Role of integrin \(\alpha_1\beta_1\) in the regulation of renal medullary osmolyte concentration

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Moeckel, Gilbert W., Li Zhang, Xiwu Chen, Michele Rossini, Roy Zent, and Ambra Pozzi. Role of integrin \(\alpha_1\beta_1\) in the regulation of renal medullary osmolyte concentration. Am J Physiol Renal Physiol 290: F223–F231, 2006. First published August 16, 2005; doi:10.1152/ajprenal.00371.2004.—The mechanism by which cells sense extracellular tonicity and trigger the accumulation of protective organic osmolytes is poorly understood. It has been proposed that changes in cell volume following alteration of extracellular tonicity are important initiators of signaling events that lead to osmolyte accumulation. Because the extracellular matrix receptors integrins are linked to the cytoskeleton and can transduce signals that alter cell behavior, we investigated the role of these receptors in the modulation of osmolyte accumulation in the kidney medulla under different osmotic conditions. We show that integrin \(\alpha_1\)–null mice have impaired ability to accumulate organic osmolytes in the inner medulla due to altered signaling and decreased induction of osmolyte transporters or aldose reductase gene transcription. Utilizing inner medullary collecting duct cells, we demonstrate that the lack of integrin \(\alpha_1\beta_1\) results in an impaired ability to induce the toxicity enhancer-binding protein TonEBP under hypertonic conditions. Furthermore, under the same conditions, integrin \(\alpha_1\)–null cells show prolonged ERK1/2 phosphorylation and decreased inositol uptake compared with control cells. The reduction of inositol uptake is significantly reversed by treatment with the MEK inhibitor PD-98059. Finally, integrin \(\alpha_1\)-null mice develop morphological changes of early tubular necrosis and increased apoptosis of renal medullary cells following dehydration. Together, these results show that integrin \(\alpha_1\beta_1\) is an important mediator of the compatible osmolyte response in the medulla of the mammalian kidney.

volume regulation; signaling; tonEBP; MAPK

Cells in the mammalian kidney medulla are exposed to large alterations of extracellular tonicity that change with the hydration state of the animal (2). Cells respond to hypertonic stress by accumulating nonperturbing organic osmolytes such as the polyhydric alcohols \(\text{myo-inositol}\) and sorbitol, the methylamines betaine and glycerophosphorylcholine (GPC), and several amino acids (42). Intracellular concentrations of osmolytes are controlled by both rate of synthesis and intracellular accumulation. \(\text{Myo-inositol}\) and betaine are accumulated by sodiumpoy-coupled transporters, while sorbitol synthesis is catalyzed by the enzyme aldose reductase (AR). Both sodium-\(\text{myo-inositol}\) (SMIT) (41) and the betaine-GABA transporter 1 (BGT1) (38), as well as AR (35), are induced by hypertonicity in renal medullary cells both in vitro and in vivo, while GPC concentrations are regulated by reduction of the degradation enzyme GPC:choline \(p\)-hosphodiesterase (20). Increased abundance of mRNA for SMIT, BGT1, and AR in response to hypertonicity depends on the binding of a transcription factor TonE-binding protein (TonEBP/NFAT 5) (23) to toxicity enhancer element (TonE) (36).

The signaling mechanism whereby hyperosmolality induces osmolyte transporter activity and/or enzyme transcription in the mammalian kidney medulla is still poorly understood. Activation of the MAP kinases might play a role, as JNK, ERK, and p38 MAP kinase pathways are induced when the renal medulla or collecting duct cells are exposed to hypertonicity (8, 13, 22, 27). Toxicity-dependent changes in cytoskeletal organization might activate the MAP kinase pathways in a c-src-dependent fashion (1, 17).

Interestingly, integrins, transmembrane receptors for extracellular matrix components, play a critical role in regulating the cytoskeletal organization (29) as well as src-dependent activation of MAP kinases (12).

Integrin \(\alpha_1\beta_1\), a major collagen-binding receptor, is highly expressed in both glomerular and tubular cells in the kidney (19, 39). Kidneys of integrin \(\alpha_1\)-null mice are abnormal with small and dysmorphic glomeruli that become severely sclerosed following renal injury (7). In addition, mesangial cells lacking integrin \(\alpha_1\) have an altered cytoskeleton characterized by increased F-actin (Pozzi A, unpublished observations). Moreover, inner medullary collecting (IMCD) cells treated with blocking anti-\(\alpha_1\) antibodies or siRNA show decreased adhesion, migration, survival, and tubule formation on collagen I (6). Together, these data suggest that integrin \(\alpha_1\beta_1\) plays a critical role in normal renal cell morphology and physiological homeostasis.

Since integrin \(\alpha_1\beta_1\) appears to be important not only for IMCD survival in vitro (6) but also for mechanical-induced stress responses (3, 8), we determined the role of this collagen receptor in mediating protection of the host against hyperosmotic stress. We demonstrate that following dehydration integrin \(\alpha_1\)-null mice, but not their wild-type counterparts, show early focal medullary tubular and interstitial cell injury that correlates with an inability to upregulate the expression of osmolyte transporters and/or enzymes. Thus we provide evidence that integrin \(\alpha_1\beta_1\) is required for the induction of hyperosmotic stress-induced osmolyte response that might protect the kidney against renal tubular injury.

MATERIALS AND METHODS

Mouse and experimental procedure. Wild-type and integrin \(\alpha_1\)-null 129Sv/ter female mice (8 wk old, ~25 g body wt) were used for all
the experiments performed according to institutional animal care guidelines. All animal experiments in this study were reviewed and approved by the Vanderbilt University Institutional Animal Care and Use Committee protocol review board. Inner medullary osmolyte concentrations were quantified in kidney medullas of wild-type and integrin α1-null mice which were either untreated, dehydrated for 24 h, or treated with furosemide (LyphoMed, Rosemont, IL; 1 mg/kg body wt ip) twice daily for 3 days before death (25). Briefly, neutralized perchloric acid extracts from renal inner medullary tissues were prepared as described (25) and cellular organic osmolyte concentrations were quantified by an isocratic HPLC system (40). Intracellular osmolyte content was normalized to total protein concentrations in each of the samples. Protein concentrations were determined using the Bradford method (4). For collection of urine samples, mice were kept in metabolic cages and urine samples were collected over 24-h time periods. Urine osmolalities were measured using the Advanced Micro Osmometer (Advanced Instruments, Norwood, MA). Blood urea nitrogen (BUN) levels were determined using urea nitrogen kit (Sigma, St. Louis, MO). Three mice per genotype were used for single experiments. Four independent experiments were performed.

**Histological evaluation of tubular injury.** Paraffin sections of kidneys from wild-type and integrin α1-null mice untreated or dehydrated for 24 h were stained with hematoxylin and eosin or PAS and quantitatively assessed for tubular injury. Tubulointerstitial injury criteria included dilatation of lumen, flattening of epithelium, and sloughing of proximal tubule brush border. Severity of injury was graded on a scale of 1–4 (1 = less than 25%, 2 = 25–50%, 3 = 51–75%, and 4 = 76–100% of tubular profiles with injury). Three kidneys per genotype per treatment were analyzed.

Apoptosis within tubules was evaluated by staining paraffin kidney sections using ApopTag Plus Peroxidase in SITU Apoptosis Detection Kit (Serologicals). Degree of apoptosis was graded on a scale of 1–4 as indicated above. To evaluate epithelial injury in thick ascending loop of Henle (TALH), paraffin kidney sections were stained with anti-human Tamm-Horsfall protein (THP) antibody (MP biomedical #S7140) as described (5).

**Isolation of IMCD cells.** Primary IMCD cells were isolated from wild-type and α1-integrin null mice as described (30). Briefly, kidneys were removed immediately at death and processed under aseptic conditions. Kidney medullas of two to four mice were dissected and transferred to hyperosmotic enzyme buffer (120 mM NaCl and 80 mM urea with a total osmolality of 630 mosmol/kg H2O) containing 12 ml DMEM-Ham’s F-12 medium (GIBCO BRL), plus 24 mg collagenase B (Roche, Indianapolis, IN) and 8.5 hyaluronidase (Worthington Bioche-mical, Lakewood, NJ). All solutions in this process were hyperosmotic (640 mosmol/kg H2O). Inner medullas were minced and digested in enzyme solution for 90 min at 37°C under continuous agitation (300 rev/min) in a humidified incubator (5% CO2–95% O2). The resulting cell suspension was centrifuged at 160 g for 1 min, the cellular pellet washed in prewarmed, enzyme-free hyperosmotic DMEM-Ham’s F-12 medium and resuspended in hyperosmotic medium that contained 50% low glucose DMEM (Irvine Scientific, Santa Ana, CA), 50% Coo’s Improved Ham’s F-12 medium (Cellgro, Medi-tech, Herndon, VA), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin G, 100 U/ml streptomycin sulfate, 50 mM hydrocortisone, 5 mM 3,3,5-triiodo-L-thyronine, 1 mM sodium selenite, 5 mg/l transferrin, and 10% fetal bovine serum (vol/vol).

**Myo-[3H]inositol uptake study.** 25 × 10^5 IMCD cells from wild-type and integrin α1-null mice were plated on a 96-well plate in DMEM/F12 containing 10% FCS and kept under isoflurane conditions for 12 h (Costar 3598, Corning). Cells (100% confluent) were then washed with PBS and incubated in serum-free medium kept isometric or in 100-mosmol/kg H2O increments to a final osmolality of 500 mosmol/kg H2O. Cells were incubated for 60 min with different concentrations of unlabeled and [3H]labeled inositol (ICN, Boston, MA) (125 and 250 μmol/l unlabeled inositol mixed with 1 nmol of [3H]inositol, specific activity 10–20 Ci·mmol⁻¹·1⁻¹). In one experimental group, cells were pretreated with 20 μM PD-98059 (Calbiochem, La Jolla, CA) for 24 h before addition of inositol (43). Cells were then washed twice with cold iso- or hypertonic PBS, lysed in 1% SDS, and [3H]inositol uptake was measured using a β-scintillation counter (Beckman, Fullerton, CA). Total inositol uptake was calculated, after subtraction of isoinositol from hypertonic [3H]inositol uptake, using specific activity and respective labeled/unlabeled inositol ratio. Manual counts were performed in some wells to confirmed equal number of wild-type and integrin α1-null IMCD cells. Three independent experiments were performed in duplicate.

**Generation of probes for Northern blot analysis.** Total RNA from mouse inner medulla (~1 μg) was reversed transcribed using Ready To Go T-Primed First-Strand Kit (Amersham, Piscataway, NJ). cDNAs were amplified using the following selective primers: AR (545 bp): sense, 5′-GGATGTTAAAGGGGCGTGC-3′, antisense, 5′-GCTGGTGTCAAGACGTTGG-3′. The cDNA probe for SMIT was a generous gift from Dr. M. Kwon (University of Maryland, MD). All probes were 32P-labeled using Prime-it RII Random Primer Labeling Kit (Stratagene, La Jolla, CA).

Northern blot. IMCD cells were grown to confluency in DMEM/F12 containing 10% FCS and then adapted to a final hyperosmolality of 500 oS/m over a 12-h period (100-oS/m increment in DMEM/F12 containing 2% FCS). IMCD cells were then kept at 500 oS/m for further 24 h and subsequently harvested for RNA isolation.

Total RNA from mouse renal inner medulla (3 animals/genotype per experiment) or IMCD cells was isolated using guanidinium thiocyanate as described (9). Twenty micrograms of total RNA were separated in formaldehyde-containing 1% agarose gels, transferred to nylon membranes (Nytran Supercharge, Schleicher & Schuell, Keene, NH), and hybridized with the 32P-labeled cDNAs described above. Mouse GAPDH cDNA was used for normalization. Transporter or enzyme and GAPDH bands were quantified by densitometry analysis using an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA) and signals were expressed as transporter or enzyme/GAPDH.

**Western blot analysis.** Confluent IMCD cells were serum starved for 24 h and then incubated for 0, 10, 30, and 60 min in hypertonic serum-free medium. The cells were then washed with PBS, scraped, suspended in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, and centrifuged for 10 min at 14,000 rpm. Cell or tissue lysates were resolved by 4–20% gradient SDS/PAGE (50 μg total protein/lane) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated with either rabbit anti-TnEPB antiserum (1:2,000; generous gift from Dr. S. K. Woo, University of Maryland), rabbit anti-phospho-ERK (1:1,000), anti-phospho-p38 (1:1,000), and anti-phospho-AKT antibodies (all from Cell Signaling Technology, Beverly, MA). Immunoreactive proteins were visualized using a peroxidase-conjugated goat anti-rabbit and an ECL kit (Pierce, Rockford, IL). Total ERK, p38, and AKT content was verified by stripping the membranes in 50 mM Tris-HCl (pH 6.5) containing 2% SDS and 0.4% β-mercaptoethanol for 1 h at 55°C and reprobing them with rabbit anti-ERK, -p38, and -AKT antibodies (Cell Signaling Technology).

For the PKA-specific kinase assay, IMCD cells were scraped in 25 mM Tris-HCl, pH 7.4 with 0.5 mM EDTA, 0.5 mM EGTA and protease cocktail buffer (Worthington Biochemical). Cell lysates were subsequently sonicated and 5 μl lysate, corresponding to 10 μg total protein, were assayed for PKA-specific kinase activity using a specific PKA kit (Promega, Madison, WI) according to the manufacturer’s instruction. Phosphorylated and unphosphorylated substrates were separated by agarose gel electrophoresis (0.8% agarose in 50 mM Tris-HCl, pH 8.0 buffer). The gels were photographed after placing on a ultraviolet transilluminator.

**Statistical analysis.** We used the t-test for comparisons between two groups and ANOVA using Sigma-Stat software for statistical differences between multiple groups. \( P \leq 0.05 \) was considered statistically significant.
RESULTS

Tubular injury following dehydration. Kidney tissue sections from wild-type and α1-null mice, either allowed free access to fluids or dehydrated for 24 h, were examined by light microscopy for morphological evidence of tubular epithelial injury. While no significant tubular injury was observed in dehydrated wild-type mice (Fig. 1, A, C, E), integrin α1-null mice had mild focal morphological changes of early tubular necrosis in the S3 segment of outer medulla. This was characterized by tubular lumen dilatation, disintegration of individual epithelial cells, sloughing of apical brush border, and nuclear dropout involving 25–50% (grade 2) of tubular profiles (Fig. 1B). Epithelial cell sloughing and decreased staining intensity for THP was also observed in some outer medulla TALH of dehydrated α1-null mice (Fig. 1D). Both tubular epithelial and interstitial cells in the inner medulla of integrin α1-null mice showed moderate apoptosis involving 50–75% of papillary cells (Fig. 1F). No significant TUNEL positivity was seen in medullary cells of dehydrated wild-type animals (Fig. 1E). Despite these focal morphological differences, BUN levels were similar in the two genotypes.

Deficient osmolyte accumulation in integrin α1-null mice kidney. To determine whether wild-type and integrin α1-null mice accumulate the organic osmolytes sorbitol, betaine and inositol, we quantitated these osmolytes in kidney inner medulla tissue sections from untreated, dehydrated and diuretic animals. Urine osmolality was significantly decreased in α1-null mice compared with their wild-type counterparts under control conditions (water ad libitum, Table 1). Also, significant differences in sorbitol and betaine, but not inositol, concentrations were observed between wild-type and α1-null mice under control conditions (Fig. 2). As expected, significant increases in inner medullary betaine and inositol concentrations were detected in dehydrated wild-type mice compared with their untreated counterparts (Table 2 and Fig. 2). In contrast, medullary osmolyte accumulation, with exception to inositol, remained constant in dehydrated α1-null mice compared with their untreated counterparts (Table 2 and Fig. 2). Surprisingly, urine osmolarities and urine volumes were not significantly different in dehydrated α1-null mice compared with dehydrated wild-type mice (Table 1).

Because osmolyte concentrations closely follow NaCl concentrations in mammalian kidney medulla, treatment with the diuretic furosemide significantly reduces medullary osmolyte content in vivo (24). As expected, furosemide treatment in this study reduced medullary concentrations of all three organic osmolytes in wild-type mice (Fig. 3 and Table 2) compared with their untreated counterparts. The largest reduction was

Fig. 1. Tubular epithelium in wild-type mice (A, C, and E) and integrin α1-null mice (B, D, and F) following 24 h of dehydration. α1-Null mice showed sloughing of apical brush border (★ in B, PAS, ×200), a sign of early tubular epithelial cell injury that was not observed in wild-type mice (A, PAS, ×200). Tamm-Horsfall Protein (THP) immunostaining was stronger in intact thick ascending loop of Henle (TALH) in wild-type mice (C, ×400) compared with their α1-null counterparts (D, ×400). Increased apoptosis, as measured by TUNEL staining, was evident particularly in papillary interstitial cells in dehydrated α1-null animals (arrow in F, ×400) and not seen in wild-type animals (E, ×400) following dehydration.
seen for inositol (62%), whereas sorbitol and betaine concentrations were reduced to a lesser extent. Interestingly, in diuretic \( \alpha_1 \)-null mice, only inositol concentrations were significantly reduced compared with control animals (Fig. 3). Urine osmolality was significantly lower in furosemide-treated \( \alpha_1 \)-null mice compared with their wild-type counterparts (575 ± 31 vs. 818 ± 17 mosmol/kg\( \text{H}_2\text{O} \), respectively; Table 1).

Integrin \( \alpha_1 \)-null mice are deficient in osmolyte transporter and AR enzyme expression following hypertonic conditions. We previously reported that hyperosmotic conditions lead to increased transcriptional activity of osmolyte transporters and enzymes both in vivo and in vitro (24, 26). To determine whether failure of integrin \( \alpha_1 \)-null mice to accumulate osmolytes following dehydration was due to impaired transcriptional regulation of osmolyte transporter and/or enzyme genes, we analyzed mRNA levels of SMIT and AR in renal medullary sections of wild-type and integrin \( \alpha_1 \)-null mice. Furthermore, in the same tissue sections, we determined protein levels of TonEBP, the predominant transcription factor that regulates osmolyte transporters and enzymes in the kidney (23).

Dehydration significantly increased SMIT mRNA levels in wild-type but only marginally in \( \alpha_1 \)-null mice compared with their respective controls (Fig. 4, A and B). Diuresis, on the other hand, did not change SMIT mRNA levels in either wild-type or \( \alpha_1 \)-null mice compared with their untreated controls. Wild-type and integrin \( \alpha_1 \)-null mice showed similar AR mRNA levels under control conditions, while dehydration led to significantly increased AR mRNA levels in wild-type mice (Fig. 4, A and B) compared with controls. In contrast, increase in AR mRNA levels was significantly less in dehydrated integrin \( \alpha_1 \)-null mice compared with their untreated counterparts. On the other hand, diuresis significantly reduced the AR mRNA levels in wild-type, but not in \( \alpha_1 \)-null animals (Fig. 4, A and B). Increase in TonEBP levels was reduced by 50% in dehydrated \( \alpha_1 \)-null mice compared with dehydrated wild-type animals. Furosemide diuresis significantly decreased TonEBP in wild-type animals only (see Fig. 5, A and B).

Integrin \( \alpha_1 \)-null IMCD cells are unable to modulate TonEBP levels and inositol uptake following hypertonic exposure. To better understand the underlying cellular mechanisms that lead to deficient osmolyte uptake in the renal medulla of integrin \( \alpha_1 \)-null mice, we utilized primary IMCD cell cultures from both wild-type and \( \alpha_1 \)-null mice. As TonEBP is the predominant transcription factor that regulates osmolyte transporters and enzymes in the kidney (23), we assessed the levels of TonEBP in IMCD cells following exposure to isotonic or hypertonic conditions. No significant differences in TonEBP levels were observed between wild-type and integrin \( \alpha_1 \)-null IMCD cells cultured under isotonic conditions (Fig. 6, A and B). However, under hypertonic conditions, TonEBP levels increased 62% in wild-type, but only 31% in \( \alpha_1 \)-null IMCD cells compared with their isotonic control cells (Fig. 6, A and B).

As MAP kinase and ERK pathways are induced when collecting duct cells are exposed to hypertonicity (13, 16, 22, 27) and integrin \( \alpha_1 \beta_1 \) can stimulate activation of the ERK pathway in certain cell types (28), we investigated ERK signaling in wild-type and integrin \( \alpha_1 \)-null IMCD cells exposed to hyperosmotic conditions. Furthermore, we investigated AKT and PKA activity, as these kinases have recently been shown to be involved in osmolyte regulation (29, 30). As expected, we found that treatment of wild-type IMCD cells with furosemide significantly decreased AKT and PKA activity (31). As AKT and PKA activity are involved in activation of the MAP kinase and ERK pathways (32, 33), we investigated whether these pathways were also activated in wild-type IMCD cells exposed to hyperosmotic conditions. We found that treatment of wild-type IMCD cells with furosemide significantly increased MAP kinase and ERK activity (34). In contrast, we found that treatment of \( \alpha_1 \)-null IMCD cells with furosemide significantly decreased MAP kinase and ERK activity (35).

The data presented in Table 1 and Table 2 show that hyperosmotic conditions lead to increased transcriptional activity of osmolyte transporters and enzymes in wild-type mice, while dehydration significantly reduced SMIT and AR mRNA levels in wild-type mice.

### Table 1. BUN, urine osmolality, and urine volume in wild-type and \( \alpha_1 \)-null mice compared with their wild-type counterparts

<table>
<thead>
<tr>
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<th>Wild-Type</th>
<th>( \alpha_1 )-null</th>
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<tbody>
<tr>
<td>BUN, mg/dl</td>
<td>21±2</td>
<td>18±1</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kg( \text{H}_2\text{O} )</td>
<td>1,373±103</td>
<td>945±41*</td>
</tr>
<tr>
<td>Urine volume, µl</td>
<td>96±14</td>
<td>80±18</td>
</tr>
</tbody>
</table>

All values are means ± SD of \( n = 3–6 \). CO, control (water ad lib); DH, 24-h dehydration; FUR, furosemide treatment. *Significant differences (\( P < 0.05 \)) between wild-type and integrin \( \alpha_1 \)-null mice within the same treatment group.

### Table 2. Differences in medullary osmolyte concentration in wild-type and \( \alpha_1 \)-null mice

<table>
<thead>
<tr>
<th>Osmolyte</th>
<th>( \Delta \text{OO}_{\text{hol}} )</th>
<th>( \Delta \text{OO}_{\text{O}} )</th>
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<tr>
<td>Sorbitol</td>
<td>92±18</td>
<td>3±20</td>
</tr>
<tr>
<td>Betaine</td>
<td>672±104</td>
<td>ND</td>
</tr>
<tr>
<td>Inositol</td>
<td>741±34</td>
<td>−191±20</td>
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Values represent means ± SD of 4 experiments. All values are pmol/µg protein in medullary kidney tissue. \( \Delta \text{OO}_{\text{hol}} \), change in organic osmolyte (OO) concentrations in dehydrated wild-type and \( \alpha_1 \)-null mice compared with animals under control conditions (water ad lib). \( \Delta \text{OO}_{\text{O}} \), change in OO concentrations in furosemide-treated wild-type and \( \alpha_1 \)-null animals compared with controls. ND, no difference.
either play a role in TonEBP activation (PKA) or induced under hyperosmotic conditions (11, 37). As shown in Fig. 7, increased activation of ERK, as evaluated by analyzing the levels of phosphorylated protein, was observed in wild-type IMCD cells within 20 min of hypertonic stress, but rapidly returned to baseline within 40 min of onset of hyperosmotic conditions. In contrast, \( \alpha_1 \)-null IMCD cell activation of ERK peaked at 40 min posthyperosmotic stress induction and remained elevated over the entire study period. In contrast, while no or minimal activation of p38 was observed in \( \alpha_1 \)-null IMCD exposed to hypertonic stress, increased levels of p38 phosphorylation were observed at 40 and 60 min posthyperosmotic stress in wild-type cells. There was no significant difference in AKT phosphorylation between the two cell types treated with hypertonic medium (Fig. 7). Similarly, no PKA activity was evident in either cell type cultured under normal and hyperosmotic conditions (Fig. 8).

TonEBP has previously been shown to regulate SMIT mRNA levels and inositol uptake in kidney cells (14). We therefore measured \([^3]H\)inositol uptake in wild-type and integrin \( \alpha_1 \)-null IMCD cells cultured under hyperosmotic conditions. Although \([^3]H\)inositol uptake increased proportional to rising medium inositol concentrations (125 and 250 \( \mu \)mol/l) in both wild-type and \( \alpha_1 \)-null cells, inositol uptake in the \( \alpha_1 \)-null IMCD cells was markedly decreased compared with wild-type cells (Fig. 9).

To determine whether the prolonged increased ERK activation might be a cause for impaired inositol uptake, we performed inositol uptake experiment in the presence of the MEK inhibitor PD-98059. Incubation with PD-98059 restored inositol uptake in \( \alpha_1 \)-null IMCD cells to levels close to those observed in wild-type cells (Fig. 9). No differences in inositol uptake were observed between wild-type cells untreated or treated with the MEK inhibitor. Thus prolonged ERK activation in integrin \( \alpha_1 \)-null IMCD cells affects inositol uptake from the surrounding culture medium.

**DISCUSSION**

Our study was aimed at investigating the hypothesis that integrin \( \alpha_1 \beta_1 \) is an important mediator in renal medullary osmolyte regulation. We show that integrin \( \alpha_1 \beta_1 \) plays a regulatory role in hypertonicity-mediated osmolyte accumulation in the kidney inner medulla both in vivo and in vitro. Mice lacking integrin \( \alpha_1 \beta_1 \) not only have less osmolyte accumulated under normal conditions but also show impaired regulation of osmolytes in states of dehydration and diuresis. These deficiencies in \( \alpha_1 \)-null mice appear to be based on inability of these animals to induce adequate p38 activation under dehydration conditions, while ERK activity at the same time is exaggerated. These changes in hyperosmotically-induced signaling path-
ways lead to impaired expression of TonEBP, the transcription factor that regulates the osmolyte transporter SMIT and the sorbitol-catalyzing enzyme aldose reductase in the kidney.

This impairment of the compatible osmolyte response results in mild, focal tubular cell injury in the α1-null mice following 24-h dehydration. Our findings are consistent with a previously published study showing that impaired inositol uptake induces cell death in TALH cells in dehydrated animals in vivo (18). Surprisingly, BUN levels did not increase significantly in acutely dehydrated α1-null mice. Moreover, although dehydrated α1-null animals showed focal injury of TALH segments, no impairment of urinary concentration capability was noticed. The reason for this lack of physiological significant injury is that only 25–50% of the assessed renal parenchyma showed
signs of mild, early acute tubular necrosis in S3 and TALH segments. Longer periods of dehydration or administration of additional injurious agents would produce more extensive injury with impairment of physiological renal function.

Furthermore, findings of a recent study by Lam et al. (21) showed that urinary concentration defect in transgenic mice that overexpressed OREBP/TonEBPdn (a dominant negative form of TonEBP) was most likely due to reduced expression of aquaporin AQP2 and the urea transporter UT-A1 and UT-A2 mRNAs, rather than reduction in intracellular osmolyte concentrations.

We demonstrate that integrin α1 null mice, unlike wild-type animals, are less able to upregulate expression of TonEBP in vivo following dehydration. Similarly, increases in TonEBP expression in α1-null IMCD cells exposed to hypertonic medium are not of the same magnitude as those seen in wild-type IMCD cells. In both experiments, the impairment of TonEBP expression was 50%. A previous study by Sheikh-Hamad et al. (34) showed the importance of β1-integrin in cellular hyperosmotic stress response.

The authors argue that signaling pathways in osmotically stressed cells may be initiated from the HB-EGF/CD9/β1-integrin protein complex. Our results support their hypothesis and further indicate that inefficient signaling in integrin α1-null IMCD cells leads to deficient TonEBP expression and subsequently decreased osmolyte accumulation. Together, these findings suggest that integrin α1, which is one of the 12 β1-integrins (15), may be required for mediating normal physiological signaling responses in renal medullary collecting duct cells following hypertonic stress.

We demonstrated that the lack of ability of α1-null IMCD cells to accumulate osmolytes correlates with a persistence of ERK signaling. In addition, treatment with the MEK inhibitor PD-98059 rescues the ability of α1-null cells to accumulate inositol. These results suggest that the persistence of ERK signaling is at least one of the abnormal signaling events that prevent osmolyte accumulation under hyperosmotic conditions in α1-null IMCD cells. A previous study suggested that both p38 and ERK might play a role in osmolyte accumulation and that inhibition of p38 correlates with upregulation of other MAPKs (32). Our findings support this hypothesis as α1-null IMCD cells showed very little p38 activation but strong and persistent ERK phosphorylation under hyperosmotic conditions. This activation pattern was different from wild-type cells, which showed both ERK and p38 activated following hypertonic stress. These findings indicate that integrin α1β1 might be an important regulator of signal responses to hyperosmotic stress.

A recent study indicated a role of PKA signaling in TonEBP regulation and osmolyte accumulation (11). Furthermore, AKT is activated following hypertonic conditions (37). Surprisingly, we did not observe significant difference in AKT activation between wild-type and α1-null IMCD cells. This indicates that AKT is not a major pathway by which integrin α1β1 regulates osmolyte accumulation under hyperosmotic conditions. Also, we could not detect PKA activity in our wild-type or null IMCD cells following hyperosmotic stimulation, suggesting that PKA is not involved in α1β1-dependent regulation of osmolyte accumulation.

In conclusion our study suggests that integrin α1β1 is an important regulator of signaling events that lead to osmolyte accumulation in mammalian kidney inner medulla cells, both in vivo and in vitro. Integrin α1β1-mediated signaling might be important for stimulating the transcription factor TonEBP expression and subsequently for inducing osmolyte transporter and enzyme gene expression in kidney medulla. Furthermore, α1-mediated ERK1/2 phosphorylation appears to be a critical regulatory step for the maintenance of intracellular osmolyte

Fig. 8. Measurement of PKA activity in IMCD cells from α1-null and wild-type animals, with (HS) and without (CO) hyperosmotic (HS) exposure. PKA assay was performed according to the manufacturer’s instructions by using Pep-Tag assay protein kinase kits.

Fig. 9. Concentration dependent myo-inositol uptake in wild-type and integrin α1-null IMCD cells cultured under hyperosmotic conditions in the absence or presence of PD-98059 (20 μM) as described in MATERIALS AND METHODS. All values represent hyperosmotic uptake minus uptake under isotonic conditions and represent means ± SD from 4 experiments. Differences between wild-type and integrin α1-null IMCD cells (*) or between α1-null cell untreated or treated with MEK inhibitor (#) were significant with P < 0.05.
content in IMCD cells. Lack of integrin α1 predisposes mice kidney medullary cells to apoptosis, likely due to impaired osmolyte accumulation and impaired protection against hyperosmotic stress.

GRANTS

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