Cold-Induced Microtubule Disruption and Relocalization of Membrane Proteins in Kidney Epithelial Cells

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Abstract. Cold preservation of kidneys is commonly used in human transplantation and in vitro studies. However, although disruption of the cytoskeleton by cold has been demonstrated in cultured cells, the effect of cold treatment on intact kidney is poorly understood. In this study, specific antibodies were used to examine the effect of hypothermia on the cytoskeletal network and the trafficking of some membrane proteins in the urinary tubule. Rat kidneys were cut into thin slices (approximately 0.5 mm) that were divided into several groups: (1) some were immediately fixed in paraformaldehyde, sodium periodate, and lysine (PLP); (2) some were stored at 4°C for 15 min or 4 h before being fixed in cold PLP; or (3) after 4 h cold treatment, some slices were rewarmed to 37°C for 15, 30, and 60 min in a physiologic solution, pH 7.4, and were then fixed in warm PLP. Immunofluorescence staining revealed an almost complete disruption of the microtubule network in proximal tubules after 15 min cold treatment, whereas microtubules in other segments were affected after 4 h. A partial recovery of the microtubule network was observed after 60 min rewarming. In contrast, actin filaments seemed to be resistant to cold treatment. gp330, aquaporin-2, H⁺-ATPase, and the AE1 anion exchanger were all relocated into numerous vesicles that were distributed throughout the cytoplasm after hypothermia followed by rewarming, whereas Na-K-ATPase retained its basolateral localization. The vasopressin-stimulated insertion of aquaporin-2 water channels into the apical membrane was inhibited during the initial rewarming period after cold exposure. Thus, cold preservation of tissues might impair, at least transiently, the polarized membrane expression and function of some transport proteins in renal epithelial cells. (J Am Soc Nephrol 9: 155–166, 1998)

The complex and highly regulated functions of the kidney require the coordinated participation of a variety of specialized epithelial cells. Each nephron segment contains one or more specific cell types that use unique combinations of membrane transport proteins to regulate body electrolyte, water, and acid-base balance (1–3). Vectorial transepithelial transport results from the polarized insertion of these membrane proteins into their appropriate apical, lateral, or basal membrane domains. Numerous studies on epithelial cell function have used micro-dissected kidney tubules in vitro, and much of our knowledge of polarized transepithelial transport derive from such functional data (4–6). A common aspect of these studies is the use of cold preservation of kidneys to provide fresh tissue from which urinary tubules are dissected and subsequently rewarmed for in vitro examination. For human kidney (and other organs) transplantation, cold preservation is also a procedure that allows the organs to be stored, in certain cases for many hours, before surgery.

However, whereas cold treatment clearly prevents some cellular injury and degeneration, other kidney functions might be compromised by this maneuver. For instance, disruption of the cytoskeleton by cold has been demonstrated in cultured kidney epithelial cells (7), cultured pulmonary endothelial cells (8), arterial smooth muscle cells (9), and hepatocytes (10). The membrane-associated cytoskeletal proteins ankyrin and fodrin, as well as the Na-K-ATPase, are initially redistributed into the cytoplasm in proximal tubules from human transplanted kidneys (11), and this loss of cell polarity is accompanied by an impairment of the allograft function. An intact cytoskeleton is required for functional epithelial cell polarity to occur (12,13). Microtubules and microfilaments influence the movement of organelles and transporting vesicles and are thus involved in the establishment and maintenance of cell polarity. Despite this important function, the effect of cold preservation on the cytoskeleton and the trafficking of membrane proteins in kidney cells in situ is poorly understood.

In the present study, the cytoskeleton (actin filaments and microtubules) and the localization of some membrane proteins were examined in rat kidney after cold treatment followed by rewarming. The following transport proteins were examined: the highly glycosylated protein gp330 (megalin) located in the apical membrane of proximal tubule cells (14), the vasopressin-regulated water channel aquaporin-2 (AQP2) in principal cells of the collecting duct (15,16), and the vacuolar H⁺-ATPase located in intercalated cells of the collecting duct and in proximal tubule cells (17,18). These proteins were selected for study because our previous data showed that they
are redistributed in their respective epithelial cells after colchicine-induced microtubule depolymerization (16,18,19). Finally, the distribution of the basolateral proteins, Na-K-ATPase, and the AE1 anion exchanger was also examined.

Our results show that exposure to cold followed by rewarmed induces a marked disruption of the microtubule network, whereas the actin cytoskeleton appears to be unaffected. gp330, AQP2, H^+ATPase, and AE1 were all partially redistributed into cytoplasmic vesicles, while collecting duct Na-K-ATPase retained its basolateral localization. This study indicates that cold preservation of tissue might interfere, at least transiently, with normal kidney function.

Materials and Methods

Tissue Preparation

Male Sprague Dawley rats were anesthetized with sodium pentobarbital, and the kidneys were briefly perfused via the abdominal aorta with Hank's balanced salt solution (HBSS) at 37°C (bubbled with a 5% CO_2/95% O_2 gas mixture to reach pH 7.4). Kidneys were removed, and thin slices (approximately 0.5 mm) were made using a Stadie-Riggs slicer (Thomas Scientific, Swedesboro, NJ) and divided into four groups. Some slices were immediately fixed by immersion in a warm (37°C) fixative containing 2% formaldehyde, 10 mM sodium periodate, and 70 mM lysine (PLP). Some slices were stored at 4°C in a preservative solution containing 56 mM Na_2HPO_4, 13 mM NaH_2PO_4, and 140 mM sucrose (4) either for 15 min or for 4 h before being fixed in cold PLP. After the 4-h cold treatment, some slices were rewarmed to 37°C for 15 and 30 min in HBSS kept at pH 7.4, and were then fixed in warm PLP. This protocol was repeated four times, using one rat each time, so that every experimental condition could be compared to its own control (paired experiments).

In two separate series of experiments, kidney slices were first incubated at 37°C for 30 min in HBSS containing 1 μM vasopressin before being divided into five groups. Some slices were fixed in warm PLP after being exposed to 10 μM forskolin for 10 min (control). Some slices were stored at 4°C for 4 h in the same preservative solution as that described above, and were then fixed in cold PLP. After cold exposure, some slices were rewarmed to 37°C in Hank's buffer containing 1 μM vasopressin for 15, 30, and 60 min. Ten minutes before fixation, 10 μM forskolin was added to the buffer.

All slices were fixed by immersion for an additional 6 h before rinsing and storage in phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.4).

Immunostaining

Kidney slices were cut again at a thickness of either 4 or 1.5 μm for immunocytochemistry. For 4-μm sections, tissues were cryoprotected in 30% sucrose before freezing in liquid nitrogen and sectioning with a Reichert Frigocut microtome (Leica, Deerfield, IL) using disposable knives. For 1.5-μm sections, tissues were cryoprotected in 2.3 M sucrose before freezing in liquid nitrogen and sectioning with glass knives mounted on a Reichert FC4D ultracryomicrotome, as described previously (20). Sections were picked up on Fisher Superfrost Plus slides and stored at 4°C before immunofluorescence staining.

After being rehydrated in PBS for 5 min, sections were incubated in PBS containing 1% bovine serum albumin for 15 min to block nonspecific staining. Primary antibody diluted as described below was applied for 1.5 h at room temperature, followed by two washes of 5 min each in high-salt PBS (containing 2.7% NaCl) to reduce background staining and one wash in normal PBS. Goat anti-mouse IgG, goat anti-rabbit IgG conjugated with FITC (20 and 8 μg/ml, respectively; Kirkegaard and Perry, Gaithersburg, MD), goat anti-rabbit IgG conjugated with indocarbocyanine (Cy3) (2 μg/ml; Jackson Immuno Research, West Grove, PA), or donkey anti-mouse IgG conjugated with Cy3 (0.6 μg/ml; Jackson ImmunoResearch) was applied for 1 h at room temperature and then rinsed as for the primary antibody. For FITC-labeled sections, counterstaining in 0.01% Evan's blue was performed followed by three rinses in PBS. Sections were mounted in Vectashield diluted 2:1 in 0.1 M Tris-HCl, pH 8.0. In some cases (for H^+ATPase and Na-K-ATPase detection in proximal tubules), a 5-min incubation with 1% sodium dodecyl sulfate (SDS), followed by three washes in PBS, was performed before the 1% bovine serum albumin incubation to enhance fluorescence staining, as described previously (21).

To visualize the microtubule network, a monoclonal antibody against α-tubulin (Sigma) was used at a dilution of 1:100. Other antibodies used were: (1) a monoclonal antibody against the 31-kD subunit of the vacuolar H^+ATPase (provided by Stephen Gluck, Washington University, St. Louis, MO) was used undiluted; (2) a polyclonal antibody against gp330/megalin was used at a dilution of 1:200; (3) a polyclonal, affinity-purified antibody against the C-terminal 14 amino acids of the AQP2 water channel was used at a 1:4 dilution; (4) a monoclonal antibody against the AE1 Cl/HCO_3 exchanger (provided by Seth Alper, Beth Israel Hospital, Boston, MA) was used at a 1:1600 dilution; and (5) a mouse monoclonal antibody against the Na-K-ATPase (provided by Doug Fambrough, Johns Hopkins University, Baltimore, MD) was used at a 1:50 dilution. All of these antibodies have been described extensively in previous studies (16,18,19,20,22).

Some 1.5-μm sections were double-stained for H^+ATPase and AQP2 to examine the relative localization and redistribution of these proteins in the same tubule. For this purpose, sections were incubated first with the anti-AQP2 antibody followed by goat anti-rabbit IgG coupled to FITC, and second with the anti-H^+ATPase antibody followed by goat anti-mouse IgG coupled to Cy3. Fluorescent localization of actin filaments was performed using FITC-phalloidin conjugate on 4-μm sections. After rehydration in PBS for 5 min, sections were incubated for 15 min with FITC-phalloidin (0.4 μg/ml; Sigma), followed by three washes in PBS and mounting in Vectashield diluted in Tris-HCl.

Sections were photographed in black and white on Kodak Tmax 400 film at 1600 ASA, and on color Kodak Ektachrome 400 Elite film at 3000 ASA. An Optronics 3-bit charge-coupled device color camera attached to a Nikon FXA photomicroscope was also used to capture images directly; they were stored on an Apple Macintosh Power PC 8500, using IP Lab Spectrum software (Scanalytics, Vienna, VA). The digitized images were printed on a Tektronix Phaser 440 dye sublimation color printer.

Results

Actin Filaments

Localization of filamentous actin (F-actin) using FITC-phalloidin revealed an intense labeling of the brush border of proximal tubule cells (Figure 1a). Dense bundles of actin were also present around the periphery and basolateral pole of proximal tubule cells and collecting duct cells. As shown in Figure 1, b and c, neither cold preservation nor rewarmed affected the appearance of actin filaments.
Cold-Induced Microtubule Disruption

Under control conditions (Figure 2A), microtubules are organized in longitudinal arrays that extend between the apical and the basal poles of epithelial cells in all tubule segments. This pattern of staining is typical of the extensive microtubule cytoskeleton described previously in kidney epithelial cells (13). An almost complete disruption of the microtubule network occurred in proximal tubules after a short (15 min) incubation of tissue slices at 4°C, whereas microtubules in collecting tubules and cortical thick ascending limbs of Henle were still unaffected (not shown). After 4 h cold treatment, the typical apical/basal microtubule arrangement was disrupted in all tubule segments, leading to a complete disappearance of staining in proximal tubules or a diffuse cytoplasmic staining in other segments (Figure 2B). Indeed, we have shown previously, using the microtubule-depolymerizing agent colchicine, that intact microtubules are the major contributors to the indirect immunofluorescence signal in proximal tubules and that tubulin monomers produce a considerably weaker signal (22). In the present study, therefore, the cold-induced reduction of intracellular tubulin staining probably indicates an almost complete microtubule depolymerization, whereas the diffuse cytoplasmic staining observed in some tubule segments might indicate partial microtubule depolymerization. Little or no apparent repolymerization was seen in proximal tubules, whereas some collecting duct cells showed a partial recovery after 15 min rewarming to 37°C in a physiologic solution (Figure 2C).

After 30 min rewarming, a partial recovery of the microtubule cytoskeleton occurred in some epithelial cells (not shown). After 60 min rewarming, microtubules showed a brighter staining, indicative of progressing repolymerization in some tubule segments, but some proximal tubule and collecting duct cells still had not recovered (Figure 2D).

gp330 (Megalin)

In sections of control kidneys, gp330 antibodies revealed an intense labeling of brush-border membranes of proximal tubule cells (Figure 3a), as shown previously (19,23). After 4 h cold treatment, gp330 was partially redistributed into many cytoplasmic vesicles (Figure 3b). No recovery was observed after 15 min rewarming to 37°C (not shown). After 60 min rewarming, the gp330 distribution had partially recovered and showed a strong brush-border membrane staining with fewer labeled intracellular vesicles (Figure 3c).

H⁺-ATPase

Under control conditions, the pattern of staining using anti-H⁺-ATPase antibodies was identical to our previous findings (17,18). In proximal tubules, H⁺-ATPase was located at the apical pole of the cells and formed a tight band of staining at the base of the microvilli, whereas the tips of the microvilli were unstained (Figure 4A). After 15 min exposure of kidney slices to 4°C, H⁺-ATPase-containing vesicles were already detectable in the subapical region of the cytoplasm (not shown). After 4 h cold exposure, these vesicles became more numerous and were scattered more deeply into the cytoplasm (not shown). During rewarming, a rapid and marked redistribution of H⁺-ATPase toward the tip of the brush border was
Figure 2. Immunofluorescence staining of microtubules, using an anti-α-tubulin antibody in rat kidney cortex. (A) Under control conditions, microtubules are arranged in longitudinal arrays between the apical and basal poles of epithelial cells. (B) After 4 h cold treatment, proximal tubules (PT) are negative, whereas other segments (thick ascending limbs of Henle, collecting duct) show a diffuse cytoplasmic staining, indicating microtubule depolymerization. (C) After 15 min rewarming to 37°C following 4 h cold treatment, little or no microtubule repolymerization is seen in proximal tubule cells, whereas some collecting duct cells show a partial recovery (arrowheads). (D) After 60 min rewarming, the microtubule network shows a normal appearance in some epithelial cells, but some proximal tubule and collecting duct cells are still unstained (arrows). Bar, 20 μm.

observed after 15 min (Figure 4B). After 30 min rewarming, the brush-border microvilli were still labeled in some proximal tubules, but in other tubules there was an increase in staining at the base of the microvilli, indicating a partial recovery of the baseline H^+ATPase distribution (Figure 4C).

In control cortical collecting ducts (Figure 5A), H^+ATPase was located in the apical pole of type A-intercalated cells (A-cells) and in the basolateral pole of type B-intercalated cells (B-cells), as described previously (17). In some B-cells, a few intracellular vesicles located either in the apical pole or scat-
Figure 3. Localization of gp330 (megalin) in rat kidney cortex. (a) Under control conditions, proximal tubule brush border is intensely stained, and a few cytoplasmic vesicles are labeled. (b) After 4 h cold treatment, gp330 shows a partial relocalization into numerous cytoplasmic vesicles. (c) After 60 min rewarming to 37°C following cold treatment, fewer intracellular vesicles are seen, indicating that gp330 has been reinserted back into the brush-border membrane. Bar, 20 μm.

Figure 4. H^+ATPase immunostaining of rat kidney cortex (green), counterstained with Evan's blue (red). (A) Under control conditions, proximal tubule cells show a tight band of staining at the base of the microvilli (arrows), whereas the tips of the microvilli are negative. (B) After 15 min rewarming following 4 h cold treatment, a marked relocalization of H^+ATPase toward the tip of the brush-border microvilli is observed, leading to a disappearance of the tight band of staining at the base of the microvilli (arrows). (C) After 30 min rewarming, some proximal tubules have a normal appearance of H^+ATPase staining, with negative microvilli and tight subapical labeling (arrows), but some proximal tubules still had not recovered and show brush-border staining (arrowheads). Bar, 10 μm.
Figure 5. (A) Two examples of cortical collecting duct (CCD) immunostained for H\(^+\)ATPase (green) and counterstained with Evan's blue (red), showing type A and B intercalated cells (IC). H\(^+\)ATPase is located at the apical pole of A-IC (arrows), whereas B-IC show basolateral staining with a few intracellular vesicles (arrowheads). Some B-IC also have a bipolar or diffuse pattern of staining. (B) After 15 min rewarming following 4 h cold treatment, several IC show an almost complete redistribution of H\(^+\)ATPase into cytoplasmic vesicles, leading to a loss of H\(^+\)ATPase polarity. This is particularly apparent in B-IC (arrowheads), whereas some A-IC seem to remain relatively unaffected (arrows). (C) After 30 min rewarming, most IC still have a diffuse pattern of staining for H\(^+\)ATPase, but a partial H\(^+\)ATPase repolarization is seen in some A-IC (arrow) and B-IC (arrowheads). Bar, 15 μm.
relocalization into numerous vesicles scattered throughout the cytoplasm (Figure 5B). This effect was more pronounced in B-cells, in which H^+ATPase-containing vesicles were present in all regions of the cell, whereas in A-cells, vesicles were still restricted to the apical pole. No significant recovery was seen after up to 30 min rewarming in most B-cells, but a partial relocalization of H^+ATPase toward the basolateral pole was observed in some B-cells, and a tight apical staining was also observed in some A-cells, indicating that a partial recovery started to occur at this time point (Figure 5C).

The pattern of staining using antibodies against H^+ATPase and α-tubulin on consecutive sections of a cortical collecting duct shows that after 30 min rewarming following 4 h cold treatment, the microtubule network in principal cells seemed to have recovered, whereas microtubules in intercalated cells were still not repolymerized (Figure 6, A and B). This absence of polymerized microtubules in intercalated cells was accompanied by a diffuse H^+ATPase staining that was more intense in the basolateral pole, identifying these cells as B-cells.

In medullary collecting ducts, only A-cells with apical H^+ATPase were observed (Figure 7A), as shown previously (17,18). Cold exposure did not affect the polarity of the H^+ATPase (Figure 7B), but rewarming to 37°C for a period of 15 min markedly redistributed H^+ATPase into cytoplasmic vesicles (Figure 7C). A significant recovery of H^+ATPase toward the apical pole of medullary A-cells was seen after 30 min rewarming (Figure 7D).

**Water Channel AQP2**

As shown in Figure 7A, the vasopressin-regulated water channel AQP2 is located, under control conditions, mainly in the apical pole of principal cells of the collecting duct (16). The polarized distribution of AQP2 was not significantly affected during cold exposure, although a stronger basolateral staining started to appear after 4 h cold treatment (Figure 7B). As for the H^+ATPase, rewarming to 37°C led to the redistribution of AQP2 into numerous endocytotic vesicles after 15 min (Figure 7C). A partial recovery leading to relocalization of AQP2 toward the apical pole was observed in some cells after 30 min rewarming (Figure 7D).

To determine whether the redistribution of AQP2 during the rewarming period was attributed to a lack of hormonal stimulation, a separate series of experiments was performed in the presence of 1 μM vasopressin in the rewarming buffer. In addition, 10 μM forskolin was added 10 min before fixation to induce a maximal cAMP increase. Under these conditions, AQP2 water channels underwent the same redistribution after 15 min rewarming as that observed in the absence of vasopressin (not shown). After 30 and 60 min rewarming, AQP2 was localized toward the apical pole and formed, in some cases, a tight apical membrane staining indicating recovery of the vasopressin response. It thus appears that the vasopressin-stimulated insertion of AQP2 water channels into the apical membrane is transiently impaired during the rewarming period after cold exposure.

**Na-K-ATPase**

As shown in Figure 8, A and B, neither cold exposure nor rewarming affected the bright basolateral staining observed using Na-K-ATPase antibody in all tubule segments.

**AE1 Anion Exchanger**

Under control conditions, the Cl/HCO3 exchanger AE1 was located in the basolateral membrane of A-cells (Figure 8C). During the rewarming period after cold treatment, AE1 was markedly redistributed into numerous cytoplasmic vesicles; this effect was more pronounced in A-cells from the outer and inner medulla (Figure 8D).
Figure 7. Double staining to detect \( \text{H}^+ \text{ATPase} \) (red) and aquaporin-2 (AQP2; green) in the inner stripe of the outer medulla. (A) Under control conditions, \( \text{H}^+ \text{ATPase} \) and AQP2 are located at the apical pole of collecting duct IC and principal cells, respectively. (B) After 4 h cold treatment, the apical polarity of \( \text{H}^+ \text{ATPase} \) and AQP2 is preserved, although a faint AQP2 basolateral staining is also seen in principal cells (arrows). (C) After 15 min rewarming following 4 h cold treatment, a marked redistribution of \( \text{H}^+ \text{ATPase} \) in IC, and AQP2 in principal cells, is observed and is accompanied by a complete disappearance of apical membrane staining. (D) After 30 min rewarming, a significant recovery of \( \text{H}^+ \text{ATPase} \) toward the apical pole of IC is seen (arrowheads), whereas only a partial recovery of the apical AQP2 localization occurs in some principal cells. Bar, 15 \( \mu \text{m} \).

Discussion

Cold preservation of tissues is commonly used for human organ transplantation and for physiological studies on \textit{in vitro} preparations. However, although hypothermia allows longer \textit{in vitro} survival of tissues, the effect of cold \textit{per se} has not been investigated on intact kidney. The aim of the present study was to examine the cytoskeleton and the distribution of some key transport proteins in proximal and collecting tubules after cold preservation and rewarming to 37°C.

Effect of Cold Preservation and Rewarming on the Cytoskeleton

Actin Filaments. The pattern of actin staining of all epithelial cell types examined remained unchanged after up to 4 h cold treatment alone and during rewarming. In contrast, Hall \textit{et al.} have shown a cold-induced depolymerization of actin filaments in cultured pulmonary endothelial cells (8) and freshly dissected pulmonary arterial smooth muscle cells (9). The extent of depolymerization in endothelial cells varied with the duration of cold exposure and the type of fluid in which the cells were cooled (8). From the present study, it appears that actin filaments in kidney epithelial cells are either more resistant to cold or that the preservation solution that was used helped to prevent significant actin filament depolymerization.

Microtubules. Cold exposure induced a marked depolymerization of the microtubule network in all nephron segments. Proximal tubules were the most sensitive to cold treatment and showed a more rapid and severe disruption of the microtubule cytoskeleton. These results differ from those obtained using colchicine treatment, after which a bright subapical band of residual tubulin staining is always seen in proximal tubules (22). Thus, it appears that cold treatment may depolymerize the apical cap of microtubules that are colchicine-resistant.

Partial recovery of the microtubule cytoskeleton began during the rewarming period in all tubule segments, but some cells remained unstained, indicating either permanent damage or that a longer period of recovery was required. Previous studies have also reported a partial recovery of the cytoskeleton that required rewarming periods varying between 15 and 60 min, and have suggested that up to 20 h was necessary for complete repolymerization in cultured endothelial cells (7,8).
In another report, addition of polyethylene glycol to the cooling solution preserved actin filament and microtubule morphology in hepatocytes during cold treatment at 4°C for up to 24 h (10).

We have shown previously a similar disruption of the microtubule network by ischemia followed by reperfusion in the kidney (22). In that study, proximal tubules were also more sensitive, with the most severe damage seen in the S3 segment. Cortical thick ascending limbs, collecting ducts, and distal tubules did not show significant alteration of the microtubule network. This segmental difference in microtubule stability after ischemic injury is reproduced in the present study during cold treatment, although hypothermia seems to be more effective than ischemia in depolymerizing microtubules. Nevertheless, a partial depletion of cellular ATP that could be responsible for microtubule depolymerization and membrane protein redistribution might also have occurred during hypothermia. Although we cannot rule out the possibility that some degree of ischemic injury contributed to the results found in the present report, no effects were seen on the actin cytoskeleton, which is severely perturbed in other models of ischemic renal injury in which intracellular ATP is depleted (24). In addition, high concentrations of intracellular calcium have been shown to induce microtubule depolymerization either directly, or indirectly via calmodulin (25,26). In the present study, an elevation of intracellular calcium appears less likely because the tissue was cooled in a solution containing no added calcium. The microtubule-associated STOP protein, which confers cold stability to microtubules via a calmodulin-dependent mechanism, has recently been cloned from rat brain (27). A similar protein is also expressed in other mammalian tissues and thus could be involved in the dynamic remodeling of the microtubule network (28). GTP is also required for both initiation and maintenance of microtubule polymerization after cold disruption (29). Other mechanisms, including a decrease in intracellular pH (30) and activation of microtubule-severing proteins similar to p56 (31) or the ATPase katanin (32), may also be involved in microtubule depolymerization. Whether these mechanisms
are responsible for the variable stability of microtubules upon cold treatment observed in the different nephron segments is still a matter of speculation.

Apical Membrane Proteins

Cold preservation followed by rewarming induced a marked redistribution of gp330, H\(^+\)ATPase, and AQP2 into endocytotic vesicles. We have reported previously that in vivo microtubule disruption by colchicine induced a marked relocation of gp330, H\(^+\)ATPase, and AQP2 that was accompanied by an almost complete disappearance of membrane staining, indicating that endocytosis was not initially inhibited (16,18,19). Microtubule depolymerization probably reduces the rate of exocytosis by impairing the delivery of intracellular transport vesicles to the plasma membrane. During cold treatment, no significant redistribution of most proteins tested was observed, as long as the tissue was kept at 4\(^\circ\)C, consistent with reduced rates of endocytosis at this temperature. For gp330, however, redistribution was observed even in the absence of rewarming, indicating either the presence of recycling mechanisms even at 4\(^\circ\)C or a rapid redistribution that occurred during the cooling down process of proximal tubule cells. Upon rewarming, gp330 redistribution became more accentuated, leading to the formation of numerous intracellular vesicles, but the apical membrane remained stained. Despite an apparently complete microtubule disruption by cold, the extent of gp330 redistribution was less marked than after colchicine-induced microtubule depolymerization (19), indicating a potential time dependence of gp330 redistribution. Indeed, up to 6 h colchicine treatment was necessary to induce a significant modification of gp330 staining, whereas the microtubule network was disrupted after only 2 h treatment (our unpublished results). The partial reinsertion of gp330-carrying vesicles back into the apical membrane observed after 60 min rewarming indicates restoration of active endocytosis that was probably associated with the partial microtubule repolymerization observed at this time point.

The cause of the apparent H\(^+\)ATPase migration along the plasma membrane upon rewarming after cold exposure is unknown, but might reflect a complex series of events, because H\(^+\)ATPase, as well as other brush-border proteins, can be inserted only at the base of microvilli by vesicular trafficking. An as yet poorly understood relocation in the plane of the plasma membrane must then occur to allow proteins to relocate to the microvillar tip. Microtubules are unlikely to be directly involved in this process because microvilli do not contain microtubules. In addition, we have previously shown a complete disappearance of apical H\(^+\)ATPase staining in proximal tubule cells after microtubule disruption by colchicine (18). However, a potential role of the actin cytoskeleton, which was not apparently perturbed in our experiments, remains to be determined. In intercalated cells, the H\(^+\)ATPase relocalization observed after rewarming was more accentuated in A-cells from the medulla than from the cortex, indicating a more dynamic recycling machinery in medullary A-cells.

We addressed the question of whether a lack of hormonal stimulation might be responsible for the loss of polarity of AQP2 in principal cells during the rewarming period. Under normal conditions, vasopressin stimulation induces AQP2 insertion into the apical membrane from a pool of specialized vesicles, leading to urine concentration in the collecting duct (16,33). Microtubule disruption by cold followed by rewarming in the presence of nocodazole has been shown to inhibit the vasopressin-induced stimulation of sodium transport in A6 cells (7). In the present study, inhibition of the vasopressin-induced insertion of AQP2 in the apical membrane was observed during the first 15 min of rewarming. This inhibition was probably due to the lack of polymerized microtubules and to the resulting scattering of cytoplasmic vesicles, which reduced the number of vesicles beneath the apical membrane. In addition, the more complete response observed upon vasopressin stimulation after 30 and 60 min rewarming, when microtubules are partially repolymerized, argues in favor of a role for the microtubule network in this process.

Basolateral Membrane Proteins

Previous studies have suggested that microtubules are involved in the efficient delivery of membrane proteins to the apical membrane, and in basol-to-apical transcytosis (12,13). However, we have shown previously that microtubule disruption not only affects the localization of apical H\(^+\)ATPases (in A-cells), but also impairs the basolateral insertion of this protein in B-cells (18). The present study provides further evidence of the involvement of microtubules in the insertion of basolateral H\(^+\)ATPases. In fact, basolateral H\(^+\)ATPases were among the most affected proteins of all tested in this study, with a more diffuse distribution of cytoplasmic vesicles observed in B-cells compared with cortical A-cells. This might be due to a more labile microtubule network in B-cells. The fact that microtubules were still depolymerized in most B-cells after a recovery period of 30 min at 37\(^\circ\)C supports this hypothesis. However, a few B-cells showed a strict basolateral H\(^+\)ATPase localization after this time. Whether microtubules had at least partially repolymerized in this subpopulation of B-cells or whether other factors are involved in the reestablishment of basolateral H\(^+\)ATPase polarity requires further investigation.

In contrast to all other proteins tested in this study, the basolateral distribution of Na-K-ATPase remained unchanged during cold exposure followed by rewarming. Na-K-ATPase is anchored to the basolateral membrane via its association with the cytoskeleton and might be excluded from dynamic recycling pathways (34). However, recent evidence has indicated that Na-K-ATPase activity might be modulated via recycling mechanisms (4,35–37). The absence of Na-K-ATPase redistribution after cold preservation observed in the present study might reflect a slower rate of recycling compared with gp330, H\(^+\)ATPase, AQP2, or AE1, leading to a longer half-life in the plasma membrane. Indeed, whereas a significant redistribution of Na-K-ATPase in proximal tubules has been reported from human transplanted kidneys that had been exposed to cold ischemia for an average of 20 h, no significant redistribution was observed after an average of 10 h of cold ischemia (11). In addition, Na-K-ATPase electrophysiological activity can still be detected on isolated proximal tubules perfused in vitro after...
preservation of the kidney at 4°C for up to 8 h (4), supporting the notion that this protein is not initially affected by cold treatment followed by rewarming. It is interesting to note that an internalization of Na-K-ATPase was observed after ATP depletion in Madin–Darby canine kidney cells, and that this redistribution was not microtubule-dependent but rather appeared to correlate with cortical actin network depolymerization (38). It is therefore possible that, in the present study, the integrity of Na-K-ATPase insertion into the basolateral membrane was maintained because the actin cytoskeleton was not modified. Interestingly, despite its similar anchoring to the basolateral cytoskeleton, the AE1 anion exchanger was markedly redistributed in A-cells during the rewarming period. This may reflect a differential sensitivity of principal and intercalated cells to the cold incubation performed in this study. The question of whether AE1 recycling might also be involved in the regulation of this transporter under normal conditions requires further investigation.

**Effect of Cold Preservation on Kidney Function**

The present study indicates that cold preservation of kidneys affects the microtubule network and the localization of some membrane proteins, while other proteins seem unaffected. The extent to which protein redistribution is induced might depend on the rate of recycling, with proteins that recycle most rapidly being the most affected by cold treatment followed by rewarming. These results thus suggest an impairment of some kidney functions, at least transiently, by hypothermia.

In the context of human transplantation, for which organs are cooled to 4°C during transportation to the recipient, the effect of cold described in the present study might contribute to the initial malfunction of freshly transplanted kidneys, requiring hemodialysis during the initial recovery period. For *in vitro* studies, which also involve cold preservation of tissues, an initial period of recovery is often required for the restoration of some tubule transport processes to occur. It is also possible that some transport mechanisms might have remained undetected because a longer period of recovery was necessary for certain proteins to reestablish their normal polarized distribution. The establishment of better tissue preservation conditions could, therefore, be important not only for human transplantation, but also for our understanding of basic transport properties of the urinary tubule.

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