0031-3998/92/3206-0710$03.00/0
PEDIATRIC RESEARCH

Vol. 32, No. 6, 1992
Printed in U.S.A.

Intracellular Cystine Loading Causes Proximal Tubule Respiratory Dysfunction: Effect of Glycine

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ABSTRACT. The present study examined proximal tubular respiration in control proximal tubules and proximal tubules loaded with cystine using 2 mmol/L cystine dimethyl ester. Basal oxygen consumption was significantly less in cystine-loaded tubules (20.6 ± 0.5 *versus* 12.1 ± 0.6 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, $p < 0.001$). In the presence of 10^{-4} mol/L ouabain, an inhibitor of the NaK ATPase, oxygen consumption was 10.2 ± 0.7 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in control tubules and 11.4 ± 1.0 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in cystine-loaded tubules. Thus, proximal tubular intracellular cystine loading specifically inhibits oxygen metabolism directed toward transport. Compared with control proximal tubules, cystine-loaded proximal tubules also had a lower rate of O_2 consumption when the cells were permeabilized to sodium with nystatin and when mitochondrial respiration was uncoupled. Glycine, an amino acid that is cytoprotective to hypoxic proximal tubule injury, ameliorated the repiratory dysfunction observed in cystine-loaded tubules. (*Pediatr Res* 32: 710–713, 1992)

Abbreviations

NaK ATPase, sodium-potassium ATPase
CCCP, *p*-chloromethyoxyphenylhydrazone
CDME, cystine dimethyl ester

Cystinosis is an autosomal recessive disease characterized by high intracellular concentrations of the amino acid cystine (1–3). Cystine accumulates predominantly in lysosomes where a defect in cystine transport has been demonstrated (2–6). Cystine accumulation leads to dysfunction of several organs, including the kidney, where it is the most common inherited cause for the Fanconi syndrome in children (1). There is no animal model for the Fanconi syndrome of cystinosis. However, cells can be loaded with high concentrations of intracellular cystine using CDME (7, 8). CDME diffuses across cell membranes where intracellular esterases liberate the relatively impermeable cystine in high concentrations. Foreman *et al.* (9) have demonstrated that proximal tubules can be loaded with cystine using CDME to levels comparable to that in kidneys of patients with cystinosis.

We have recently demonstrated that intracellular cystine loading using CDME produces a decrease in volume absorption, transepithelial potential difference, glucose, and bicarbonate transport in proximal convoluted tubules perfused *in vitro* (10). There was no change in the permeability of proximal convoluted tubules to either mannitol or bicarbonate, indicating that the decrease in transport was due to an inhibition in active transport (10). Although intracellular cystine loading in proximal convoluted tubules did not affect V_{max} of NaK ATPase activity, it produced a profound decrease in intracellular ATP concentration (11). The purpose of the present study was to further characterize the metabolic defect in cystine-loaded proximal tubules and to examine if glycine, an amino acid that is cytoprotective to hypoxic proximal tubule injury, ameliorates the defect in proximal tubule respiration in cystine-loaded tubules.

Received June 10, 1992; accepted August 7, 1992.
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Supported by an American Heart Association grant (890664).

MATERIALS AND METHODS

Rabbit proximal tubule suspensions were prepared using a method similar to that described by Weinberg *et al.* (12, 13). Briefly, kidneys were removed immediately after the rabbits were killed and placed in an ultrafiltrate-like solution at 4°C containing (in mmol/L) 115 NaCl, 2.1 KCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $MgSO_4$, 1.2 $CaCl_2$, and 2.4 KH_2PO_4. This and other solutions were initially bubbled at 37°C with 95% O_2/5% CO_2.

The renal cortex was then minced on an ice-cold glass Petri dish using a razor blade. The tissue was placed in a previously silicone-coated flask (Sigma Coat, Sigma Chemical Co., St. Louis, MO) with 24 mL of buffer containing (in mmol/L) 115 NaCl, 3.3 KCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $MgSO_4$, 2.4 $CaCl_2$, 25 mannitol, 5 glucose, 4 Na lactate, 1 alanine, 1 Na butyrate, and 1.2 KH_2PO_4. The solution also contained 2.5 mg/mL albumin (Sigma, Fraction V) and 1 mg/mL collagenase (Boehringer, Mannheim, Indianapolis, IN). The solution was gassed with 95% O_2/5% CO_2 at 37°C, and the tissue was then incubated in a sealed container at 37°C for 35 min in a shaker bath. After incubation, 75 mL of cold ultrafiltrate-like solution (4°C) was added to stop the collagenase activity, and the suspension was filtered through four layers of gauze. The preparation was centrifuged at 800 rpm for 2 min at 4°C to separate the tubules and glomeruli from the debris. The pellet was washed two more times with 50 mL of the ultrafiltrate-like solution and finally in 20 mL of an ultrafiltrate-like solution containing 5 g/dL BSA (all at 4°C).

The pellet was then resuspended in a 50% Percoll solution (Pharmacia, Piscataway, NJ) containing (in mmol/L) 100 NaCl, 26 $NaHCO_3$, 3.4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 2.6 $CaCl_2$. The preparation was centrifuged at 13 000 rpm at 4°C for 30 min, which separated the denser proximal tubules from distal tubules and glomeruli. The proximal tubules were washed three times with an ultrafiltrate-like solution. The tubules were then resuspended in an ultrafiltrate-like solution that contained (in mmol/L) 4 Na lactate, 10 Na butyrate, 1 alanine, 5 glucose, and 0.6% dextran to yield a tubule suspension with a concentration of 5–6 mg protein/mL. (Less NaCl was added to this solution to maintain an osmolality of 290 mosmol/kg water.) The protein

concentration was measured using the Lowry method with BSA as the standard (14). The tubule suspension was kept at 4°C.

O_2 consumption studies were performed in a 3-mL chamber using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The tubule suspension was first warmed to 37°C, stirred, and gassed with 95% O_2 and 5% CO_2 for 10 min. In studies in which the effect of proximal tubular cystine loading was measured, 2 mmol/L CDME (Sigma) was added to the tubule preparation at the time of incubation. The chamber was then sealed and O_2 consumption was measured in nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. After the basal O_2 consumption rate was measured, either nystatin (200 μg/mg protein), ouabain (10^{-4} mmol/L), or 15 μmol/L CCCP were added to the proximal tubule suspension, and the rate of O_2 consumption was again measured. Nystatin permeabilizes the proximal tubule membranes to Na and increases the intracellular Na concentration. This allows determination of the maximal rate of coupled respiration. Ouabain inhibits the NaK ATPase, allowing determination of O_2 consumption not used for transport. CCCP is a mitochondrial uncoupler and provides a measure of the maximal rate of tubular respiration.

The next series of experiments was designed to examine if glycine was cytoprotective to cystine-loaded proximal tubules. In these studies, the purified proximal tubules were resuspended in a solution containing either 5 mmol/L glycine, or 2.5 mmol/L NaCl. NaCl was added as a control to keep the osmolality constant. O_2 consumption studies were then performed in cystine-loaded tubules as described above. In addition, studies were performed to examine if glycine had an effect on proximal tubule respiration in the absence of cystine loading.

Data are expressed as mean ± SEM. Statistical analysis was performed using the unpaired *t* test and analysis of variance.

RESULTS

The results of O_2 consumption studies in control and cystine-loaded proximal tubules are shown in Table 1 and Figure 1. Basal O_2 consumption in control proximal tubules was 20.6 ± 0.5 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. This value comprises total cellular respiration used for transport and nontransport functions. To determine the component of cellular respiration used for nontransport functions (*i.e.*, non-NaK ATPase respiration) 10^{-4} mol/L ouabain was added to the tubular suspension. The non-NaK ATPase respiration was 10.2 ± 0.7 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, which was significantly less than the basal O_2 consumption ($p < 0.001$). O_2 consumption directed to NaK ATPase or transport activity was 10.4 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, the difference between these values. Nystatin permeabilizes proximal tubule membranes to Na and maximizes NaK ATPase activity, allowing measure of the maximal NaK ATPase activity (the difference between the nystatin- and ouabain-treated O_2 consumption rates). Nystatin increased O_2 consumption over basal activity to 30.0 ± 1.7 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ ($p < 0.001$). The maximal NaK ATPase activity was 19.8 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. Uncoupled mitochondrial respiration was measured using CCCP, which provides a measure of maximal O_2 consumption. O_2 consumption was 64.0 ± 3.3 in control tubules, which was higher than basal O_2 consumption ($p < 0.001$).

Table 1. *Proximal tubular respiration**

	Basal	Ouabain	Nystatin	CCCP
Control	20.6 ± 0.5 (129)	10.2 ± 0.7† (*n* = 11)	30.0 ± 1.7† (*n* = 18)	64.0 ± 3.3† (*n* = 27)
2 mM CDME	12.1 ± 0.6 (*n* = 88)	11.4 ± 1.0 (*n* = 18)	17.6 ± 1.3†‡ (*n* = 57)	24.3 ± 4.7†‡ (*n* = 11)
2 mM CDME, 5 mM glycine	16.5 ± 1.8§‖ (*n* = 43)	11.4 ± 1.5 (*n* = 16)	21.9 ± 2.2§ (*n* = 14)	36.9 ± 6.3†‡ (*n* = 13)

* Values expressed as nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.
† Different from basal of same group, $p < 0.001$.
‡ Different from comparably treated control, $p < 0.001$.
§ Different from comparably treated control, $p < 0.01$.
‖ Different from 2 mM CDME, $p = 0.01$.

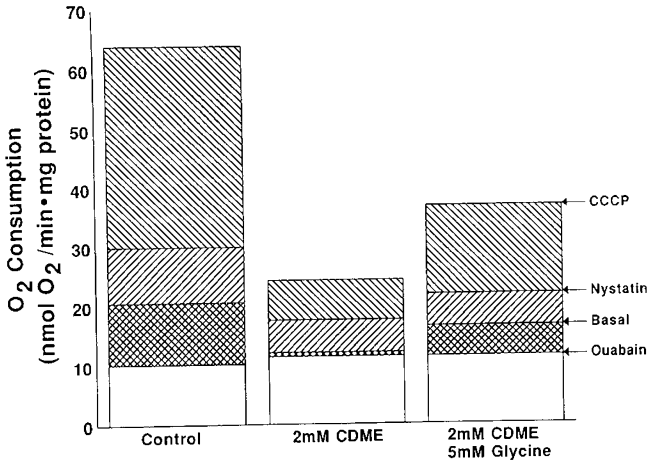

Fig. 1. O_2 consumption in control tubules, cystine-loaded tubules, and cystine-loaded tubules in the presence of glycine. □, O_2 consumption in the presence of ouabain; ▩, difference in O_2 consumption between basal and ouabain-treated tubules; ▨, difference in O_2 consumption between nystatin-treated tubules and basal O_2 consumption; and ▧, difference in O_2 consumption between CCCP- and nystatin-treated tubules. O_2 consumption used for transport (NaK ATPase activity) is the difference between the basal and ouabain values. The difference in the nystatin value and that in the presence of ouabain is the maximal NaK ATPase activity. Uncoupled respiration was measured in the presence of CCCP.

Cystine-loaded tubules had a basal O_2 consumption of 12.1 ± 0.6 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, which was significantly less than the basal rate of control tubules ($p < 0.001$). In cystine-loaded tubules, O_2 consumption was 11.4 ± 1.0 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ when 10^{-4} mol/L ouabain was added to the tubule suspension. Thus, the non-NaK ATPase respiration was not different in control and cystine-loaded tubules. The O_2 consumption directed to NaK ATPase activity was only 0.7 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. Thus, the difference in basal O_2 activity between control and cystine-loaded tubules was entirely due to a decrease in transport-directed O_2 consumption. In spite of the low O_2 consumption used for NaK ATPase activity, nystatin did increase O_2 consumption in cystine-loaded tubules to 17.6 ± 1.3 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. This value was significantly greater than basal O_2 consumption, but less than that measured in control tubules in the presence of nystatin ($p < 0.001$). The maximal NaK ATPase activity was 6.2 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in cystine-loaded tubules, compared with 19.8 in control tubules. Cystine-loaded tubules treated with CCCP had an O_2 consumption of 24.3 ± 4.7 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, which was greater than the basal rate of O_2 consumption, but significantly less than control tubules treated with CCCP ($p < 0.001$).

Glycine has been shown to be cytoprotective to hypoxic injury in proximal tubules (13, 14). Inasmuch as an underlying defect in both models is a decrease in cellular ATP (12, 13, 15) and the effect of ischemia on the above proximal tubular metabolic profile was similar to our present results in cystine-loaded tubules (16), the effect of 5 mmol/L glycine on cystine-loaded tubules was examined. In these studies, glycine was added when the proximal tubules were resuspended. In control tubules, 2.5 mmol/L NaCl was added to maintain a constant osmolarity. Glycine had no effect on basal respiration when added to control tubules ($n = 29$, 20.0 ± 1.1 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$). The effect of glycine on respiration in cystine-loaded tubules is shown

in Table 1 and Figure 1. Basal O_2 consumption in cystine-loaded tubules incubated with 5 mmol/L glycine was 16.5 ± 1.8 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, which was significantly higher than that of cystine-loaded tubules incubated without glycine. There was no difference in O_2 consumption in cystine-loaded tubules in the presence or absence of glycine after addition of either ouabain or CCCP. Cystine-loaded tubules incubated with nystatin had an O_2 consumption of 21.9 ± 2.2 in the presence of glycine. Although this was not significantly different than the 17.6 ± 1.3 in all of our cystine-loaded tubules incubated with nystatin without glycine, control and cystine-loaded tubules were always run during each protocol to ensure the consistency of our preparation. The cystine-loaded, nystatin-treated tubules (containing 2.5 mM NaCl) run at the time of the glycine experiments had an O_2 consumption of 14.6 ± 2.5 ($n = 11$), which was lower than the cystine-loaded tubules treated with nystatin in the presence of glycine ($p < 0.05$). Thus, glycine partially ameliorated the basal and nystatin-stimulated O_2 consumption in cystine-loaded tubules.

DISCUSSION

This study examined the effect of intracellular cystine loading on proximal tubule O_2 consumption. Although cystine-loaded tubules had a lower rate of basal O_2 consumption, there was no difference in O_2 consumption when NaK ATPase activity was inhibited with ouabain. Intracellular cystine loading also decreased O_2 consumption in the presence of nystatin and CCCP. Glycine partially ameliorated the decrease in basal and nystatin-stimulated O_2 consumption in cystine-loaded tubules.

The generalized proximal tubular defect in the Fanconi syndrome of cystinosis could potentially be due to a number of mechanisms. The proximal tubule reabsorbs most of the filtered bicarbonate, phosphate, amino acids, and glucose. The Fanconi syndrome could be due to a generalized decrease in active transport, or it could result from an increase in the permeability of the proximal tubule to these solutes, allowing greater diffusion from the blood into the tubular lumen. Potential mechanisms that could cause a generalized inhibition of active transport include a direct inhibition of the NaK ATPase on the basolateral membrane, a decrease in ATP production, or a generalized inhibition of the transports on the apical or basolateral membrane responsible for solute transport. These mechanisms have been examined using cystine-loaded tubules (9–11, 17). We have previously shown that the permeability of the proximal convoluted tubule to bicarbonate and mannitol is not affected by cystine loading consistent with an inhibition in active transport (10). Foreman *et al.* (17) found that the rate of proline uptake into brush border membrane vesicles obtained from cystine-loaded tubules was not different than that in control tubules. Thus, cystine loading does not directly affect the transporters on the apical membrane. We have previously demonstrated that cellular cystine loading did not affect the V_{max} of NaK ATPase activity (11). Intracellular cystine loading, however, produced a substantial fall in intracellular ATP concentration (11). The results of the present study, which demonstrated that cellular cystine loading inhibits the rate of O_2 consumption, are consistent with these previous results.

The approach used in the present study to examine the metabolic defect in cystine-loaded tubules has been previously used in the study of ischemic acute renal failure (16). In this study, rats had an ischemic insult to their kidney followed by various periods of reperfusion. There was a decrease in intracellular ATP concentration after the ischemic injury, but V_{max} NaK ATPase activity was not affected. In O_2 consumption studies, proximal tubules harvested after the ischemic insult and 15 min of reperfusion had a lower rate of basal, nystatin-stimulated, and mitochondrial uncoupled O_2 consumption compared with control proximal tubules. O_2 consumption was the same in control and ischemic tubules in the presence of ouabain.

The present study demonstrates that 50% of O_2 consumption in proximal tubules is used for NaK ATPase-dependent transport and the remainder for other cellular functions. As with ischemic injury, cystine loading almost totally and selectively inhibited O_2 consumption directed to transport. The preservation of nontransport O_2 consumption likely allows the proximal tubule cell to maintain essential cellular functions for survival and cell reparation. These data suggest that the proximal tubule cell is able to inhibit transport by inhibiting apical solute entry, the NaK ATPase activity, or the ATP pool directed for transport. The fact that nystatin resulted in a substantial, although brief, increase in cellular respiration suggests that the entry of solutes across the apical membrane could be inhibited when the intracellular ATP concentration falls.

The proximal tubular injury due to cystine loading shares many similarities with the proximal tubule injury due to hypoxia and ischemia (13, 16, 18). Glycine has been shown to be cytoprotective against hypoxic injury to proximal tubules in suspension (13, 15). The cytoprotective effect of glycine is only apparent when glycine is present at the time of injury, but not when removed from the media before injury or when added after the tubules have been exposed to hypoxia (13). The mechanism of the cytoprotective effect of glycine is unknown. It does not act by increasing glutathione or ATP concentrations or via an action against reactive O_2 metabolites (13, 15). Glycine had no effect on basal O_2 consumption or ATP levels in control tubules (13). In the present study, glycine did not affect O_2 consumption in control tubules. In cystine-loaded tubules, glycine increased basal and nystatin-stimulated O_2 consumption in comparison to cystine-loaded tubules without glycine. Unlike hypoxic tubules, glycine did not significantly increase uncoupled tubule respiration (13). Although the mechanism is unknown, glycine is cytoprotective in these two types of proximal tubular injuries.

In summary, this study further characterized the defect in cystine-loaded proximal tubules. Consistent with our previous findings, the underlying defect is secondary to an alteration in cellular metabolism. This study demonstrates that the proximal tubule preferentially inhibits O_2 consumption utilized for transport purposes, leaving nontransport-directed O_2 consumption intact. Glycine is partially cytoprotective against the injury due to cellular cystine loading.

Acknowledgments. The authors thank Drs. Normal Siegel, Karen Gaudio, and Joel Weinberg for their helpful suggestions and Janell McQuinn for her able secretarial assistance.

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