Role of dipeptidyl peptidase IV in regulating activity of Na\(^{+}/H^{+}\) exchanger isoform NHE3 in proximal tubule cells

Adriana C. C. Girardi,\(^1\) Felix Knauf,\(^1\) Hans-Ulrich Demuth,\(^2\) and Peter S. Aronson\(^3\)

\(^1\)Department of Internal Medicine and Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520-8029; and \(^2\)Probiodrug AG, Halle (Saale) D-06120, Germany

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Girardi, Adriana C. C., Felix Knauf, Hans-Ulrich Demuth, and Peter S. Aronson. Role of dipeptidyl peptidase IV in regulating activity of Na\(^{+}/H^{+}\) exchanger isoform NHE3 in proximal tubule cells. Am J Physiol Cell Physiol 287: C1238–C1245, 2004.—We recently reported that NHE3 exists in multimeric complexes with dipeptidyl peptidase IV (DPPIV) in renal brush-border membranes. To examine the possible role of DPPIV in modulating NHE3 activity, we evaluated whether specific competitive inhibitors that bind to the active site of DPPIV affect NHE3 activity in the OKP line of opossum kidney proximal tubule cells. The DPPIV inhibitors diprotin A and P32/98 significantly reduced NHE3 activity, whereas the inactive isomer P34/98 had no effect. DPPIV inhibitors did not reduce the activity of another brush-border transport process, Na-phosphate cotransport. Effects of DPPIV inhibitors on NHE3 activity were not associated with detectable changes in amount or apparent molecular weight of NHE3 or in NHE3 surface expression. To investigate the signaling mechanisms involved in modulation of NHE3 activity by DPPIV, we used inhibitors of protein kinase pathways known to regulate NHE3. Whereas the PKA inhibitor H-89 failed to block the effect of DPPIV inhibitors, the tyrosine kinase inhibitor genistein alone caused a decrement in NHE3 activity very similar in magnitude to that caused by P32/98. We also found that the effects of genistein and P32/98 on NHE3 activity were not additive. In contrast, forskolin/IBMX and P32/98 had additive inhibitory effects on NHE3 activity. These findings suggested that the effect of DPPIV inhibitors to reduce NHE3 activity results from inhibition of a tyrosine kinase signaling pathway rather than by activation of PKA. We conclude that DPPIV plays an unexpected role in modulating Na\(^{+}/H^{+}\) exchange mediated by NHE3 in proximal tubule cells.

sodium/hydrogen exchange; diprotin A; P32/98; tyrosine kinase

\(^{1}\)Department of Internal Medicine and Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520-8029; and \(^{2}\)Probiodrug AG, Halle (Saale) D-06120, Germany

Address for reprint requests and other correspondence: P. S. Aronson, Section of Nephrology, Dept. of Internal Medicine, Yale Univ. School of Medicine, PO Box 208029, New Haven, CT 06520-8029 (E-mail: peter.aronson@yale.edu).

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REGULATION OF NHE3 BY DPPIV

METHODS

Materials. Reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. OKP cells, a clonal subtype of the opossum kidney cell line originally described by Cole et al. (13), were provided by Dr. Orson Moe (University of Texas Southwestern Medical School, Dallas, TX). Dulbecco’s modified Eagle’s medium (DMEM), heat-inactivated fetal bovine serum, sodium pyruvate, and penicillin/streptomycin were purchased from Life Technologies (GIBCO BRL, Gaithersburg, MD). 22Na and [32P]phosphoric acid were obtained from NEN Life Science (Boston, MA). EZ-Link sulfo-NHS-SS-biotin as well as immunopure immobilized streptavidin were purchased from Pierce (Rockford, IL). Monoclonal antibodies directed to opossum NHE3 (3H3) and megalin (6A6) were generously provided by Dr. Daniel Biemesderfer (Yale School of Medicine, New Haven, CT). A polyclonal antibody (rabbit IgG) to DPPIV/CD26 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). This antibody was raised against a recombinant protein corresponding to amino acids 261–530 of human DPPIV/CD26 (42). Phospho-tyrosine monoclonal antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY). DPPIV inhibitors were obtained as follows: diprotin A (Ile-Pro-Ile) (46) was purchased from Bachem (Philadelphi, PA); P329R (isouleucyl thiazolidine fumarate) (38) and its inactive isomer, P349R (3-isouleucyl thiazolidine fumarate), were obtained from Probioburg (Halle (Saale), Germany).

Cell culture. OKP cells were maintained in 75-cm² tissue culture flasks in DMEM containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were incubated at 37°C in a humidified 5% CO2–95% air atmosphere. Cells were subcultured using Ca2+/Mg2+-free phosphate-buffered saline and 0.25% trypsin-EDTA. The medium was replaced every 2 days. For experiments, cells were seeded onto tissue culture plates and were used 2 days after reaching 100% confluence.

Immunoprecipitation. OKP cells grown in six-well plates were solubilized at 4°C in Tris-buffered saline (TBS) buffer (pH 7.4) containing 1% Triton X-100 and the protease inhibitors pepstatin A (0.7 µg/ml), leupeptin (0.5 µg/ml), and PMSF (40 µg/ml). The samples were subjected to centrifugation (15,000 g for 10 min) using a table-top centrifuge (Hermle model Z230M; National Labnet, Woodbridge, NJ). Primary antibodies (50 µg) were added to the supernatants, and the samples were incubated at 4°C for 1 h. Immune complexes were collected using 5 mg/sample of protein G-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were washed five times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting. Protein samples were solubilized in SDS sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 100 mM KH2PO4, 10 mM HEPES-Tris, pH 7.4). The surface of the gel was blocked with 5% triethanolamine, 2 mM CaCl2, and 2 mg/ml EZ-Link sulfo-NHS-SS-biotin. The beads were washed twice with PBS (pH 7.4) and then solubilized for 1 h by adding a buffer containing 125 mM potassium acetate, 25 mM HEPES, 15 mM sodium pyrophosphate, 1% Triton X-100, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, and 40 µg/ml PMSF, pH 7.4. The samples were centrifuged at 15,000 g for 10 min, and 50 µl of streptavidin-coupled agarose were added to the supernatants. After 1 h of incubation, the beads were washed five times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.

Analysis of protein tyrosine phosphorylation. Tyrosine kinase activity was measured in the OKP-DPPIV immune complex by Western blotting using an anti-phosphotyrosine antibody (17). OKP cells grown to confluence in six-well plates were incubated with 24–48 h and subsequently incubated for 30 min at 37°C with either DPPIV inhibitors or diluent. All of the following manipulations were performed at 4°C. Cells were washed twice with PBS containing 0.1 mM CaCl2 and 1.0 mM MgCl2 (PBS-Ca-Mg). The surface membrane proteins were then biotinylated by incubating the cells twice for 25 min with 2 ml of biotinylation buffer (150 mM NaCl, 10 mM triethanolamine, 2 mM CaCl2, and 2 mg/ml EZ-Link sulfo-NHS-SS-biotin). The cells were washed twice for 20 min with a quenching buffer (PBS-Ca-Mg/100 mM glycine) and then solubilized for 1 h by adding a buffer containing 125 mM potassium acetate, 25 mM HEPES, 15 mM sodium pyrophosphate, 1% Triton X-100, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, and 40 µg/ml PMSF, pH 7.4. The samples were centrifuged at 15,000 g for 10 min, and 50 µl of streptavidin-coupled agarose were added to the supernatants. After 1 h of incubation, the beads were washed five times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.
Modulation of NHE3 activity by DPPIV inhibitors in OKP cells. We previously demonstrated that the brush border Na+/H+ exchanger NHE3 associates with DPPIV in rabbit renal proximal tubule (18). DPPIV, also known as CD26, is a multifunctional protein that, in addition to its proteolytic properties, acts as a binding protein and signaling molecule. The emerging evidence for regulation of transporters by proteases led us to investigate whether DPPIV modulates NHE3.

For our experiments we used OKP cells, a line of opossum proximal tubule cells that has transport properties very similar to those of the native mammalian proximal tubule (13). We first verified whether NHE3 existed in protein complexes with DPPIV in OKP cells. Figure 1 shows the results of an experiment in which solubilized OKP cell proteins were immunoprecipitated with a commercial antibody to DPPIV (42). The immune complexes were prepared for immunoblotting and probed with antibodies against DPPIV, NHE3, and megalin. All three proteins were detected in the OKP cell lysate. DPPIV and NHE3 were coprecipitated by the anti-DPPIV antibody. In contrast, anti-DPPIV did not coprecipitate megalin, confirming the specificity of the NHE3-DPPIV interaction. This observation is in agreement with our previous studies performed in rabbit proximal tubule that revealed that the pools of NHE3 complexed with DPPIV and megalin are largely distinct (18).

To examine the possible role of DPPIV in modulating NHE3, we evaluated whether specific inhibitors that bind to the active site of DPPIV affect NHE3 activity in OKP cells. The following inhibitors were used: diprotin A (H-Ile-Pro-Ile-OH), P32/98, and its inactive optical isomer, P34/98, as a negative control. Table 1 shows the concentration dependence for diprotin A, P32/98, and P34/98 inhibition of DPPIV enzymatic activity in OKP cells. We found that maximal (>99%) inhibition of DPPIV activity in OKP cells was achieved by 1 mM diprotin A and 10 μM P32/98. As expected, P34/98 did not affect DPPIV catalytic ability.

We next evaluated whether the concentration of each inhibitor that maximally affected DPPIV enzymatic activity would affect NHE3 transport activity. NHE3 activity was assayed as...
22Na uptake in the absence or presence of 1 mM diprotin A, 10 μM P32/98, and 10 μM P34/98 after OKP cells were acid loaded using the ammonium prepulse technique. As shown in Fig. 2A, the control rate of 22Na uptake measured under these conditions was inhibited 90% by 100 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA), consistent with NHE3 activity. Both diprotin A and P32/98 significantly inhibited 22Na uptake. In contrast, the inactive isomer P34/98 did not affect 22Na influx. None of the inhibitors affected the small component of 22Na uptake that was EIPA insensitive. The effects of the inhibitors on the EIPA-sensitive component of 22Na uptake are shown in Fig. 2B, which indicates that both diprotin A and P32/98 inhibited NHE3 activity by ∼40%.

We then examined the concentration dependence of the effects of the DPPIV inhibitors on NHE3 activity to allow correlation with inhibition of DPPIV enzymatic activity. The effects of increasing concentrations of diprotin A, P32/98, and P34/98 on EIPA-sensitive uptake of 22Na after an acid load are shown in Fig. 3. The dose-response curves for inhibition of NHE3 activity correlate with the effects of the inhibitors on DPPIV catalytic activity in that P32/98 is ∼100-fold more potent than diprotin A for inhibiting both DPPIV activity and NHE3 activity. However, there were two striking differences between the dose-response curves for inhibition of DPPIV activity and NHE3 activity. First, maximum inhibition of NHE3 activity reached a plateau of ∼45%, whereas DPPIV activity could be completely inhibited. This finding indicates that only a fraction of NHE3 activity is dependent on DPPIV activity. Second, the dose-response curves for inhibition of NHE3 activity by diprotin A and P32/98 are shifted to the right by a factor of 10 compared with those for inhibition of DPPIV activity. For example, concentrations of diprotin A (1 μM) and P32/98 (0.01 μM) that caused >30% inhibition of DPPIV activity caused no detectable inhibition of NHE3 activity. These findings indicate that only a small fraction of residual DPPIV activity is required to fully stimulate the component of NHE3 activity that is dependent on DPPIV activity. Thus near maximal inhibition of DPPIV activity is required to reduce NHE3 activity. This is qualitatively analogous to the dependence of proximal tubule bicarbonate reabsorption on carbonic anhydrase activity, in which case enzyme activity must be inhibited >99% to observe a significant physiological effect (32).

To verify the specificity of the effect of DPPIV inhibitors on NHE3 activity, we evaluated whether these agents would affect the activity of another brush-border membrane sodium-dependent transporter, the Na-Pi cotransporter, which we had previously found not to be associated with DPPIV (18). Na-Pi cotransporter activity was measured as 32P-radiolabeled phosphate uptake in the presence of sodium. As shown in Fig. 4A,
Effect of DPPIV inhibitors on stability of the NHE3-DPPIV complex. We next investigated whether the effect of DPPIV inhibitors to reduce NHE3 activity is due to a change in either total or surface NHE3 expression. Figure 6A displays the results of a typical Western blot analysis of total cell lysate. Total NHE3 protein expression did not change when these cells were treated with 10 μM P32/98. Figure 6A also shows that DPPIV does not seem to be involved in processing of NHE3, because there was no detectable shift in apparent molecular weight of NHE3 when cells were treated with DPPIV inhibitor.

In OKP cells, it has been well documented that NHE3 is regulated by trafficking between intracellular pools and the apical surface (14). Following protocols for surface biotinylation of NHE3 that have been successfully adapted to OKP cells (2, 56), we examined whether NHE3 surface expression would be altered by DPPIV inhibition. Surface proteins were biotinylated and precipitated with streptavidin. The precipitate was then probed for NHE3 by immunoblotting. As shown in Fig. 6B, DPPIV inhibition did not change NHE3 surface expression. On the basis of the results shown in Fig. 6, we conclude that DPPIV inhibitors do not alter either the total amount or apparent molecular weight of NHE3 or change NHE3 surface expression.

Role of protein kinases in mediating the effect of DPPIV inhibitors on NHE3 activity. The signal transduction cascade mediating the acute effect of NHE3 agonists and antagonists involves multiple pathways. Given that cAMP is one of the major intracellular messengers mediating inhibition of NHE3 in OKP cells (8, 54), we examined whether NHE3 modulation by DPPIV inhibitors would occur through activation of this second messenger system. The involvement of a cAMP/PKA-mediated pathway in the regulation of NHE3 by DPPIV inhibitors was examined by pretreating OKP cells for 30 min with the specific PKA inhibitor H-89. OKP cells were then incubated with 100 μM forskolin/1.0 mM IBMX, 10 μM P32/98, or vehicle. NHE3 transport activity was then measured. As expected, exposure to 10 μM H-89 completely blocked the inhibitory effect of forskolin/IBMX on Na+/H+ exchange (Fig. 7). However, 10 μM H-89 did not attenuate the effects of DPPIV inhibitors on NHE3 activity in OKP cells, indicating that PKA does not mediate the effect of DPPIV inhibitors.

Several studies have demonstrated that tyrosine kinase signaling pathways can stimulate NHE3 activity (26, 45, 53). We
then considered the possibility that the effect of DPPIV inhibitors to reduce NHE3 activity in OKP cells might arise from inhibition of tyrosine kinase signaling. To address this issue, we tested whether the tyrosine kinase inhibitor genistein would reduce NHE3 activity in OKP cells and, if so, whether its effect would be additive to that of the DPPIV inhibitor P32/98. As shown in Fig. 8, genistein caused a decrement in NHE3 activity very similar in magnitude to that caused by P32/98, and the effects of P32/98 and genistein were not additive. In contrast, as also shown in Fig. 8, P32/98 and forskolin/IBMX produced an additive inhibitory effect on NHE3 activity, consistent with our previous conclusion that DPPIV inhibitors inhibit NHE3 activity via a PKA-independent mechanism.

To further investigate whether DPPIV inhibitors actually decrease cell tyrosine kinase activity in OKP cells and thereby mimic the effect of genistein, we analyzed the content of tyrosine-phosphorylated proteins present in the DPPIV immune complex from OKP cells. Cells incubated in presence of genistein, P32/98, or vehicle were solubilized, and the resulting supernatant was immunoprecipitated with anti-DPPIV. The phosphorylation on tyrosine residues of DPPIV-associated proteins was analyzed by immunoblotting with an anti-phosphotyrosine MAb. As shown in Fig. 9, genistein and P32/98 each induced a significant decrease in the tyrosine phosphate residues of a single protein with a high apparent molecular mass (>212 kDa). As expected, addition of the phosphatase inhibitor pervanadate enhanced the levels of tyrosine phosphorylation of this protein compared with control. The findings shown in Fig. 9 provide direct evidence that DPPIV inhibitors affect tyrosine kinase signaling in OKP cells. Finally, it may be noted that tyrosine phosphorylation of NHE3 itself was not detected in the anti-DPPIV immunoprecipitate.

DISCUSSION

We have used two highly specific DPPIV inhibitors to examine the role of this peptidase in regulating the activity of NHE3 in OKP cells. As mentioned earlier, DPPIV is a serine protease that cleaves NH2-terminal dipeptides from proteins with a penultimate proline or alanine residue (16, 25). Diprotin A (Ile-Pro-Ile) is a tripeptide substrate that binds in the active site of DPPIV but is only slowly cleaved (39, 43). Based on the
structural similarity of thiazolidine to proline, P32/98 (isoleucine-thiazolidine) is a dipeptide product analog with high affinity for binding to the active site and inhibiting DPPIV (38, 41). Diprotin A and P32/98 are respectively 10 and 80 times more potent for inhibiting DPPIV than for inhibiting the functionally related proline-specific protease DPPII (29, 41).

We now report that these two DPPIV inhibitors significantly decrease NHE3 activity in OKP cells, suggesting that the catalytic site of this peptidase has a role in modulating NHE3. Previously documented mechanisms that mediate the acute regulation of NHE3, including alteration of surface expression and phosphorylation by PKA, do not appear to be involved in the downregulation of NHE3 by DPPIV inhibitors. In addition, we could not detect any change in the apparent molecular weight of NHE3 resulting from possible DPPIV-mediated proteolysis of the transporter. However, we have found that the decrement in NHE3 activity induced by the DPPIV inhibitor P32/98 is not additive with that caused by the tyrosine kinase inhibitor genistein, suggesting that DPPIV inhibitors may affect NHE3 by a tyrosine kinase signaling pathway.

It is therefore of interest that DPPIV (CD26) regulation of cell proliferation and cytokine production in lymphocytes has been associated with changes in tyrosine kinase signaling (20, 22–24, 36). Indeed, it has been specifically demonstrated that DPPIV inhibitors affect tyrosine phosphorylation of multiple proteins in lymphocytes (23, 24). Similarly, we found that the DPPIV inhibitor P32/98 decreased tyrosine phosphorylation of a high apparent molecular mass protein (>212 kDa) that coprecipitates with DPPIV, indicating a role for DPPIV in regulating tyrosine kinase signaling in OKP cells.

The molecular mechanisms by which DPPIV inhibitors affect protein tyrosine phosphorylation in lymphocytes or other cell types are not known. The cDNA sequence of DPPIV predicts a type II membrane protein, which is anchored to the cell surface by a single hydrophobic segment and has a short cytoplasmic region consisting of only six amino acids (42). Therefore, DPPIV is very unlikely to signal by itself. One possibility is that binding of DPPIV inhibitors to the catalytic site results in a conformational change that, in turn, affects tyrosine kinase signaling mediated by associated proteins. For instance, in T lymphocytes, DPPIV interacts with CD45 (22, 44), a tyrosine phosphatase that plays an important role in T cell activation. Another possibility is that DPPIV inhibitors affect cell signaling by blocking DPPIV catalytic activity. Known substrates for DPPIV include cytokines, chemokines, growth factors, and hormones (28). Processing by DPPIV activity can lead to either activation of proforms or peptide degradation. Thus DPPIV catalytic site inhibitors could alter cell signaling and protein phosphorylation by blocking DPPIV-mediated activation or inactivation of peptide ligands that bind to cell surface receptors.

In summary, we have found that DPPIV inhibitors significantly decrease NHE3 activity in OKP cells, most likely by inhibiting a tyrosine kinase signaling pathway. Thus our studies reveal an unexpected role for DPPIV in modulating NHE3 activity in proximal tubule cells.

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