presence of other potential acceptor amino acids in the tubular fluid. Under physiological conditions, the total filtered load of amino acids far exceeds that of glutathione, and thus one can hardly expect glutathione to be accessible for direct translocation into renal cells. The physiological importance of plasma glutathione for maintenance of renal GSH level is therefore difficult to evaluate quantitatively. On the other hand, beneficial effects such as improvement of rheological parameters as well as of tubular function has been reported to result from perfusion of kidneys removed for transplantation with glutathione [11], and similar studies are now in progress.

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