Effects of pathophysiological concentrations of albumin on NHE3 activity and cell proliferation in primary cultures of human proximal tubule cells

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Lee, E. M., C. A. Pollock, K. Drumm, J. A. Barden, and P. Poronnik. Effects of pathophysiological concentrations of albumin on NHE3 activity and cell proliferation in primary cultures of human proximal tubule cells. Am J Physiol Renal Physiol 285: F748–F757, 2003. First published June 10, 2003; 10.1152/ajprenal.00442.2002.—The progression of renal disease correlates strongly with hypertension and the degree of proteinuria, suggesting a link between excessive Na+ reabsorption and exposure of the proximal tubule to protein. The present study investigated the effects of albumin on cell growth and Na+ uptake in primary cultures of human proximal tubule cells (PTC). Albumin (1.0 mg/ml) increased cell proliferation to 134.1 ± 11.8% (P < 0.001) of control levels with no change in levels of apoptosis. Exposure to 0.1 and 1.0 mg/ml albumin increased total 22Na uptake to 119.1 ± 6.3% (P = 0.005) and 115.6 ± 5.3% (P < 0.006) of control levels, respectively, because of an increase in Na+/H+ exchanger isoform 3 (NHE3) activity. This was associated with an increase in NHE3 mRNA to 161.1 ± 15.1% (P < 0.005) of control levels in response to 0.1 mg/ml albumin. Using confocal microscopy with a novel antibody raised against the predicted extracellular NH2 terminus of human NHE3, we observed in nonpermeabilized cells that exposure of PTC to albumin (0.1 and 1.0 mg/ml) increased NHE3 at the cell surface to 115.4 ± 2.7% (P < 0.0005) and 122.4 ± 3.7% (P < 0.0001) of control levels, respectively. This effect was paralleled by significant increases in NHE3 in the subplasmalemmal region as measured in permeabilized cells. These albumin-induced increases in expression and activity of NHE3 in PTC suggest a possible mechanism for Na+ reabsorption in response to proteinuria.

Proteinuria; sodium-hydrogen exchange; sodium retention

Proteinuria and Na+-dependent hypertension are well known to occur in the setting of renal disease. It has been considered that proteinuria is primarily due to glomerular pathology and that excessive tubular Na+ reabsorption is a “normal” response to reduced plasma volume. However, in conditions such as diabetes mellitus (34), microalbuminuria, hypertension (8), and increased proximal tubular Na+ reabsorption occur in the presence of volume expansion and are the initial manifestations of nephropathy. Similarly, in primary proteinuric renal disease, Na+ retention, which can result in edema and/or hypertension, may occur in the presence of a relatively normal serum albumin concentration. These observations suggest a more direct relation between proteinuria and Na+ reabsorption that is independent of the circulating plasma volume.

Under normal conditions, the kidneys filter ~180 liters of filtrate and reabsorb 1.7 kg of NaCl per day (21), a function principally performed by the Na+/H+ exchanger (NHE) isoform 3 (NHE3) in the luminal membrane (3). Several lines of experimental evidence suggest that increases in NHE3 activity are linked to hypertension. Elevated levels of NHE3 protein and activity have been observed in freshly isolated tubular cells and isolated intact tubules from spontaneously hypertensive rats (22). In NHE3 knockout mice, systolic and arterial blood pressures are reduced, suggesting a key role for NHE3 in maintaining these parameters (27), whereas there is an increase in NHE3 activity in purmorphycin aminonucleoside-induced nephrotic rats that may contribute to the increased Na+ retention in these animals (4). Significantly, a study in hypertensive patients revealed that proximal tubule Na+ reabsorption was an independent determinant of the blood pressure response to salt-induced hypertension (8). It is important to keep in mind that although animal models of diabetes may develop kidney disease that displays features in common with human diabetic nephropathy, no single animal model develops renal changes identical to those observed in humans (37). As a result, it is critical to determine the response of cells of human proximal tubular origin to exposure to conditions that mimic the proteinuric state.

In addition to Na+ reabsorption, it is estimated that several grams of albumin enter the proximal tubules on a daily basis, yet the urinary excretion of albumin is normally <50 mg/day; this suggests constitutive reabsorption of albumin (28). This reabsorption occurs in the proximal tubule via receptor-mediated endocytosis involving the scavenger receptor megalin (10). Album-
min itself has been shown to exert a number of effects on the proximal tubule. Exposure of opossum kidney (OK) cells to pathophysiological concentrations of albumin has been shown to stimulate cellular proliferation (12) and also to impair albumin endocytosis by reducing the number of binding sites ($V_{max}$) at the plasma membrane (19), whereas exposure to albumin has been shown to induce apoptosis in LLC-PK1 cells (16).

NHE3 has been recently demonstrated to play a critical role in receptor-mediated albumin uptake in cell cultures. Studies in OK cells have shown that failure to acidify the early endosome (18) or inhibition of NHE3 with amiloride analogs significantly reduces albumin uptake (17), an observation that is supported by the finding that albumin uptake is abolished in NHE3-deficient OK cells (20). Thus changes in the regulation of endosomal pH may play a significant role in tubular dysfunction (29). In rabbit renal cortical membrane fractions, a substantial fraction of the cellular pool of NHE3 has been shown to be associated with the scavenger receptor megalin via interaction with its COOH-terminal tail (6), and NHE3 in the megalin-associated pools in the rabbit brush border was inactive (8). These data indicate a dual role for NHE3 in Na$^+$ reabsorption and albumin uptake. In contrast to the other proximal tubule NHE isozymes 1 and 2, NHE3 is known to exist primarily in endosomes, with only ~15% of the total cellular pool of NHE3 located in the plasma membrane (2, 7). Rapid alterations in the activity of NHE3 are accomplished by changes in the rates of deployment of NHE3 from the endosomes or retrieval of NHE3 from the plasma membrane (13). Furthermore, recent evidence shows that NHE3 exists in lipid rafts in rabbit ileal brush borders and that stimulation with epidermal growth factor (EGF), which increases NHE3 activity, results in a preferential increase in the amount of NHE3 in the lipid raft fraction (26).

From these data, it is clear that NHE3 exists in functionally distinct pools at the plasma membrane, for example, pools associated with Na$^+$ reabsorption and others involved in receptor-mediated albumin endocytosis. Thus there may be different retrieval/insertion mechanisms to and from these pools, such that the rates of insertion of NHE3 into the plasma membrane may occur independently of the rate of the NHE3 internalization in conjunction with receptor-mediated albumin endocytosis. This raises the intriguing possibility that increased tubular albumin may have two separate actions: 1) it may reduce the capacity of the albumin uptake pathway, thereby increasing levels of protein in the urine; and 2) if the rates of insertion of NHE3 into the different pools are not directly linked to the rate of receptor-mediated endocytosis, levels of functional NHE3 may be increased, resulting in increased Na$^+$ retention by the proximal tubule. Such a model may provide an explanation for the link between hypertension and proteinuria in diabetic nephropathy. The aims of the present study were, therefore, to determine the effects of albumin on the expression, distribution, and activity of NHE3 in human proximal tubule cells (PTC) and to correlate these effects with changes in cellular growth.

**METHODS**

**Primary culture of human PTC.** Segments of macroscopically and histologically normal renal cortex were obtained from patients undergoing nephrectomy for small (<6 cm) tumors. Patients were accepted for inclusion into the study if there was no history of renal or systemic disease known to be associated with tubulointerstitial pathology. Written informed consent was obtained from each patient before surgery, and ethical approval for the study was obtained from the Royal North Shore Hospital Human Research Ethics Committee. The methods for primary culture of human PTC are described in detail elsewhere (25). Briefly, tubular fragments were derived from segments of renal cortex by collagenase digestion and isolated by centrifugation in 45% Percoll (Pharmacia, Uppsala, Sweden). The PTC were resuspended in serum-free hormonally defined media consisting of a 1:1 (vol/vol) mixture of Dulbecco’s modified Eagle’s and Ham’s F-12 media (DMEM-F-12; ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 10 ng/ml EGF (Collaborative Research, Bedford, MA), 5 mg/ml human transferrin, 5 mg/ml bovine insulin, 0.05 mM hydrocortisone, 50 mM prostaglandin E1, 50 mM selenium, 5 PM triiodothyronine (all from Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.292 mg/ml l-glutamine (Invitrogen, New York, NY). At confluence, the cells were harvested using dispase (Integrated Sciences, Sydney, Australia) and stored in liquid nitrogen. When required, the cells were thawed, and after reaching confluence, they were harvested using dispase and subcultured. These cells were designated passage 2. The experiments in this study were performed on primary cell cultures derived from the kidneys of 16 patients.

**Experimental protocol.** All experiments were carried out on passage 2 PTC. Cells were made quiescent by incubation in DMEM-F-12 containing 5 μg/ml human transferrin and 5 mM D-glucose without growth factors for 48 h. The cells were then cultured for a further 48 h in medium containing 5 mM D-glucose (control) or 5 mM D-glucose containing 0.1 or 1.0 mg/ml delipidated bovine serum albumin (Sigma Chemical).

**Growth studies.** Experiments to measure cell number and cell protein were performed in parallel, with the results obtained for total protein being adjusted to the total cell number in each of the treatments. Cell numbers in response to each treatment were determined by manual cell counts on trypanized cells using a hemocytometer. The total protein content of cells was determined as a marker of cellular hypertrophy. Cells were solubilized with 0.2 M NaOH, and the protein was measured using a protein assay (Bio-Rad, Hercules, CA).

22Na$^+$ uptake. 22Na$^+$ uptake into cells was measured on the basis of the method of Rindler et al. (35). PTC were grown to confluence in 48-well culture plates, quiesced for 48 h, and then exposed to 0.1 or 1.0 mg/ml albumin or control conditions for 48 h. Cells were washed twice in HEPES-buffered saline (in mmol/l: 136 NaCl, 5.4 KCl, 1.2 CaCl₂, 0.8 MgCl₂, 10 acetic-HEPES, and 5 glucose, pH 7.4) and incubated with Na$^+$-free solution (HEPES-buffered saline with NaCl replaced by N-methyl-D-glucamine) for 15 min to deplete intracellular Na$^+$. All uptake solutions contained the corresponding amounts of albumin. The cells were preincubated with 100 μM ouabain with or without the NHE blocker ethylisopropylamiloride (EIPA, 100 μM) in Na$^+$-free medium for a further 30 min. The Na$^+$ solution was replaced with the
uptake solution containing $^{22}$Na$^+$ tracer (1 μCi/ml; New England Nuclear Geneworks, Boston, MA) in glucose-free HEPES-buffered saline for 20 min. At the end of the $^{22}$Na$^+$ uptake period, the cells were washed rapidly three times with ice-cold 0.1 M MgCl$_2$ and solubilized in 0.1 M NaOH. Cell lysate was mixed with scintillation fluid and counted in a beta scintillation counter (LKB Wallac, Turku, Finland). Parallel cell counts were performed, and the total $^{22}$Na$^+$ uptakes were adjusted to cell number in each treatment and expressed as a percentage of the control values.

**Cell cycle analysis.** PTC were grown to confluence in six-well plates and exposed to 0.1 or 1.0 mg/ml albumin or control conditions for 48 h. The cells were then trypsinized, placed in 1.5-ml tubes, washed in 0.5 ml of phosphate-buffered saline (PBS), centrifuged (1,000 rpm, 10 min, 4°C), and fixed in 70% (vol/vol) ethanol at −20°C for ≥3 h. Cells were washed in PBS and permeabilized in 0.5 ml of PBS with 0.1% Triton X-100 on ice for 30 min. Cells were then centrifuged, and the pellet was resuspended in 0.5 ml of fluorochrome solution (propidium iodide (50 μg/ml), RNase (1 mg/ml), and Triton X-100 (0.1% vol/vol) in PBS and incubated for 1 h at 4°C. Cells were then scanned on the relative intensities of stage SE flow cytometry system (Becton Dickinson, San Diego, CA). The propidium iodide fluorescence of individual nuclei and the forward and side scatter were measured using identical instrument settings with ≥20,000 events.

**Competitive RT-PCR.** Competitive RT-PCR was performed to determine the changes in NHE3 expression level induced by exposure to albumin. PTC were grown to confluence in six-well plates and exposed to 0.1 mg/ml albumin for 48 h before RNA extraction. Total RNA was extracted using TRIzol reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s instructions. RNA was reverse transcribed using the Superscript II reverse transcriptase kit (GIBCO BRL).

Competitive RT-PCR was performed using primers specific to the COOH-terminal tail of human NHE3 (GenBank accession no. U28043). The primer sequences were 5′-GGTCTTCACCCGTGCATCCTCCA (sense, bp 1345–1365) and 5′-CTGAGAAAAATGTCAAGGCT (antisense, bp 1769–1789), and the competitor primer sequence was 5′-CTGAGAGAAAATGTCAGGCCTGCAATCAGTGGCC (bp 1637–1655). These primers produced products of 445 and 311 bp, respectively. Products were sequenced and confirmed to be human NHE3. Competitive RT-PCR against β-actin was used as a control. Primers were designed against the human β-actin gene (GenBank accession no. M10277). The primer sequences were 5′-CAGTCAGTGCTGATCCAG (sense, bp 2058–2076) and 5′-CGCAACTAAGTCTAGTCG (antisense, bp 3005–3021), and the competitor primer sequence was 5′-CGCAACTAAGTCTAGTCG (bp 2889–2906). These primers produced products of 964 and 849 bp, respectively.

PCR were performed on cDNA using the sense and control primers, and the products were gel purified and quantitated. For the PCR, the competitor cDNA was used at 0.2, 0.5, 1.5, 4.6, and 13.7 fM. The reactions were for 35 cycles with an annealing temperature of 60°C using the Expand High-Fidelity PCR System (Roche, Mannheim, Germany). The products were run on a 2% agarose gel stained with ethidium bromide and photographed. The photograph was then scanned into a computer, and the relative intensities of the individual bands were quantitated using NIH Image software (version 1.60). To normalize the data to the levels of the housekeeping gene β-actin, the ratios of the concentration of competitor primer for NHE3 to the concentration of the β-actin primers at the equivalence point were determined. The equivalence point is defined as the point at which the ratio of the intensity of the bands for the competitor to native cDNA is unity, that is, the point at which the concentration of the competitor is equivalent to the concentration of the message for the target gene.

**Polyclonal antibody to the extracellular domain of human NHE3.** The peptide sequence of human NHE3 (GenBank accession no. NP_004165) was screened for potential extracellular epitopes. The polypeptide GGEVEVPGAGHGESGGF was selected. This peptide corresponds to amino acids 26–42 of the predicted human sequence, a region that is within the predicted first extracellular loop. The SignalP algorithm was used to predict the position of any putative signal peptide cleavage site in the NH$_2$-terminal region of human NHE3 (31). The peptide was synthesized, and an NH$_2$-terminal cysteine was added to the epitope for conjugation via dithyliar toxin using maleimidocaproyl-N-hydroxysuccinimide (Chirion Mimotopes, Clayton, Victoria, Australia). Polyclonal antibodies were raised in rabbits, and the immune serum IgG was affinity purified.

**Confocal immunofluorescence.** Confocal microscopy was performed on PTC grown on Cell-Tak (BD Biosciences, Bedford, MA)-coated coverslips as follows. PTC were fixed and permeabilized with 4% paraformaldehyde in PBS for 2 min. Where required, cells were permeabilized with 0.1% DMSO in 2% normal horse serum, 0.1% Triton X-100, and 0.1% bovine serum albumin in PBS for 2 min. Permeabilized and nonpermeabilized cells were blocked with 20% normal horse serum in PBS for 20 min. The cells were then incubated with the anti-NHE3 antiseraum (1:100) for 2 h at room temperature, washed, and incubated with a Cy3 anti-rabbit antibody (1:200; Jackson Immunochemicals) for a further 45 min. Slides were sealed with mounting medium (Dako, Carpinteria, CA) and visualized under a Leica TCS NT laser confocal microscope (Leica, Solms, Germany) with excitation at 488 nm and emission at 570 nm.

To demonstrate the specificity of the polyclonal anti-NHE3 antibody, 80 μM epitope peptide was preincubated with the antibody for 10 min to block the binding of the antibody-binding sites. Surface NHE3 distribution was determined on PTC quiesced in 5 mM glucose medium for 48 h and then exposed to 0.1 or 1.0 mg/ml albumin for 48 h. Cells were nonpermeabilized to determine surface levels of NHE3 or permeabilized to determine total NHE3 in the same apical plane used to determine the surface levels. Images were processed with Adobe Photoshop (version 5.02), and pixel densities at the apical pole of the cells were quantitated using NIH Image software (version 1.60).

**Statistical analysis.** Experiments were performed at least in triplicate on a minimum of four different cell culture preparations. Unless otherwise stated, results are expressed as percentage of control values (cells grown in the absence of albumin for the experimental period). Statistical comparisons between groups were made by analysis of variance or paired t-tests where appropriate. Analyses were performed using the software package Statview (version 4.5, Abacus Concepts, Berkeley, CA). P < 0.05 was considered significant.

**RESULTS**

**Growth studies.** Micropuncture experiments in animals yielded concentrations of albumin in the postglomerular filtrate of 10–200 μg/ml (28). We therefore used albumin at 0.1 mg/ml to reflect an upper level of normal and 1.0 mg/ml to reflect a pathological level in the proximal tubule. We found that exposure of PTC to 0.1 mg/ml albumin for 48 h had no significant effect on...
cell growth parameters. Cell number was 104.0 ± 4.7% (n = 12; Fig. 1A) and cellular protein content 102.7 ± 5.1% (n = 12; Fig. 1B) of control values. In contrast, exposure of PTC to 1.0 mg/ml albumin for 48 h caused a pronounced increase in cell number to 134.1 ± 11.8% (n = 9) of control values (P < 0.0005; Fig. 1A), consistent with a proliferative response. This was accompanied by a significant reduction in protein per cell (76.9 ± 5.8%, n = 9, P = 0.0005) compared with control values (Fig. 1B).

Apoptosis. Fluorescein-activated cell sorter analysis revealed that, under control conditions, 0.77 ± 0.16% of cells were in the pre-G1 peak, which represents apoptotic cells. No significant changes were observed in the numbers of cells in the pre-G1 peak after exposure to 0.1 or 1.0 mg/ml albumin for 48 h: 0.85 ± 0.37 and 0.71 ± 0.25%, respectively. These data indicated that exposure to albumin did not increase the levels of apoptosis of PTC.

$^{22}$Na$^+$ uptake. Total $^{22}$Na$^+$ uptake was significantly increased in PTC after exposure to albumin for 48 h at 0.1 and 1.0 mg/ml: 119.1 ± 6.3% (n = 13, P = 0.0005) and 115.6 ± 5.3% (n = 11, P < 0.006) of control levels, respectively (Fig. 2). Incubation of the control cells with 100 μM EIPA reduced $^{22}$Na$^+$ uptake to 42.4 ± 2.8% (n = 13) of baseline levels (Fig. 2). Similarly, incubation of the albumin-treated cells with EIPA reduced $^{22}$Na$^+$ uptake to the level observed in control cells: 38.6 ± 4.3% (n = 13) in 0.1 mg/ml albumin and 47.9 ± 3.7% (n = 11) in 1.0 mg/ml albumin (Fig. 2). Thus the increase in total $^{22}$Na$^+$ uptake was abolished by treatment with EIPA, and these data show that albumin exposure resulted in significant increases in the EIPA-sensitive component of $^{22}$Na$^+$ uptake to 131.0 ± 6.3% (P = 0.0001) for 0.1 mg/ml albumin and 124.7 ± 8.6% (P < 0.005) for 1.0 mg/ml albumin compared with control cells not exposed to albumin (100%). Importantly, 1.0 μM EIPA had no effect on $^{22}$Na$^+$ uptake (data not shown), indicating that this component of $^{22}$Na$^+$ uptake was not occurring via NHE isoform 1 (32). Furthermore, no effects were observed on $^{22}$Na$^+$ uptake in cells exposed to lower concentrations of albumin (0.001 or 0.01 mg/ml) for 48 h (data not shown).

Competitive RT-PCR. Competitive RT-PCR was performed to determine whether the increases in $^{22}$Na$^+$ uptake observed in response to exposure to 0.1 mg/ml albumin were paralleled by an increase in NHE3 mRNA expression levels. A representative gel of the products of the competitive RT-PCR is shown in Fig. 3A, and logarithmic plots of the ratio of the target to competitor bands against competitor concentration for NHE3 and the housekeeping gene β-actin are shown in Fig. 3B. There is a small but significant shift in the equivalence point for NHE3, whereas no change is observed with β-actin. The NHE3 ratios were standardized to the β-actin ratios to account for any changes in total mRNA. Overall, there was a significant increase in the levels of mRNA for NHE3 to 161.4 ± 15.1% (n = 6, P < 0.005) relative to the total mRNA pool (as reflected by β-actin) in PTC exposed to albumin for 48 h (Fig. 3C). These data confirm that exposure of PTC to 0.1 mg/ml albumin, a concentration that increases NHE3 activity, also results in a parallel increase in NHE3 message levels.
Confocal immunoﬂuorescence. Surface labeling was performed on nonpermeabilized and permeabilized PTC. When a confocal x-y scan was taken through the upper (subapical) part of the cells, nonpermeabilized cells exhibited a punctate distribution of labeling, principally at the cell periphery (Fig. 4A), whereas permeabilized cells also exhibited signiﬁcant levels of intracellular staining (Fig. 4B). z-Axis scans on nonpermeabilized and permeabilized cells revealed that NHE3 was primarily localized to the apical domain of the cells (Fig. 4C and D). These staining patterns are consistent with the known distribution of NHE3 in other cell types (2, 9, 33). To conﬁrm the antibody speciﬁcity, the antiserum was preincubated with the antigenic peptide before the cells were labeled. This resulted in a dramatic decrease in the ﬂuorescent signal (Fig. 4E, F), indicating that the antibody staining was indeed speciﬁc for the epitope on NHE3. A similar decrease in ﬂuorescence was also observed with a lower peptide concentration (10 μM; data not shown).

After exposure to albumin for 48 h, there was a signiﬁcant increase in the levels of cell surface NHE3 (Fig. 5A and B). Similarly, when x-y scans were performed on permeabilized cells viewed through the...
same optical plane, albumin also induced an increase in total NHE3 (Fig. 5, C and D). Measurements of relative pixel intensities in individual cells revealed a significant increase in the level of fluorescence intensity with both concentrations of albumin. In cells exposed to 0.1 mg/ml albumin, the level of fluorescence at the apical surface was 115.4 ± 2.7% (n = 20, P < 0.0001) compared with control levels (Fig. 6A). A similar increase in fluorescence (118.1 ± 3.2%, n = 17, P < 0.0001 compared with control) was observed in cells exposed to 1.0 mg/ml albumin (Fig. 6A). A similar analysis of pixel density at the apical pole was performed on permeabilized cells to determine the amounts of NHE3 on the cell surface relative to the

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**Fig. 4.** Representative images of confocal immunofluorescence microscopy showing distribution of NHE3 in PTC. Images are representative of ≥6 separate experiments. A: nonpermeabilized cells; x-y scan through the subapical pole of the cells shows punctate distribution of NHE3 at the cell periphery. B: corresponding view on permeabilized cells showing intracellular distribution. C: x-z scan of nonpermeabilized cells showing NHE3 at the apical pole of the cells. D: corresponding view on permeabilized cells showing that NHE3 is primarily located in the subapical region of the cell. E: antibody specificity shown as control permeabilized cells labeled with NHE3 antibody. F: cells labeled with NHE3 antibody preincubated with 80 μM epitope peptide showing a pronounced reduction in staining, thereby confirming the antibody specificity. Images are representative of 3 separate experiments. Scale bars, 5 μm.
intracellular pool in the immediate vicinity of the apical membrane. In response to albumin, there was an increase in total NHE3 at the apical membrane increasing to 118.7 ± 2.4% (n = 19, P < 0.0001) and 109.8 ± 2.7% (n = 20, P < 0.02), respectively (Fig. 6B). When the pixel intensities for the nonpermeabilized cells were standardized to the permeabilized cells, these data revealed that 76.1 ± 2.1% of the total apical NHE3 pool was located at the cell surface (Fig. 6C). Significantly, although the total amount of NHE3 at the cell surface increased, the proportion of NHE3 at the cell surface relative to the total subapical pool did not change with albumin exposure: 74.0 ± 1.7% and 81.9 ± 2.2% of total NHE3 for 0.1 and 1.0 mg/ml albumin, respectively (Fig. 6C). These data suggest that the increase in NHE3 activity we observed in response to prolonged exposure to albumin resulted from an increase in total NHE3 that translated into a parallel increase in the plasma membrane, rather than altered rates of insertion from submembrane stores.

DISCUSSION

This study describes the effects of exposure of primary cultures of human PTC to pathophysiological concentrations of albumin on cell growth and NHE3 expression and activity. In addition to demonstrating altered cell growth parameters, we have shown that exposure of PTC to pathophysiological levels of albumin results in significant increases in Na\(^+\) reabsorption through an increase in NHE3 activity. This occurs in association with increases in NHE3 gene and protein expression at the apical membrane of the PTC.

Our demonstration that albumin at 1.0 mg/ml resulted in a pronounced proliferative effect is consistent with findings in OK cells, where exposure to albumin increased cell number and total thymidine uptake, with a maximal effect at 1.0 mg/ml albumin (12). The fact that we observed no effects on cell growth at the lower concentration of albumin (0.1 mg/ml) may simply reflect a difference in the cell types studied. These data show that delipidated albumin alone, at a concentration in the high pathophysiological range and in the absence of growth factors, is able to induce cell proliferation. It has been shown that, after induction of experimental diabetes in a rat model, nephromegaly is preceded by an initial hyperplastic phase over the first few days (23). Consequently, this initial hyperplasia may, in part, be accounted for by the proliferative effect of elevated albumin.
Albumin alone (at >0.5 mg/ml) has been shown to protect cultured murine PTC from apoptosis by a mechanism involving the scavenging of reactive oxygen species (24). In the present study, we observed no significant changes in the levels of apoptosis in the presence and absence of albumin; thus our data are consistent with a renoprotective effect of albumin. These findings contrast with those reported in LLC-PK1 cells, where exposure to albumin induced apoptosis; however, in these studies, much higher concentrations of albumin were used (>5 mg/ml) (16). An in vivo study in protein-overload rats showed an increase in the number of proliferating cells as determined by in situ hybridization for histone mRNAs. However, this increase was counteracted by an even greater increase in the number of apoptotic cells, with tubular atrophy being the net result (36). Furthermore, it has been shown in LLC-PK1 cells (42), OK cells (14), and primary cultures of rat PTC (38) that exposure to pathophysiological levels of albumin results in an activation of NF-κB that results in enhanced cytokine/chemokine production. Thus the increases in PTC number that we observe in culture may result in vivo in the increased cytokine/chemokine production that underlies the inflammatory phase of tubulointerstitial pathogenesis (41).

NHE3 has been shown to exist primarily in subapical endosomal pools and a juxtanuclear compartment in OK cells (2) and AP-1 cells transfected with NHE3 (15). It has been demonstrated that as little as 15% of the total cellular NHE3 is present in the plasma membrane (1, 2, 7). To directly monitor changes in levels of NHE3 at the cell surface, we developed an antibody to a predicted extracellular epitope in the first extracellular loop of human NHE3 on the basis of the model of NHE3 with 12 transmembrane loops and intracellular NH₂ and COOH termini. The epitope recognized by our antibody corresponds to amino acids 26–42 of the published human NHE3 sequence (GenBank accession no. NP_004165). A recent topological analysis of rabbit NHE3, however, showed that the first 30 amino acids of NHE3 form a signal peptide that is cleaved during processing, resulting in an extracellular NH₂ terminus (40). We found a predicted signal peptide cleavage site between Gly27 and Val28 of the human sequence comparable to the predicted cleavage site in rabbit NHE3 between Gly29 and Ala30 (40). Thus our antibody recognizes the first 16 amino acids of the NH₂ terminus of human NHE3 after signal peptide cleavage, and the binding of our antibody to the surface of nonpermeabilized PTC confirms that this region of human NHE3 is extracellular.

Surface labeling of PTC revealed a punctate distribution of NHE3 similar to that observed in OK cells (2, 39). In permeabilized PTC, there was considerably more NHE3 in the cytosol, and this intracellular NHE3 appeared to be associated largely with endosomal compartments in the subapical region of the PTC (Fig. 4). Therefore, these data obtained from confocal sections taken through the cell body are in agreement with the overall cellular distribution of NHE3 in other cell types, e.g., PS120 and OK cells (2, 15), where only 10–15% of the total cellular NHE3 pool was reported to be inserted at the cell surface (1, 2, 7). Interestingly, our data, derived from confocal sections taken in the

**Fig. 6.** Graphical representation of images in Fig. 5. A: pixel intensity analysis of cell surface NHE3 showed a clear increase in NHE3 in response to exposure to 0.1 and 1.0 mg/ml albumin (n = 20 and 17, respectively). B: pixel intensity analysis of total NHE3 in permeabilized cells labeled with NHE3 antibody and viewed through the same optical plane as in A (n = 19 and 20, respectively). Data show a clear increase in total NHE3 after exposure to albumin. C: proportion of surface NHE3 relative to total NHE3 in the same optical plane. Data show that changes in cell surface expression parallel changes in total NHE3 (n = 12, 19, and 20 for control and 0.1 and 1.0 mg/ml albumin, respectively). Results are standardized to control levels (100%). Values are means ± SE. *P < 0.05; **P < 0.0001.
plane of the apical cell surface on permeabilized and nonpermeabilized cells (Figs. 5 and 6), suggest that a significant proportion (~70%) of the total NHE3 in the immediate direct vicinity of the cell membrane is present at the cell surface. However, because of the difficulties in precise quantitation of relative intensities due to overlapping stores of NHE3 visualized under permeabilized and nonpermeabilized conditions, it is possible that this method may overestimate the proportion of total NHE3 at the cell surface. Nevertheless, our data support the study in OK cells showing that NHE3 exists in different functional domains and is present in large complexes at the apical surface of OK cells, with the intracellular pools acting as reservoirs for membrane-recruitable NHE3 (2). It has been reported that NHE3 exists as an active oligomer in the microvillar domain and as an inactive megalin-associated form in intermicrovillar domains (5) and that NHE3 levels increase preferentially in lipid rafts after stimulation with EGF (26). Thus the increase in surface staining for NHE3 and increased NHE3 activity that we observed after exposure to albumin may reflect an enhanced association with the plasma membrane domains involved in mediating Na+ reabsorption. This model, on the basis of the present data, is consistent with a recent finding in nephrotic rats, where the increase in NHE3 activity was associated with a shift from the inactive megalin-associated pool to the active pool in the brush border of proteinuric rats (4), potentially contributing to increased Na+ retention. Our findings are also in agreement with a report that albumin increased NHE3 activity and immunoreactivity in OK cells, although higher concentrations of albumin (~5 mg/ml) were required to elicit a response (30).

The increase in NHE3 expression and activity suggests that exposure to albumin increases the Na+ reabsorptive capacity of the human proximal tubule. Significantly, albumin induced increases in Na+ reabsorption at concentrations below which it exerted its proliferative effect. High concentrations of albumin are reported to enhance the proliferation of PTC (11); however, the differential effects we observed at lower concentrations indicate a specific role of albumin in regulating Na+ reabsorption.

In summary, the present study demonstrates the concentration-dependent uptake of albumin by primary cultures of human PTC and shows that albumin uptake is associated with an increase in the activity of NHE3. A sustained 10–20% increase in the transcription and activity of NHE3 in response to elevated albumin may lead to a significant increase in Na+ retention, contributing to the development of hypertension, whereas a concomitant reduction in the tubular absorption of albumin in the presence of elevated albumin would result in the increased excretion of albumin manifest as proteinuria. Thus the data in the present study present a possible mechanism to explain the link between reduced albumin uptake and increased Na+ retention as observed in diabetic nephropathy.

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DISCLOSURES

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