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Thiol redox and phosphate transport in renal brush-border membrane. Effect of nicotinamide

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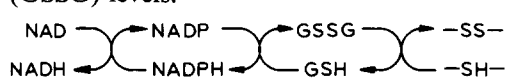
In the present study, the effect of thiol redox and its possible role in the inhibitory effect of nicotinamide on renal brush-border membrane (BBM) phosphate uptake was examined. Addition of thiol reducing agent, dithiothreitol (DTT, 5 mM), caused an increase, while addition of thiol oxidant, diamide (DM, 5 mM) caused a reversible decrease in sodium-dependent BBM phosphate uptake. Kinetic analyses revealed an increase in both V_{max} and K_m by DTT, and a decrease in V_{max} by DM. These results suggest that thiol redox influences BBM phosphate uptake with sulfhydryl (SH) groups relate to its capacity and disulfide (SS) groups to its affinity for phosphate. Since changes in cytosolic NAD levels may affect BBM thiol redox through changes in redox states of NADP and glutathione systems, we have examined such possibility by studying the effect of nicotinamide (NM). Incubation of proximal tubules with NM (10 mM) induced an oxidative effect on redox states of cytosolic NAD, NADP systems as inferred from decreased cellular lactate/pyruvate, malate/pyruvate, respectively. Measurements of cytosolic glutathiones and BBM thiols also revealed that NM pretreatment shifted the cytosolic glutathione redox (GSH/GSSG) and BBM thiol redox (SH/SS) toward more oxidized state. On the other hand, incubation of proximal tubules with NM suppressed phosphate uptake by the subsequently isolated BBM vesicles. The lower phosphate uptake by NM-pretreated BBM vesicles was reversed by DTT and was resistant to the inhibitory effect of DM. These results thus suggest that BBM thiol oxidation may be involved in the inhibitory effect of NM on BBM phosphate uptake.

Introduction

Thiol groups play important roles in protein functions [1]. This includes the transport function of plasma membrane proteins. Thiol-modifying agents have been shown to affect membrane transport of sugar [1–5], Na^+/H^+ exchange [6] and organic solutes [7–10], suggesting the presence of thiol groups at or near these transport sites.

The possibility that thiol groups may participate in membrane phosphate transport has been suggested from studies with the inner membrane of mitochondria [11–13], and studies with the duodenum where the active component of phosphate transport across the luminal brush-border membrane (BBM) was shown to be inhibited by $HgCl_2$ [14]. Whether or not thiol groups also participate in renal (BBM) phosphate transport is not known. In conjunction with the previously proposed inhibitory effect of cytosolic NAD on BBM phosphate transport [15], such possibility could have physiological implication since an increase in cellular NAD content is expected to lead to thiol oxidation of cellular proteins through increases in NADP and oxidized glutathione (GSSG) levels.



To test such possibility, we have studied the effect of thiol redox on BBM phosphate transport and examined its role in the effect of nicotinamide (NM), an agent known to increase cellular NAD content and inhibits BBM phosphate transport in renal proximal tubules [15].

Methods

Experimental animals. New Zealand white male rabbits, weighing 1.5–2.5 kg, were used in these studies.

Abbreviations: BBM, brush-border membrane; DM, diamide; DTT, dithiothreitol; NM, nicotinamide.

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The animals were maintained on an ad lib diet of standard rabbit chow, containing 0.57% inorganic phosphorus and 1.39% calcium, with free access to tap water for drinking.

BBM vesicles preparation. BBM vesicles were isolated from renal cortex by the conventional calcium-precipitation method. In those experiments where the in vitro effect of NM was examined, purified proximal tubule suspension was first prepared from renal cortex by homogenization and sieving processes as described previously [16] and incubated with NM in a solution containing (mM): NaCl 125, $NaHCO_3$ 25, KCl 5.0, $CaCl_2$ 1.5, $MgSO_4$ 1.2, NaH_2PO_4 2, L-alanine 5 and glucose 5, preequilibrated with 95% O_2/5% CO_2 (v/v) at 37°C (pH 7.4). The preincubation was carried out in a shaker bath (40 cycles/min) at 37°C, and the BBM vesicles were then isolated from these pretreated proximal tubules. The final BBM vesicles were suspended in 300 mM mannitol and 5 mM Hepes-Tris (pH 7.4). The enrichment of BBM enzyme marker, alkaline phosphatase [17], averaged 13.8 ± 0.87 ($n = 12$)-fold from the crude renal cortex or 8.8 ± 0.43 ($n = 24$)-fold from the proximal tubule suspension. The basolateral membrane enzyme marker, Na^+/K^+-ATPase [18], endoplasmic reticulum enzyme marker, glucose-6-phosphatase [19], and mitochondria enzyme marker, cytochrome-*c* reductase [20], were reduced to $86 \pm 2\%$, $66 \pm 8\%$ and $30 \pm 5\%$ ($n = 12$), respectively, of either crude renal cortex or purified proximal tubules. Protein concentration was assayed using Coomassie brilliant blue G250 with bovine serum albumin as the reference protein [21].

Measurements of BBM phosphate and glucose uptake. Transport studies were performed at room temperature using a Millipore rapid-filtration procedure under an inward Na gradient of 100 mM. The uptake solution for phosphate or glucose uptake was composed of 100 mM NaCl, 100 mM mannitol, 5 mM Hepes-Tris (pH 7.4) and 0.1 mM [^{32}P]phosphoric acid (2.5 μCi/ml) or 0.1 mM [U-^{14}C]glucose (2.5 μCi/ml), respectively. The [^{32}P]phosphoric acid or D-[^{14}C]glucose retained on the filter membrane was transfered into the liquid scintillation fluid (Aquasol, New England Nuclear, Boston, MA) and counted in a liquid scintillation counter (LS 5801, Beckman, Palo Alto, CA). The sodium-dependent uptake of phosphate and glucose were calculated from the difference between uptakes with an dwithout sodium in the uptake solution and expressed as pmol/mg protein per uptake time. In these studies, sodium-independent phosphate or glucose uptake was constantly below 5% of the total uptake and was not affected by different experimental conditions. The direct effects of thiol reagents on BBM phosphate and glucose uptake were examined by preincubating the BBM vesicles with these agents at 4°C prior to uptake measurement.

Assays of intracellular metabolites. Proximal tubules were incubated with different reagents in the same medium as for BBM preparation. After incubation, proximal tubules were separated from the medium by rapid centrifugation at 4°C and frozen immediately in liquid nitrogen till the assay. After the wet weight of each sample was measured, tissue extracts were obtained with perchloric acid at 4°C followed by centrifugation and the pH of the supernatant was neutralized with KOH. The contents of pyruvate, lactate and malate in the supernate were measured fluorometrically with a fluorometer (J4-7439A, Aminco, Silver Spring, MD) as described [22–24]. For pyruvate measurement, the supernatant was run through a Florisil column to remove the tissue fluorescence before the assay. The results were calculated and expressed as nmol/mg tissue wet weight.

Glutathiones were measured with cytosolic fractions by using the fluorometric method as described [25]. In brief, proximal tubules (5–10 mg) were homogenized in a solution (0.2 ml) containing sodium phosphate (0.1 M) and EDTA (5 mM) to which 50 μl of 25% H_3PO_4 solution was added as the protein precipitant. After centrifugation at $100\,000 \times g$ for 30 min at 4°C, 80 μl of the supernatant was mixed with 80 μl of Na_3PO_4 (15%, w/v). For GSH assay, 40 μl of this mixture was added to 1 ml of homogenization solution and added 0.1 ml of *o*-phthalaldehyde in methanol (1 mg/ml). After 15 min incubation at room temperature, the fluorescence was measured at excitation 350 nm and emission 420 nm. For GSSG assay, 80 μl of the same mixture solution was added to 40 μl of an aqueous solution containing *N*-ethylmaleimide (2 mM). After 30 min incubation at room temperature, 1 ml of NaOH (0.1 M) and 0.1 ml of *o*-phthalaldehyde in methanol (1 mg/ml) were added and incubated at room temperature for another 15 min. The fluorescence was measured at excitation 350 nm and emission 420 nm. Standards of GSH and GSSG were used for calibration.

BBM thiol assay. Thiol assays were performed with the proteolipid extract of BBM vesicles [26] where SH group was measured by using Ellman's reagent, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) [27] and SS group determined from the difference of SH group levels before and after reduction by sodium borohydride [28]. In brief, BBM (1 mg) suspended in 2 ml of Tris buffer (0.2 M, pH 8.2) containing EDTA (0.02 M) was added to 10 ml of absolute methanol containing 0.01 M DTNB. After 15 min incubation at room temperature in test tubes stoppered with rubber caps, samples were centrifuged at $3000 \times g$ for 15 min and the absorbance of the supernatant was read with a spectrophotometer at 412 nm. GSH was used as the standard for calibration. All buffers and solutions were gassed with nitrogen prior to their use so to prevent spontaneous oxidation. Reduction with sodium borohydrate was performed by adding same amount of BBM suspended in 0.5 ml of the same Tris buffer to 0.1 ml of solution containing sodium

versenate (0.1 M) and urea (1.44 g) and incubated with 1 ml of freshly prepared 2.5% sodium borohydrate at 38°C for 30 min. 0.5 ml of 0.2 M hydrochloric acid containing monopotassium phosphate (1 m) and 2 ml of acetone were then added to destruct the remaining sodium borohydrate. After being gassed with nitrogen, the amount of SH group present was determined by using DTNB as described above.

Materials. Radioisotopes were purchased from Amersham Inc. (Arlington Heights, IL). Chemicals used were of reagent grades and obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics. Data are expressed as means ± S.E. and analyzed with Student's *t*-tests for paired or unpaired data as appropriate.

Results

Effects of thiol agents on BBM phosphate and glucose uptake

To examine if changes in thiol redox affect BBM phosphate uptake, effects of dithiothreitol (DTT, a reducing agent which converts SS group to SH group) and diamide (DM, an oxidant which converts SH to SS) were studied. In separate studies, it was found that addition of these agents did not affect pH of the uptake solution, and that maximum effects of these agents were obtained at 5 mM concentration for 15 min incubation. The following studies were thus performed with the maximal effective incubation time and concentration. As is shown in Fig. 1, DTT enhanced the initial sodium-dependent phosphate uptake from 522 ± 42 to 634 ± 30 pmol/mg per 15 s ($n = 7$, $P < 0.05$). In contrast, DM suppressed the initial sodium-dependent phosphate uptake from 493 ± 33 to 393 ± 48 pmol/mg per 15 s ($n = 7$, $P < 0.01$) (Fig. 1). The inhibitory effect of DM was reversible by further incubation with DTT (473 ± 34 pmol/mg per 15 s, $n = 7$) (Fig. 1). Kinetic

TABLE I

Kinetic parameters of effects of dithiothreitol (DTT, 5 mM), diamide (DM, 5 mM) and nicotinamide (NM, 10 mM) on BBM sodium-dependent phosphate transport

Mean ± S.E., $n = 7$ each, * $P < 0.05$ vs. control.

	K_m (μM)	V_{max} (pmol/mg per 15 s)
Control	92 ± 6	878 ± 46
DTT	135 ± 7 *	1240 ± 65 *
Control	89 ± 8	846 ± 53
DM	81 ± 10	552 ± 47 *
Control	94 ± 7	881 ± 52
NM	99 ± 11	539 ± 64 *

TABLE II

Effects of dithiothreitol (DTT, 5 mM), diamide (DM, 5 mM) and nicotinamide (NM, 10 mM) on BBM sodium-dependent glucose uptake

Mean ± S.E., $n = 5$ each.

	BBM glucose uptake (pmol/mg)			
	15 s	1 min	2 min	90 min
Control	125 ± 21	631 ± 48	587 ± 42	220 ± 12
DTT	130 ± 19	648 ± 53	602 ± 44	218 ± 9
DM	118 ± 18	627 ± 44	568 ± 38	224 ± 16
Control	148 ± 23	588 ± 51	572 ± 47	210 ± 17
NM	161 ± 27	603 ± 62	566 ± 55	216 ± 21

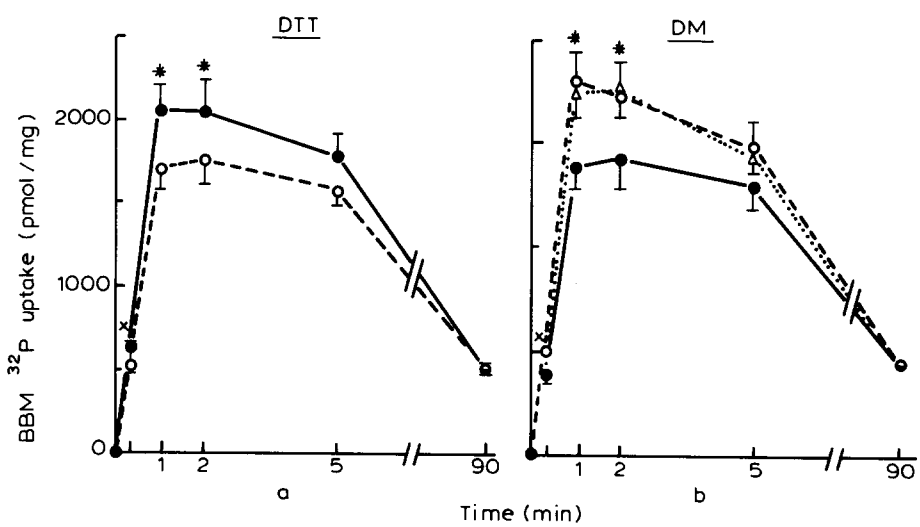

Fig. 1. Effects of dithiothreitol (DTT) and diamide (DM) on BBM sodium-dependent phosphate uptake. (Left) Incubation of BBM vesicles with DTT (5 mM) at 4°C for 15 min increases BBM sodium-dependent phosphate uptake (●———●) as compared to control vesicles (○------○). (Right) incubation of BBM vesicles with DM (5 mM) at 4°C for 15 min suppressed BBM sodium-dependent phosphate uptake (●———●) as compared to control vesicles (○------○). Further incubation of DM-treated vesicles with DTT (5 mM) for 15 min reversed the BBM phosphate uptake (△·····△) to control levels. (Mean ± S.E., $n = 7$ each, * $P < 0.05$).

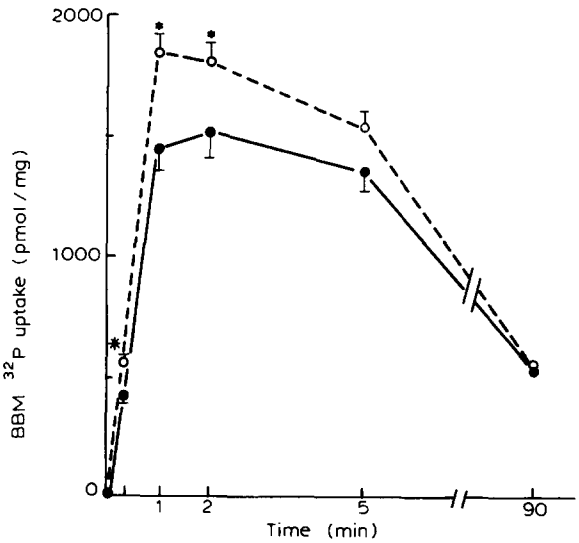

Fig. 2. Effect of nicotinamide (NM) on BBM sodium-dependent phosphate uptake. Preincubation of proximal tubules with NM (10 mM) at 37°C for 30 min suppressed the sodium-dependent phosphate uptake by the subsequently isolated BBM vesicles (●——●) as compared to vesicles isolated from proximal tubules preincubated without NM (○------○). (Mean ± S.E., $n = 5$, * $P < 0.05$).

analyses were performed with phosphate concentration in the uptake solution varied from 0.01 to 1 mM. As is shown in Table I, DTT had dual effects to increase both the K_m and V_{max} while DM reduced the V_{max} without affecting K_m. Results of BBM glucose uptake are shown in Table II. Both DTT and DM did not affect the sodium-dependent BBM glucose uptake.

Effect of nicotinamide (NM) on BBM phosphate uptake

As is shown in Fig. 2, preincubation of proximal tubules with NM (10 mM) for 30 min at 37°C suppressed the sodium-dependent phosphate uptake by the subsequently isolated BBM vesicles (from 577 ± 35 to

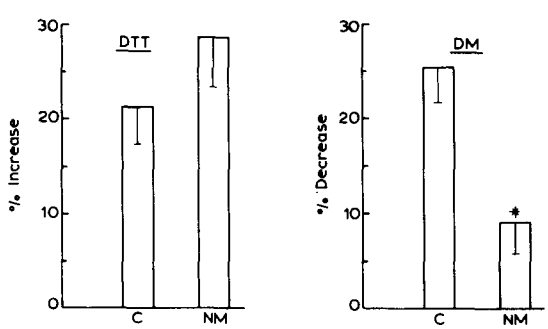

Fig. 3. Effects of dithiothreitol (DTT) and diamide (DM) in nicotinamide (NM)-pretreated BBM vesicles. BBM vesicles were isolated from proximal tubules incubated with or without NM (10 mM) at 37°C for 30 min and were incubated with DTT (5 mM) or DM (5 mM) at 4°C for 15 min prior to uptake measurements. (Left) The stimulatory effect of DTT on phosphate uptake was augmented in NM-pretreated BBM vesicles. (Right) The inhibitory effect of DM on phosphate uptake was diminished in NM-pretreated BBM vesicles. (Mean ± S.E., $n = 5$ each, * $P < 0.05$).

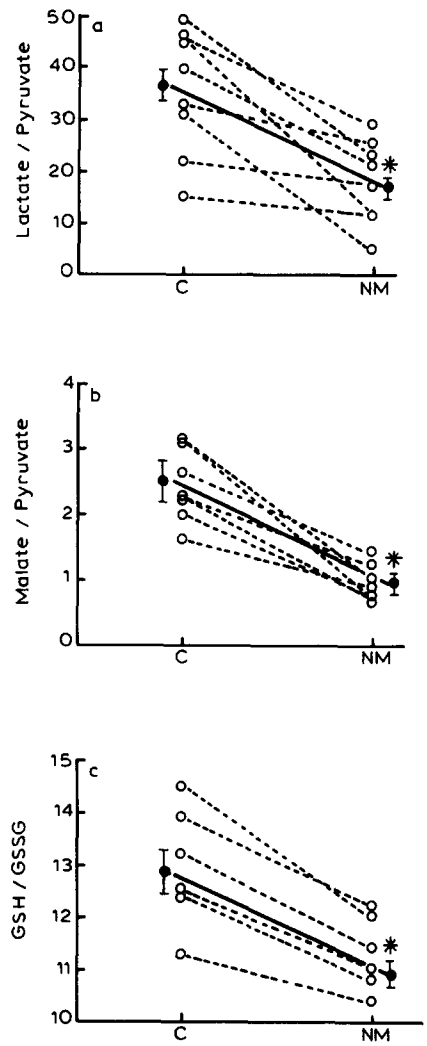

Fig. 4. Effect of nicotinamide (NM) on proximal tubular lactate/pyruvate, malate/pyruvate and GSH/GSSG ratios. Cellular metabolites were measured from extract of proximal tubules incubated with and without NM (10 mM) at 37°C for 30 min. NM decreased ratios of lactate/pyruvate (a) and malate/pyruvate (b), indices of cytosolic NADH/NAD and NADPH/NADP ratios, respectively. NM also decreased cytosolic ratio of GSH/GSSG (c). (○------○ indicates the result of a single experiment and ●——● indicates the mean ± S.E. of those results. * $P < 0.05$).

426 ± 36 pmol/mg per 15 s, $n = 5$, $P < 0.02$) due to a decrease in V_{max} (Table I). NM pretreated BBM, however, exhibited altered sensitivities to both DTT and DM. Addition of DTT (5 mM) reversed the suppressed phosphate uptake by NM pretreated BBM from 426 ± 36 to 547 ± 33 pmol/mg per 15 s ($n = 5$, $P < 0.01$). The percentage increase in phosphate uptake by DTT was thus augmented in NM pretreated BBM vesicles as compared to nontreated BBM vesicles (28.6 ± 5.3% vs. 21.3 ± 4.0%) (Fig. 3). In contrast, addition of DM to NM pretreated BBM failed to cause further significant decrease in BBM phosphate uptake (387 ± 34 pmol/mg per 15 s, $n = 5$, $P > 0.05$). Thus, the percentage inhibition on phosphate uptake by DM was diminished in

NM pretreated BBM vesicles as compared to non-treated BBM vesicles (9.0 ± 3.4% vs. 25.4 ± 3.8%, $P < 0.01$) (Fig. 3). As shown in Table II, NM pretreatment did not affect the sodium-dependent BBM glucose uptake.

Effect of NM on intracellular metabolites

Effect of NM on cytosolic NAD and NADP redox systems was assessed by monitoring changes of intracellular metabolites [29,30]. For the redox ratio of cytosolic free [NADH]/[NAD], substrate and product concentrations of lactate dehydrogenase, i.e., [lactate]/[pyruvate] were measured. For the redox ratio of cytosolic free [NADPH]/[NADP], substrate and product concentrations of malic enzyme, i.e., [malate]/[pyruvate] were measured. As is shown in Fig. 4, incubation with NM (10 mM) for thirty minutes at 37°C lowered both [lactate]/[pyruvate] and [malate]/[pyruvate] ratios in proximal tubular cells. Effect of NM on cytosolic glutathione redox, i.e., GSH/GSSG was assessed by direct measurement of GSH and GSSG in the cytosolic fraction of the proximal tubules. As is shown in Fig. 4, NM pretreatment also lowered cytosolic GSH/GSSH ratio in the proximal tubular cells.

Effect of NM on BBM thiol redox

To confirm the effect of NM on BBM thiol redox, we have measured SH and SS groups of BBM vesicles isolated from proximal tubules pretreated with NM. Fig. 5 depicts the results from these studies. The SH/SS ratio was significantly lower in BBM vesicles isolated from proximal tubules preincubated with NM (1.32 ± 0.09 vs. 1.60 ± 0.07, $n = 6$, $P < 0.01$).

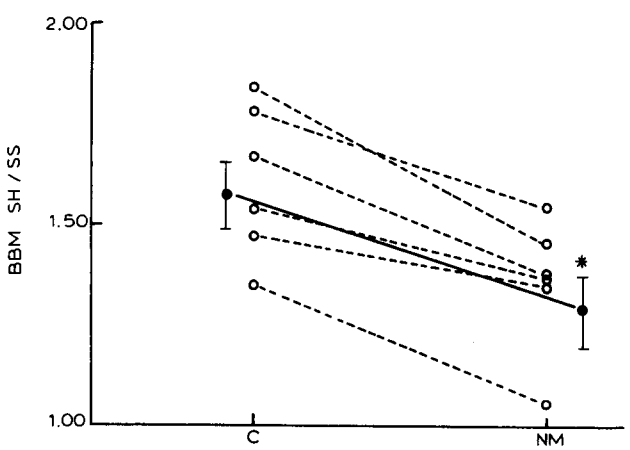

Fig. 5. Effect of nicotinamide (NM) on BBM thiol redox. Thiol redox ratio, SH/SS, was decreased in BBM isolated from proximal tubules pretreated with NM (10 mM). (o------o indicates the result of a single experiment and ●———● indicates the mean ± S.E. of those results. * $P < 0.05$).

Discussion

Renal BBM phosphate uptake represents the first and probably the rate-limiting step of phosphate reabsorption by renal proximal tubule [31,32]. Experimental evidence to date favors the view that BBM phosphate uptake is under the regulation of multifarious processes including protein phosphorylation [33,34]. ADP-ribosylation [35–37] and membrane lipid modification [38,39]. In addition, results of the present study suggest that BBM thiol redox may serve as another mechanism whereby BBM phosphate uptake can be regulated. Oxidation of BBM SH-groups by DM suppressed, while reduction of BBM SS-groups by DTT enhanced BBM phosphate uptake. It seems unlikely that these effects of DTT and DM are due to nonspecific alterations in membrane properties because another sodium-cotransport system, i.e., BBM sodium-dependent glucose uptake was not affected by these agents. The lack of DTT effect on BBM glucose uptake found in these studies may seem at variance with previous studies reported by Turner and George [4,5] where SS-groups were demonstrated to be essential for BBM glucose transporter. However, Turner and George [4] reported a time-dependent biphasic effect of DTT, i.e., an initial (5–10 min incubation with DTT) increase following by a decrease in sodium-dependent phlorizin binding to BBM vesicles. It is possible that the 15-min incubation time employed in the present study could have masked the effect of DTT.

Kinetic analyses showed that DM caused a decrease in V_{max} while DTT caused an increase in both V_{max} and K_m for phosphate. It thus appears that thiol groups are present at or near BBM phosphate transporter where SH-groups affect its capacity whereas SS-groups affect its affinity for phosphate. Although results of the present study do not define the exact location of thiol groups affected by the thiol reagents used, they do indicate that the function of BBM phosphate transporter can be influenced by the balance in the prevalence of SH vs. SS groups, i.e., the redox states of BBM thiol groups. These results are analogous to the phosphate transporter of mitochondria inner membrane where SH-groups were proposed to serve as the binding site for phosphate [11–13]. While the enzyme marker results negate against significant contamination from mitochondria membranes, the extent to which such contamination could have contributed to these results is not clear from the present study.

In the present study, incubation of proximal tubules with NM lowered cellular [lactate]/[pyruvate] and [malate]/[pyruvate] ratios, suggesting an oxidative effect of NM on cytosolic NAD and NADP systems, respectively. It is likely that such oxidative effect of NM was indirectly transmitted to BBM thiol redox through cytosolic glutathione redox system as was evidenced

from the results that NM also lowered the cytosolic GSH/GSSG and BBM SH/SS ratios. On the other hand, incubation of proximal tubules with NM suppressed the phosphate uptake by the subsequently isolated BBM vesicles. The decrease in phosphate uptake by NM pretreated BBM was reversed by DTT and could not be lowered further by DM, suggesting that NM may share same mechanism whereby DM inhibits BBM phosphate uptake, i.e., oxidation of BBM thiol groups. Taken together, these results suggest that the oxidative effect of NM on BBM thiols contributes, at least partly, to its inhibitory effect on BBM phosphate uptake.

In summary, results of the present study show that thiol redox status modulates BBM phosphate transport and suggest that thiol oxidation may serve as one of the mechanisms whereby cytosolic NAD inhibits BBM phosphate uptake. In conjunction with the proposed role of cytosolic NAD on BBM phosphate transport regulation in general [15], it is tempting to speculate a broad implication of thiol redox in these processes. However, in view of the moderate extent to which thiol redox status affects BBM phosphate transport, it is unlikely that this alone plays a central role in the regulation of BBM phosphate transport. Rather, it is more likely that together with other cellular mechanisms such as protein phosphorylation, ADP-ribosylation and membrane lipid modification, changes in thiol redox status may concertedly contribute to the intricate regulatory process of BBM phosphate transport. The significance of these different mechanisms in BBM phosphate transport regulation under various conditions remains to be defined in future studies.

Acknowledgments

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