AE2 isoforms in rat kidney: immunohistochemical localization and regulation in response to chronic NH₄Cl loading

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Submitted 21 November 2003; accepted in final form 26 January 2004

Frische, Sebastian, Alexander S. Zolotarev, Young-Hee Kim, Jeppe Praetorius, Seth Alper, Soren Nielsen, and Susan M. Wall. AE2 isoforms in rat kidney: immunohistochemical localization and regulation in response to chronic NH₄Cl loading. Am J Physiol Renal Physiol 286: F1163–F1170, 2004. First published January 28, 2004; 10.1152/ajprenal.00409.2003.—Three splice variants of anion exchanger (AE)2 (AE2a, b, and c) have been described in the rat, but their relative distribution in rat kidney is not known. The purpose of this study was to describe the segmental and cellular distribution of the AE2 isoforms in the rat kidney and to evaluate whether the expression levels of these AE2 isoforms are regulated independently in response to chronic NH₄Cl loading. AE2b are differentially expressed and regulated in the rat kidney. The regulation following NH₄Cl loading of AE2b in the cTAL suggests a role for AE2 in transepithelial bicarbonate reabsorption in this segment.

immunohistochemistry; collecting duct; nephron segments

ELECTRONEUTRAL Cl⁻/HCO₃⁻ exchange is a ubiquitous process that serves a number of cellular functions. The first Cl⁻/HCO₃⁻ exchanger cDNA cloned was erythroid anion exchanger (AE)1 also known as band 3 protein. In the kidney, AE1 is a truncated splice variant of the red cell band 3 and is critical in the process of net acid secretion along the collecting duct (CD) (8). Subsequently, three other Cl⁻/HCO₃⁻ exchanger cDNAs, encoding proteins of similar molecular structure (AE 2–4), have been reported. In the kidney, AE3 is observed in the afferent arteriole (6), whereas AE4 is expressed in renal intercalated cells (17, 27). However, the physiological role of AE3 and AE4 in the kidney is unknown.

AE2 is thought to be constitutively expressed in almost all mammalian cells and has accordingly been detected by RT-PCR in all rat kidney nephron segments (5). Functionally, AE2 exhibits unique properties relative to AE1. First, AE2 exchange activity increases with both increasing intracellular pH and extracellular pH (23, 24). Second, the anion exchange activity of AE2 is increased by hypertonic extracellular conditions. In Xenopus laevis oocytes, hypertonicity increases AE2-mediated Cl⁻/HCO₃⁻ exchange, which acts in tandem with increased Na⁺/H⁺ exchange to generate a regulatory volume increase (14). Third, AE2-mediated anion exchange is regulated by extracellular ammonium (13).

In addition to its functions related to cellular pH and volume regulation, AE2 may play a role in transepithelial bicarbonate transport and urinary acidification in rat kidney. Immunohistochemical studies showed AE2 immunoreactivity in varying amounts in basolateral plasma membrane domains of epithelial cells in all rat kidney nephron segments distal to the proximal tubule, with the strongest labeling in the medullary thick ascending limb (mTAL), inner medullary collecting duct (IMCD), and macula densa (2). Functional studies confirmed the presence of AE2 in the mTAL (10). AE2 mRNA has been shown to be present in rat proximal tubules by RT-PCR. However, by immunohistochemistry, AE2 protein expression is low in this segment (2). Thus AE2 likely plays a minor role in bicarbonate absorption in the proximal tubule.

In contrast to the strongly labeled basolateral regions of macula densa cells, AE2 is weakly detected in the cortical TAL (cTAL) and weakly detected or absent in distal convoluted tubule, connecting tubule, and the cortical portion of the CD (2). As the CD extends into the medulla, immunolabeling increases gradually and is most intense in the most terminal portion of the IMCD (imCD) (2). Across the apical membrane of the rat terminal IMCD, secretion of chloride and absorption of bicarbonate (28) occur in tandem with Cl⁻ uptake and HCO₃⁻ efflux across the basolateral membrane (electroneutral Na⁺-independent Cl⁻/HCO₃⁻ exchange) (22). Because the rat IMCD does not express AE1 (1), and because AE2 is highly expressed in rat IMCD, AE2 might be the gene product that mediates this electroneutral Cl⁻/HCO₃⁻ exchange (2, 25).

Three splice variants of AE2 (AE2a, b, and c), which differ in tissue and cellular distribution, have been described in the rat (29). By Northern blot analysis, mRNA of all isoforms has...
been detected in the stomach, whereas only AE2a and AE2b mRNA have been detected in the kidney (29). However, AE2c mRNA has been detected in the kidney by RT-PCR (2). In rabbit stomach, AE2 protein expression as well as DIDS-sensitive Cl⁻/HCO₃⁻ exchange are more than 10-fold higher in parietal cells than in mucus cells. Moreover, Northern blot analysis has shown that more AE2a than AE2b mRNA is present in mucus cells, whereas AE2b mRNA is the dominant isofrom in parietal cells (21). The cell type-specific expression of each AE2 isofrom may reflect the distinct functional regulation of Cl⁻/anion exchange in these gastric cell types. The comparative distribution of AE2a and AE2b protein in rat kidney remains unreported.

The purpose of this study was to describe the segmental and cellular distribution of the AE2a and AE2b polypeptides in the rat kidney and to determine whether their levels are regulated independently in response to chronic NH₄Cl loading. This was achieved by immunoblotting, immunohistochemistry, and immunoelectron microscopy using antibodies specific to the unique NH₂-terminal AE2a and to an amino acid (aa) sequence common to AE2a and b but different from AE2c.

**MATERIALS AND METHODS**

**Antibodies.** Two new polyclonal antibodies were produced, anti-AE2a (α-2–22) and anti-AE2ab (α-101–117).

AE2a (α-2–22). Polyvalent polyclonal antibodies were raised in rabbits against a synthetic peptide CSSAPRRPGASGAPSTGET that corre-
sponds to aa2–22 of the rat and mouse AE2a isofrom. This amino acid sequence is not found in rat AE2c, and only the latter five aminoacids are present in the amino acid sequences of rat AE2b and mouse AE2b.

Anti-AE2a/b (α-101–117). Polyclonal antibodies were raised in rabbits against a synthetic peptide CGPRKPRPGASGAPSTGET corres-
ponding to aa101–117 of the rat and mouse AE2a isoform and aa88–104 in the rat and mouse AE2b sequences. This peptide se-
quenote is not found in rat AE2c. Affinity purification was performed by binding antibodies from hyperimmune serum to the peptide im-
munosorbent, eluting with citrate-phosphate, pH 2.2, and neutralizing the eluate with Tris base. Sodium azide was added at 0.1% as an antimicrobial. Antibody specificity was tested on cells overexpressing recombinant mouse AE2a, AE2b, and AE2c.

Anti-AE2a, b, c (α-J224–1237, α-COOH-term). An antibody raised against aα1224–1237 of the AE2 sequence (α-COOH-term) previ-
ously described (2) detects a shared COOH terminus of all AE2 isoforms and was used to test total AE2 expression in transfected cells.

**Immunoblotting of cell lysates of ECr-293 cells expressing mouse AE2a, AE2b1, and AE2c1.** ECr-293 cells (Clontech) were grown in DMEM plus 10% FCS with penicillin, streptomycin, and Zeocin and transiently transfected (SuperFect, Qiagen) with mouse AE2a, AE2b1, and AE2c1, or no vector. The following day, cells were exposed to 5 mM p-nitroanisole. Forty-eight hours later, cells were scraped, extracted with 1% Triton X-100, 75 mM NaCl, 10 mM Na phosphate, and 10 mM Tris-HCl, pH 7.4. Cell protein was quantified by BCA assay (Pierce) before extraction. Proteins were separated by 5–20% SDS-
PAGE, transferred to nitrocellulose, and blots were developed with the indicated antipeptide antibodies. Peroxidase-conjugated anti-Ig was from Jackson ImmunoResearch; enhanced chemiluminescence detection reagents were from Pierce.

**Immunofluorescence analysis of ECr-293 cells expressing mouse AE2a, AE2b1, and AE2c1.** ECr-293 cells grown on plastic dishes were used to test transfected with AE2a cDNA. After 20 h, cells were trypsinized and replated on polylysine-coated multichamber slides. After a 5-h recovery period, cells were induced with 5 mM p-nitroanisole. ECr-293 cells plated on polylysine-coated multichamber micro-
scope slides, with stable expression of AE2b1 or AE2c1 cDNAs, were similarly induced with 5 mM p-nitroanisole. After 24 h of induction, cells were rinsed, fixed in 3% paraformaldehyde for 30 min at room temperature, quenched with 50 mM glycine in PBS, washed with PBS, incubated with 1% SDS for 15 min for epitope unmasking, and subjected to immunostaining. Primary antibody was applied for 2 h at room temperature, slides were washed, and then secondary antibodies were applied for 45 min. Slides were visualized with a Bio-Rad MRC-1024 confocal immunofluorescence microscope.

**Mouse kidney membrane fractionation and immunoblotting.** Six-week-old non-Swiss albino mice were purchased from Harlan (Ardmore, TX). The use of animals in the experiments in the study complied with institutional and National Institutes of Health standards.
lysates and immunoblotting were performed as reported previously (9). Kidneys were placed in ice-cold solution containing 250 mM sucrose, 10 mM triethanolamine, 1 μM/ml leupeptin (Sigma), and 0.1 mg/ml PMSF (US Biochemical, Toledo, OH). Kidney tissue was dissected to separate cortex, outer medulla, and IM. Tissue samples from each of these regions were homogenized with a tissue homogenizer (Omnit/tech Quest, Warrenton, VA) at 15,000 rpm on ice for 15 s. The homogenization step was repeated twice. Protein content was measured using the method of Lowry et al. (19). Membranes were solubilized for 15 min at 4°C in Laemmli sample buffer. SDS-PAGE was performed on minigels of 8% polyacrylamide. Each lane was loaded with 25 μg protein. The proteins were transferred from the gels electroelastically onto nitrocellulose membranes. After being blocked with 5 g/dl nonfat dry milk, membranes were probed with the anti-AE2a and anti-AEa/b antibodies at 1:10,000 and 1:5,000 dilutions, respectively. Donkey anti-rabbit IgG conjugated to HRP at a 1:5000 dilution (31458, Pierce, Rockford, IL) was used as a secondary antibody. Sites of antibody-antigen reaction were visualized using luminal-based enhanced chemiluminescence (KPL; Kirkegaard and Perry, Gaithersburg, MD) before exposure of X-ray film (X-OMAT AR; Eastman Kodak, Rochester, NY).

**RESULTS**

AE2a antibody specifically recognizes the AE2a polypeptide, and the AE2a/b antibody specifically recognizes the AE2a and AE2b polypeptides. ECR-293 cells overexpressing mouse AE2a, AE2b1, and AE2c1 polypeptides were used to test antibody specificity. Immunoblotting with antibody recognizing the shared COOH-terminus of AE2 (α-COOH-term) resulted in bands of the expected sizes: ~180 kDa for AE2a and AE2b and ~160 kDa for AE2c (Fig. 1, A and B). The immunizing peptide AE2a aa2–22 used for the NH2-terminal AE2a antibody included five COOH-terminal amino acid residues also present in AE2b1 as aa4–8. However, using the NH2-terminal AE2a antibody (α-NH2-term), immunoreactivity was detected only in lysates of AE2a-transfected cells (Fig. 1C). In contrast, the AE2a/b antibody (α101–117) detected both recombinant AE2a and AE2b1 (Fig. 1D). Neither of the new antibodies detected AE2c polypeptide (Fig. 1, C and D). Endogenous EcR-293 cell AE2 polypeptide was evident in nontransfected cells as a very faint band (right lane of each panel).

These immunoblot results were confirmed by immunofluorescent labeling of cells expressing the mouse AE2a (Fig. 2, A). AE2 polypeptide variants also present in AE2b1 as aa4–8 were detected using antibodies (P448, DAKO), labeling visualized by DAB technique and city of antibodies tested with overexpressed recombinant anion exchanger (AE2) polypeptides. Immunoblotting of 1% Triton X-100 extract of nonasterone A (Invitrogen) induced EcR-293 cells transiently transfected with cDNA encoding mouse AE2a (extract from 30 μg total cell protein), AE2b1 (from 15 μg total cell protein), AE2c1 (from 15 μg cell protein), or untransfected cells (30 μg cell protein). A and B: Blots were developed with antibody to AE2a amino acid (aa) 1224–1237 (α-COOH-term), which recognizes all 3 polypeptide variants. C: Antibody to AE2a aa1–22 (α-NH2-term) recognizes only the AE2a isom. D: Antibody to AE2a aa101–117 (α101–117) recognizes both AE2a and AE2b polypeptides. (A: reprobe of stripped C blot: B: reprobe of stripped D blot.)
AE2 isoforms in rat kidney

In the rat kidney, labeling with the AE2a/b (α-101–117) antibody was detected in the cortex, outer and inner stripe of outer medulla, and in the IM (Fig. 4, A–G). In the glomerulus and proximal tubule, no labeling was detected (Fig. 4, A–C). Only weak labeling was detected in descending thin limbs (DTL) and ascending thin limbs (ATL) of the loop of Henle (Fig. 4, C, E, F). The TAL of the loop of Henle showed strong labeling in the ISOM (Fig. 4D), whereas labeling was relatively weaker in the TAL of the outer stripe of the outer medulla and cortex (Fig. 4, A–C). Strong labeling was seen in basolateral domains of macula densa cells (Fig. 4A). In the CD, a gradual increase in labeling intensity was seen from the proximal to the distal regions. Very weak labeling was observed in the basolateral domains of principal cells in the ISOM, whereas strong labeling was seen in the basolateral domains of IMCD cells in the inner third of the IM (Fig. 4, E–G).

Labeling with antibodies specifically recognizing the AE2a isoform (α-NH2-term) (Fig. 4, H–N) was not found in any cortical tubule segments, except for occasional very weak labeling in basolateral domains of macula densa cells (Fig. 4, H–J). Very weak labeling was seen in the DTL and ATL. Labeling was noted in the medullary part of TAL (Fig. 4, J–M). Strong labeling was seen in the basolateral portion of IMCD cells in the inner third of the IM (Fig. 4N).

Immunoelectron microscopic localization of AE2 in IM. To determine the subcellular localization of AE2, electron microscopy using a preembedding HRP-visualized immunolabeling technique was employed (15). Antigen-antibody interactions are detected as a dark precipitate. AE2a/b immunolabeling was detected in the basolateral plasma membrane in IMCD cells in the inner third of the IM (Fig. 5).

Kidneys from NH4Cl-loaded rats exhibited increased labeling for AE2 in cTAL. Compared with controls (Fig. 6A), labeling with the AE2a/b antibody (α-101–117) in the cTAL was markedly increased. Moreover, in NH4Cl-loaded rats,
AE2\textsubscript{a/b} immunoreactivity extended to more distal portions of the cTAL (Fig. 6B). In the medulla, no differences in the labeling between control rats and NH\textsubscript{4}Cl-loaded rats could be seen using either AE2\textsubscript{a} antibody or AE2\textsubscript{a/b} antibody (not shown). HCl loading resulted in a similar increase in AE2\textsubscript{a/b} immunolabeling of the cTAL (Fig. 6C).

Immunoblotting confirmed upregulation of AE2 in the cortex in NH\textsubscript{4}Cl-loaded rats. The effect of NH\textsubscript{4}Cl ingestion on AE2\textsubscript{a/b} protein expression was quantified by immunoblot on samples from rat cortex/outer stripe of outer medulla, ISOM, and IM. By immunoblot, AE2\textsubscript{a/b} (\(\alpha\)-101–117) immunoreactivity increased 103\% in the cortex/outer stripe of outer medulla of kidneys from NH\textsubscript{4}Cl-loaded rats (\(P < 0.01\); Fig. 7). AE2\textsubscript{a/b} expression did not change in the ISOM and IM following NH\textsubscript{4}Cl ingestion. Moreover, NH\textsubscript{4}Cl intake did not change AE2\textsubscript{a} (\(\alpha\)-NH\textsubscript{2}-term) protein expression in any of these regions of the kidney (not shown).

**DISCUSSION**

AE2\textsubscript{a} and AE2\textsubscript{b} are coexpressed in the rat kidney, but the relative amounts of the variant polypeptides vary among nephron segments. In the present study, the distributions of the AE2\textsubscript{a} and AE2\textsubscript{b} isoforms were determined by comparing
immunolabeling of antibodies that recognize either AE2a only or both AE2a and AE2b. The distribution of AE2a/b labeling was identical to that described previously, which employed an antibody (α-H9251-COOH-term) that recognizes all AE2 isoforms (2). The pattern of AE2a-specific labeling in kidney differed from AE2a/b labeling in that no labeling was observed in the cortex. Moreover, relatively more of the total AE2a immunolabel was observed in the tIMCD than in the TAL. The relatively weak AE2a labeling in the TAL suggests that AE2a alone does not account for the strong labeling seen in the TAL with the AE2a/b (α-101–117) antibody. In the IM, both the AE2a and AE2a/b antibody gave rise to strong labeling in the basolateral domain of IMCD cells. This shows AE2a to be present alone or in combination with AE2b. Immunoelectron microscopy confirmed AE2 localization to the basolateral plasma membrane of renal tubular cells.

Immunoblotting of rat kidney samples confirmed the distribution of the AE2 isoforms observed by immunohistochemistry. In both the rat and mouse, greater AE2a expression was detected in the ISOM than in the IM. At first glance, results of immunoblots appear to differ from studies using immunohistochemistry, which suggest that AE2a expression is greater in IMCD than mTAL. However, basolateral membrane proteins of the mTAL comprise a particularly large fraction of the total proteins in ISOM tissue homogenates due to the deep basolateral invaginations of the mTAL cells (26). In the mTAL of the ISOM, lateral invaginations are numerous and often extend two-thirds or more of the distance from the base of the cell to the luminal border (26). Moreover, the mTAL represents more than 70% of the total protein in the outer medulla (4), whereas the tIMCD represents only 20–30% of the total protein in the IM (16). Taken together, basolateral plasma membrane proteins of the mTAL comprise a much larger fraction of the total protein content in ISOM than the fraction of IM protein from basolateral membranes of the tIMCD. Thus, if equal amounts of total protein from the outer and IM were loaded on a gel, greater basolateral membrane protein would be loaded per lane from the mTAL than from the tIMCD. The same bias may not be present by immunohistochemistry.

Alternatively, the apparent discrepancy between immunoblotting and immunohistochemistry with respect to relative intensities of immunoreactivity using the AE2a antibody may...

Fig. 6. Immunolocalization of AE2 in kidneys from NH4Cl-loaded rats. A: labeling with the AE2a/b antibody (α-101–117) in kidney cortex from control rats showed intense labeling in the basolateral domain of MD cells and weaker labeling in the basolateral domains of cells in the cortical TAL (cTAL). B: labeling with the AE2a/b antibody (α-101–117) in kidney cortex from NH4Cl-loaded rats was found to be markedly increased, and more cTAL showed immunoreactivity compared with controls. C: increased AE2a/b labeling was also observed in the cTAL from rats ingesting HCl relative to controls. Scale bars: 30 μm.

Fig. 7. Immunoblotting of samples of cortex/outer stripe of OM from NH4Cl-loaded rats. A: immunoblot using the AE2a/b antibody (α-101–117) showing an intensified 180-kDa band in samples from NH4Cl-loaded rats. B: result of densitometric analysis of the blot shown in A. The signal in NH4Cl-loaded rats is 203% of the control value.
occur because the AE2a-specific epitope, localized at the NH2-terminal of AE2a, is less accessible in the mTAL than in the tIMCD in fixed tissue, perhaps due to interactions with other proteins.

Increased cTAL immunolabeling in NH4Cl-loaded rats is likely due to upregulation of AE2b. By both immunoblotting and immunohistochemistry, AE2a/b immunoreactivity was greatly increased in the kidney cortex from NH4Cl-loaded rats relative to controls. No change in AE2a expression was detected in kidney from rats ingesting NH4Cl. Increased AE2b expression was noted by immunohistochemistry as intensified labeling within cTAL. Because AE2a immunoreactivity did not change with NH4Cl ingestion, we conclude that the increased labeling found with the AE2a/b antibody (α-101–117) is largely due to increased abundance of the AE2b isoform in cTAL following NH4Cl loading. Similar changes in AE2a/b immunolabeling were seen following 7 days of HCl loading, supporting the conclusion that the changes in AE2b expression in cTAL are involved in the renal compensation of a sustained acid load.

AE2a is widely expressed and is thought to serve general cellular functions such as regulation of intracellular pH and cell volume. However, the tissue distribution of rat AE2b mRNA, present in liver, kidney, stomach, and intestine, appears to be more limited (29). The present study demonstrates that immunolabeling of AE2a/b, but not AE2a, is increased in rat kidney cTAL following oral NH4Cl loading. Our data therefore suggest that AE2a and AE2b polypeptide levels are regulated independently. Rat AE2b mRNA is transcribed from a promoter located in intron 2 of the AE2 gene. A number of consensus binding sites for transcription factors have been identified upstream of the initiation site of rat AE2b (29). Although little is known about the signals that may induce AE2b polypeptide expression in rat cells, the human AE2b promoter can be regulated by HNF1α (20).

Functional role of AE2 in TAL and CD. Rat cTAL absorbs bicarbonate (3). Therefore, AE2 expressed in cTAL might participate in the process of bicarbonate absorption and urinary acidification in this segment, particularly following NH4Cl ingestion. Increased absorption of bicarbonate in the cTAL would help correct the metabolic acidosis that follows NH4Cl ingestion. Alternatively, increased AE2b expression might modulate Cl− excretion following NH4Cl ingestion. Chloride channels expressed on the basolateral plasma membrane differ between the cTAL and mTAL (30). Thus the mechanism of Cl− absorption and its regulation may differ between the cTAL and mTAL. Whether upregulation of AE2 in cTAL helps maintain acid-base or chloride balance (or both) following NH4Cl ingestion remains to be determined.

After ingestion of NH4Cl, net acid secretion increases in rat IMCD, both in vivo and in vitro (28). As described above, strong AE2a immunoreactivity was seen in tIMCD, and AE2a may be the only AE2 isoform present in IMCD. The results of this study do not allow any conclusions regarding the presence of AE2b in IMCD. Expression of AE2 protein in IM was not seen to change with NH4Cl loading. Thus if AE2 contributes to the increased absorption of HCO3− observed following NH4Cl ingestion, either AE2-mediated transport is not rate limiting or AE2 activity increases in this treatment model through a mechanism that does not involve changes in protein expression. NH4Cl loading is associated with a number of adaptive changes in the kidney, such as an increase in the interstitial concentration of NH4+ in the IM (12). In heterologous expression systems, AE2 is activated by increases in extracellular NH4+ concentration. Thus activation of AE2 by NH4+ might increase AE2-mediated HCO3− efflux across the basolateral membrane of the tIMCD in vivo, following NH4Cl ingestion, in the absence of changes in AE2 protein expression.

In conclusion, in the chronic NH4Cl-loading model of metabolic acidosis, AE2 protein expression is upregulated in the cortex, likely through increased abundance of AE2b polypeptide(s) in the cTAL. In the IM, a region in which the AE2a polypeptide is relatively more abundant, no change in AE2 protein expression was observed. Whether the observed up-regulation of AE2 contributes to the increase in net acid secretion observed in this treatment model remains to be determined.

ACKNOWLEDGMENTS

The authors thank G. Kall, I. M. Pausen, H. Hoyer, M. F. Vistisen, and L. V. Holbech for expert technical assistance. We also thank A. K. Stuart-Tilley for helpful assistance.

GRANTS

The Water and Salt Research Center at the University of Aarhus is established and supported by the Danish National Research Foundation (Danmarks Grundforskningsfond). Additional funding was provided by the European Commission (QLRT-2000-00987, QLRT-2000-00718). S. L. Alper was supported by National Institutes of Health Grants DK-43495 and DK-34854 (Harvard Digestive Diseases Center). S. M. Wall was supported by Grant DK-46493.

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