Epac-Rap Signaling Reduces Cellular Stress and Ischemia-induced Kidney Failure

Geurt Stokman,* Yu Qin,* Hans-Gottfried Genieser,† Frank Schwede,† Emile de Heer,‡ Johannes L. Bos,§ Ingeborg M. Bajema,‡ Bob van de Water,* and Leo S. Price*

*Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands; †Biolog Life Science Institute, Bremen, Germany; ‡Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands; §Department of Physiological Chemistry, University Medical Center, Utrecht University, Utrecht, The Netherlands

ABSTRACT
Renal ischemia-reperfusion injury is associated with the loss of tubular epithelial cell-cell and cell-matrix interactions which contribute to renal failure. The Epac-Rap signaling pathway is a potent regulator of cell-cell and cell-matrix adhesion. The cyclic AMP analogue 8-pCPT-2’-O-Me-cAMP has been shown to selectively activate Epac, whereas the addition of an acetoxymethyl (AM) ester to 8-pCPT-2’-O-Me-cAMP enhanced in vitro cellular uptake. Here we demonstrate that pharmacological activation of Epac-Rap signaling using acetoxymethyl-8-pCPT-2’-O-Me-cAMP preserves cell adhesions during hypoxia in vitro, maintaining the barrier function of the epithelial monolayer. Intrarenal administration in vivo of 8-pCPT-2’-O-Me-cAMP also reduced renal failure in a mouse model for ischemia-reperfusion injury. This was accompanied by decreased expression of the tubular cell stress marker clusterin-α, and lateral expression of β-catenin after ischemia indicative of sustained tubular barrier function. Our study emphasizes the undervalued importance of maintaining tubular epithelial cell adhesion in renal ischemia and demonstrates the potential of pharmacological modulation of cell adhesion as a new therapeutic strategy to reduce the extent of injury in kidney disease and transplantation.


Renal ischemia-reperfusion (IR) injury is a frequently occurring form of acute renal failure in patients1 and an important contributing factor to chronic allograft dysfunction after renal transplantation.2 Ischemia impairs the capacity of the tubular epithelium to maintain the integrity of its cytoskeleton and affects adherens and tight junction stability3–5 and cell-matrix interactions.6 Loss of cell adhesion is one of the earlier responses of the tubular epithelium observed during IR injury,7 preceding tubular epithelial-cell (TEC) death and inflammatory cell influx. Loss of cell-cell or cell-matrix adhesion has been found to correlate with a loss of cell function, pro-apoptotic signaling, and cell death.8,9

The second messenger cAMP controls a variety of intracellular signaling pathways including protein kinase A (PKA), ion channel activity, and, as more recent studies demonstrate, also Epac proteins (exchange proteins directly activated by cAMP).10 Upon activation by cAMP, Epac functions as an exchange factor for the small GTPase Rap1, mediating replacement of GDP for GTP, consequently leading to activation of Rap1. Among the processes known to be influenced by Rap1 are integrin-mediated adhesion to the extracellular matrix11,12 and the preservation of cell-cell con-
tacts. The cAMP analog 8-pCPT-2′-O-Me-cAMP, colloquially named “007,” selectively activates Epac with low affinity for PKA when used in the micromolar concentration range and can therefore distinguish between Epac-mediated signaling events and PKA-mediated events. A recent modification to 8-pCPT-2′-O-Me-cAMP, the introduction of an acetoxymethyl ester (8-pCPT-2′-O-Me-cAMP-AM or 007-AM), was found to greatly enhance the in vitro biologic activity of the compound presumably by facilitating cellular uptake without affecting its intracellular efficacy.

Epac is expressed by endothelial and epithelial cells with the highest expression level reported to be in the kidney. Both isoforms of Epac (Epac1 and Epac2) are expressed by all three segments of the proximal tubules and are localized in the brush border. In mice, Epac1 has been implicated in the regulation of the Na+/H+ exchanger 3 activity. The Epac-Rap pathway increases endothelial barrier function in vitro by promoting maturation of VE cadherin-mediated cell-cell adhesions, whereas inhibition of Rap1 activity prevents cadherin-based cell adhesion.

The development of Epac-selective cAMP analogs offers a novel strategy for preserving cell adhesion during ischemic injury. Here we demonstrate that 8-pCPT-2′-O-Me-cAMP-induced activation of the Epac-Rap signaling pathway protects against IR injury in a mouse model. In an in vitro hypoxia model, we show that 8-pCPT-2′-O-Me-cAMP-AM strengthens cell-cell and cell-matrix contacts and preserves the integrity and barrier function of the epithelial monolayer. These findings suggest that therapeutic strategies aimed at the preservation of TEC cell-cell or cell-matrix adhesion in general and specifically via activation of the Epac-Rap pathway during IR injury have important clinical potential.

RESULTS

Epac-Rap Signaling is Functional in Conditionally Immortalized Proximal Tubular Epithelial Cells (IM-PTEC)

We studied Epac-Rap signaling in vitro using conditionally immortalized IM-PTEC. At restrictive culture conditions, SV40 expression decreases (Figure 1A), and cells lose their immortalized status, reflected by reduced proliferation and acquisition of a characteristic “cobblestone” epithelial phenotype (data not shown). IM-PTEC cells form confluent monolayers and express the tight junction protein ZO-1 (Figure 1E). Transepts for the proximal tubular epithelial markers megalin (gp330) and sodium-glucose exchange transporters (SGLT) 1 and 2 were also detected (Figure 1B). These results show that IM-PTEC cultured under restrictive conditions are of proximal tubular origin and display the related characteristics and morphology in culture.

Transcripts for Epac1 and −2 were also detected in IM-PTEC (Figure 1B), and Epac1 expression was confirmed (Figure 1, C and F) and strongly upregulated under restrictive conditions. Consistent with expression of Epac1 protein, exposure to forskolin, 8-pCPT-2′-O-Me-cAMP, and 8-pCPT-2′-O-Me-cAMP-AM induced Rap1 activation (Figure 1G). Activation of Rap1 by 8-pCPT-2′-O-Me-cAMP-AM was more pronounced compared with that of 8-pCPT-2′-O-Me-cAMP, most likely reflecting the improved cellular uptake of the AM ester conjugate.
gate. These results demonstrate that IM-PTEC possess a functional Epac-Rap signaling pathway that can be activated either via increases in endogenous cAMP levels or directly by Epac-selective cAMP analogs.

Epac-Rap Activation by 8-pCPT-2’-O-Me-cAMP-AM Reduces Monolayer Disruption and Protects the Tubularr-Barrier Function during in Vitro Hypoxia

To mimic IR injury in vitro, the cells were submerged in paraffin oil. This hypoxia model has been shown to lead to cellular ATP depletion and induce pro-inflammatory cytokine expression. Fifty minutes of hypoxia led to cytoskeletal remodeling, loss of cell-cell contacts (Figure 2A), and stabilization of hypoxia inducible factor-1α (HIF-1α) in IM-PTEC (Figure 2B), demonstrating that this model induces bona fide hypoxia and relevant cytoskeletal and adhesive changes.

Epac-Rap signaling has been shown to promote both intercellular and cell-matrix adhesion. We tested whether activation of Epac prevents the loss of cell-cell contacts induced by hypoxia. The cells were exposed to 50 μM 8-pCPT-2’-O-Me-cAMP, 2.5 μM 8-pCPT-2’-O-Me-cAMP-AM, and 10 μM forskolin or vehicle for 30 minutes before induction of hypoxia. At regular intervals during hypoxia, the cells were fixed, stained for filamentous (F)-actin, and imaged using automated fluorescence microscopy. In vehicle-treated control cells, progressive disruption of the epithelial monolayer during hypoxia occurred, manifested as stress fiber formation and loss of cell-cell contacts resulting in gap formation (Figure 2, C and D). Monolayer disruption was determined by quantifying F-actin coverage and the coinciding gap formation in the monolayer. Monolayer integrity under normoxic conditions was used as a reference; disruption after 75 minutes of hypoxia was considered maximal (Figure 2C). Pretreatment with 8-pCPT-2’-O-Me-cAMP-AM and forskolin significantly prevented epithelial disruption, resulting in approximately 75% reduction in the total area of gaps in the monolayer. Pretreatment with 8-pCPT-2’-O-Me-cAMP-AM and forskolin significantly prevented epithelial disruption, resulting in approximately 75% reduction in the total area of gaps in the monolayer. Pretreatment with 8-pCPT-2’-O-Me-cAMP-AM did not result in significant protection against monolayer disruption and may be due to lower uptake of 8-pCPT-2’-O-Me-cAMP-AM by cells in vitro and consequent weaker activation of Rap (Figure 1B). These results suggest that activation of Epac-Rap signaling preserves the integrity of the epithelial monolayer in response to hypoxic injury in vitro.

To determine whether hypoxia-induced monolayer disruption affected the epithelial barrier function, we performed transepithelial electrical resistance (TER) measurements and examined whether protection of monolayer integrity by 8-pCPT-2’-O-Me-cAMP-AM improves epithelial barrier function. Stimulation with 8-pCPT-2’-O-Me-cAMP-AM in nonhypoxic cells increased the TER compared with controls (Figure 2E), indicating a functional effect of Epac activation on barrier function in noninjured cells. Hypoxia for 60 minutes reduced the TER of vehicle-treated cells by approximately 20% compared with steady-state, normoxic conditions. This reduction was significantly inhibited by pretreatment with 8-pCPT-2’-O-Me-cAMP-AM, resulting in a TER level equivalent to that under steady-state conditions. From these experiments, we conclude that pretreatment with 8-pCPT-2’-O-Me-cAMP-AM preserves the epithelial barrier function during hypoxia.

Exposure to 8-pCPT-2’-O-Me-cAMP-AM Prevents Loss of Epithelial Adherens Junctions during in Vitro Hypoxia

To determine whether the protective effect of 8-pCPT-2’-O-Me-cAMP-AM on monolayer disruption during hypoxia results from an effect on cell-cell junctions, the cells were stained for the adherens junction protein β-catenin and the tight junction protein ZO-1 (Figure 3A). Under normal conditions, IM-PTEC exposed to vehicle or 8-pCPT-2’-O-Me-cAMP-AM display pronounced ZO-1 and β-catenin staining at the cell membrane. After 60 minutes of hypoxia, both ZO-1 and β-catenin localization were disrupted. Treatment with 8-pCPT-2’-O-Me-cAMP-AM before hypoxia reduced the loss of β-catenin from the plasma membrane but did not significantly prevent the loss of ZO-1. We used automated segmentation and analysis of the β-catenin images to measure two different parameters of β-catenin staining; total junctional staining, determined by the product of signal intensity and total staining area (Figure 3B), and cumulative junction length of the stain (Figure 3C). Both parameters were decreased during hypoxia. Pretreatment with 8-pCPT-2’-O-Me-cAMP-AM significantly reduced the loss of β-catenin expression at the cell junctions compared with vehicle-treated hypoxic cells and suggests that 8-pCPT-2’-O-Me-cAMP-AM treatment protects adherens junctions from disassembly during hypoxia.

Adherens Junction Stabilization by 8-pCPT-2’-O-Me-cAMP-AM Is Mediated by Epac1 and Independent of PKA

To confirm that the effects of 8-pCPT-2’-O-Me-cAMP-AM on adherens junction stability are mediated by Epac1, we examined the effects of Epac1 protein knockdown by RNA interference. Mock-transfected controls and small interfering RNA (siRNA) to green fluorescence protein (GFP) were used to exclude adverse effects by the transfection procedure or nonspecific oligonucleotide effects, respectively. Expression of Epac1 was significantly reduced after exposure to mouse Epac1-specific siRNA compared with mock-treated cells and after exposure to GFP siRNA (Figure 3E). Disruption of Epac-Rap signaling blocked 8-pCPT-2’-O-Me-cAMP-AM-mediated protection against adherens junction disassembly during hypoxia but was unaffected in mock and siRNA controls (Figure 3D). Quantification of β-catenin staining supported these observations (Figure 3F). Knockdown of Epac1 by expression of short-hairpin RNA (shRNA) specific for mouse Epac1 gave similar results on 8-pCPT-2’-O-Me-cAMP-AM-induced adherens junction stability during hypoxia (data not shown).

We also examined the contribution of PKA signaling to...
Figure 2. Epac activation protects epithelial monolayer integrity during hypoxia. (A) Cells cultured under normal conditions (control, upper panel) or subjected to 60 minutes of hypoxia (lower panel) were stained for HIF-1α, F-actin, and nuclei. Note translocation of HIF-1α to nucleus in response to hypoxia. Original magnification of microscopy images, 600×. (B) Cells stained positive for HIF-1α were counted and expressed as percentages of the total number of cells. The data are expressed as the means ± SEM. *, P < 0.0001. (C) Cells were pretreated with vehicle (control), 50 μM 8-pCPT-2′-O-Me-cAMP (007), 2.5 μM 8-pCPT-2′-O-Me-cAMP-AM (007-AM), or 10 μM forskolin before start of hypoxia. The cells were fixed and stained with rhodamine-conjugated phalloidin at the indicated time.
cell-cell junction stabilization. Treatment of cells with the PKA selective activator N6-Bnz-cAMP-AM did not stabilize cell-cell junctions during hypoxia (Figure 4, B and D). A control for the AM ester, PO₄-AM, was also without effect. Inhibition of PKA with Rp-8-Br-cAMPS did not inhibit the junction stabilization induced by treatment with 8-pCPT-2′-O-Me-cAMP-AM (Figure 4, C and D), but Rp-8-Br-cAMPS prevented phosphorylation of serine 133 (pSer133) on CREB²² (data not shown). Consistent with these observations, N6-Bnz-cAMP-AM did not induce Rap activation, and Rp-8-Br-cAMPS did not inhibit Rap activation induced by 8-pCPT-2′-O-Me-cAMP-AM (Figure 4A). These results indicate that activation of PKA is not sufficient to prevent hypoxia-induced disruption of cell-cell junctions and is not required for the protective effects of 8-pCPT-2′-O-Me-cAMP-AM, and together with the results of the Epac1 protein knockdown experiments show the crucial involvement of Epac1 in mediating the activity of 8-pCPT-2′-O-Me-cAMP-AM independent of PKA.

**Exposure to 8-pCPT-2′-O-Me-cAMP-AM Reduces Loss of Epithelial Focal Adhesions during in Vitro Hypoxia**

We generated IM-PTEC with stable expression of paxillin labeled with GFP as a marker for focal adhesions²³ (Figure 5A). Hypoxia significantly reduced paxillin localization indicative of decreased matrix adhesion of cells (Figure 5B). Treatment with 8-pCPT-2′-O-Me-cAMP-AM partially preserved focal adhesions during hypoxia with a minor decrease in number of paxillin puncta compared with normoxic controls. Treatment with 8-pCPT-2′-O-Me-cAMP-AM also appeared to change the pattern of phosphorylated paxillin distribution, preserving staining preferentially in the proximity of the cell-cell junctions (data not shown).

**Intrarenal Administration of 8-pCPT-2′-O-Me-cAMP Activates Renal Rap1**

Next we explored the therapeutic significance of Epac-Rap pathway activation and the potential of 8-pCPT-2′-O-Me-cAMP as a prototype drug in a mouse model for IR injury. Expression of Epac in mouse kidney tissue and the efficacy of intrarenal 8-pCPT-2′-O-Me-cAMP treatment on Epac activation were established. In accordance with previous studies using human and rat tissue,¹⁷ Epac is expressed by the tubular epithelium including the proximal segment of the nephron (Figure 6A). We also observed expression of Epac in parietal and visceral epithelium in the glomerulus (Figure 6B). We did not detect Epac expression in capillary endothelium.

8-pCPT-2′-O-Me-cAMP was administered by intrarenal injection. To demonstrate that this approach effectively results in activation of Rap1, we clamped the renal pedicles of mice and administered either saline or 8-pCPT-2′-O-Me-cAMP. The kidneys were collected after 30 minutes and analyzed for Rap1 activation by performing a Rap1 pull-down assay on lysates of frozen kidney sections. Administration of 8-pCPT-2′-O-Me-cAMP resulted in a significant activation of Rap1 compared with saline-treated control kidneys (Figure 6, D and E), demonstrating that our approach is effective in activating renal Epac. Because the vast majority of Epac-expressing cells in the kidney are of tubular epithelial origin, activation of Rap1 after treatment with 8-pCPT-2′-O-Me-cAMP is most likely caused by activation of Epac in the tubular epithelium.

**Treatment with 8-pCPT-2′-O-Me-cAMP during Ischemia Preserves Renal Function and Reduces Tubular Epithelial-Cell Stress**

We next examined whether activation of Epac-Rap signaling can affect the pathogenesis associated with IR injury. Both renal pedicles of mice were clamped for 25 minutes, and directly 8-pCPT-2′-O-Me-cAMP or saline was administered intrarenally. The animals subjected to IR injury and treated with saline showed a significant increase in plasma urea and creatinine levels at day 1 after ischemia compared with sham-operated animals (Figure 7A), demonstrating that 25 minutes of ischemia is sufficient to induce renal failure. When the animals were treated with 8-pCPT-2′-O-Me-cAMP, a significant reduction of plasma urea was observed, suggesting that activation of Epac reduces renal failure. Renal function returned to that found in sham-operated animals at days 2 and 3. We therefore examined tissue obtained at day 1 after ischemia to determine the effect of Epac activation.

Histologic parameters of corticomedullary tubular damage were scored using periodic acid-Schiff reagent (PAS/D)-stained tissue sections. The sections of ischemic kidneys showed clear signs of tissue injury compared with sham-operated kidneys, but no difference in the degree of tubular injury between saline and 8-pCPT-2′-O-Me-cAMP-treated ischemic kidneys was detected (data not shown). Immunostainings for active caspase 3 were performed to study apoptosis. Although ischemia increased tubular epithelial apoptosis, we detected no difference in the number of positive-stained cells between control and treated ischemic kidneys (data not shown).
Figure 3. Epac activation prevents adherens junction disassembly during hypoxia. (A) IM-PTEC were treated with 2.5 μM 8-pCPT-2′-O-Me-cAMP-AM (AM) or vehicle for 30 minutes and subjected to 60 minutes of hypoxia or maintained under normal culture conditions. After 60 minutes, the cells were fixed directly. The cells were stained for β-catenin and ZO-1 and counterstained with Hoechst to visualize nuclei. Original magnification, 600×. (B) Digital-image analysis of β-catenin staining was used to quantify the total amount of fluorescence calculated as the product of the area per field occupied by β-catenin and the mean fluorescence intensity per pixel. Control-treated cells (white bars) subjected to hypoxia have significantly less cell-junction–associated β-catenin expression compared with hypoxic cells treated with 8-pCPT-2′-O-Me-cAMP-AM (black bars). *, P = 0.0005. (C) β-Catenin expression at the cell junctions was quantified using digital-image analysis and used to calculate the cumulative perimeter length as a measure for the total length in pixels of all cell junctions per field. During hypoxia, the cumulative length of cell junctions containing β-catenin in control-treated cells (white bars) was significantly lower than that found in 8-pCPT-2′-O-Me-cAMP-AM treated cells (black bars). *, P = 0.0001. (D) Immunostaining of β-catenin was performed in Epac1-deficient cells. Mock-, siEpac1-, and siRNA for GFP (siGFP)-treated cells were subjected to hypoxia and stained for β-catenin and stained for β-catenin. Mock- and siGFP-treated cells showed reduced cell-junction disassembly after incubation with 8-pCPT-2′-O-Me-cAMP-AM. The cells treated with siEpac1 showed low responsiveness to 8-pCPT-2′-O-Me-cAMP-AM as is evident by impaired junction stability during hypoxia. Interestingly, under normoxic culture conditions Epac1 deficiency did not appear to affect establishment of adherens junctions in monolayer formation when compared with controls (original magnification, 10×). (E) Four separate lysate samples of mock-, siEpac1-, and siGFP-treated cells were used for determination of Epac1 expression by Western blotting. In contrast to control mock- and siGFP-treated cells, exposure to specific siRNA reduced Epac1 expression. Tubulin (tub) expression was used as a loading control. (F) Representative quantification of junction-associated β-catenin expression by digital-image analysis expressed as the product of the area and the mean intensity of β-catenin specific signal per whole field normalized to the respective vehicle-treated normoxic control. Mock- and siGFP-treated cells displayed low β-catenin expression during exposure to hypoxia (hyp) and vehicle (white bars). Exposure to 8-pCPT-2′-O-Me-cAMP-AM (black bars) before hypoxia retained β-catenin expression to levels similar to those found
We measured expression of keratinocyte-derived chemokine (KC) as a biomarker for postischemic inflammation in mouse IR injury. Expression of KC was measured in homogenates of ischemic kidney tissue. No significant difference in the level of KC was found in homogenates of saline and 8-pCPT-2′-O-Me-cAMP-treated ischemic kidneys (data not shown).

To determine whether the protective effect of 8-pCPT-2′-O-Me-cAMP on cell-cell contacts is responsible for the reduction in loss of renal function, we stained tissue sections for catenin. Tissues from both groups of sham-operated animals showed a distinct lateral membrane staining pattern (Figure 6B). Sections from saline-treated ischemic kidneys showed an irregular and more cytoplasmic staining pattern. In contrast to this, localization of catenin in 8-pCPT-2′-O-Me-cAMP-treated ischemic kidneys resembled the pattern found in sham-operated controls.

Clusterin is an urinary marker of tubular epithelial damage. Its expression is increased by reactive oxygen species and involved in the antioxidant response. Clusterin-α expression in ischemic tissue was significantly decreased after 8-pCPT-2′-O-Me-cAMP treatment compared with controls (Figure 7, B and E). Expression of heme oxygenase-1 (HO-1) reflects the cellular response to oxidative stress.

The occurrence of intraluminal obstruction in the renal papilla was scored by determining the number of tubules located in the inner stripe of the outer medulla containing cellular debris. The incidence of intraluminal obstruction was significantly decreased in kidneys treated with 8-pCPT-2′-O-Me-cAMP compared with controls (Figure 7, F and G).

DISCUSSION

In this study, we demonstrate that activation of the Epac-Rap signaling pathway in a mouse model for IR injury reduces renal failure and tubular cell stress. Using an in vitro hypoxia model, we established that 8-pCPT-2′-O-Me-cAMP-AM-induced Epac activation prevented disruption of the epithelial monolayer and maintained tubular barrier function by preventing disassembly of adherens junctions and focal adhesion complexes. The second messenger cAMP is involved in vasopressin-induced water reabsorption in the collecting duct and renin production by juxtaglomerular cells (reviewed by Szasza´k et al.28). Activation of PKA by cAMP controls processes ranging from cellular metabolism, cycling, and growth. The discovery of Epac as an effector protein activated by cAMP has extended this range of cellular processes to include Rap-mediated events such as cell adhesion.

The phosphodiesterase inhibitors olprinone and roliparin both normoxic (nor) controls. In contrast, cells treated with siEpac1 showed a poor effect of 8-pCPT-2′-O-Me-cAMP-AM exposure on hypoxia-induced loss of junction-associated catenin. *, P = 0.048; **, P = 0.025; ns, nonsignificant. The data are expressed as the means ± SEM. The results are representative of three independent experiments.
ram$^{31}$ improve renal function and reduce inflammation after IR injury. Anas et al.$^{30}$ attribute this effect to a cAMP-PKA- or p38 MAPK-mediated decrease of NF-κB activity and IL-8 expression. We did not observe any effect on renal expression of the mouse IL-8 ortholog KC after IR injury, indicating that Epac activation during ischemia is likely not involved in inflammation.

Administration of 8-pCPT-2′-O-Me-cAMP reduced clusterin-α and HO-1 expression and prevented abnormal β-catenin distribution after ischemia. Although no signs of tubular dedifferentiation were yet observed, cytoplasmic β-catenin localization has been implicated in epithelial to mesenchymal transition.$^{32}$

In addition to promoting cadherin-mediated cell junction adhesion, activation of Rap1 by guanine nucleotide exchange factors (Rap1GEFs) such as C3G, PDZ-GEF, and Epac promotes clustering of integrins, thereby enhancing cell-matrix adhesion properties.$^{34}$ Similarly we found that Epac activation preserved the focal adhesion component paxillin during hypoxia (Figure 5A).

In accordance with previous findings describing an important role for Epac in mediating endothelial barrier function,$^{19}$ we found that activation of Epac reduced the loss of barrier...
function during hypoxia (Figure 2E). The inability of 8-pCPT-2′-O-Me-cAMP-AM to prevent tight junction disassembly during in vitro hypoxia is consistent with previous findings that Rap activation in epithelial cells stabilizes adherens junctions but not tight junctions. Previous studies have demonstrated that adherens junctions stabilization was sufficient to strengthen epithelial barrier function.

Our findings using electric cell-substrate impedance sensing (ECIS) suggest that the loss of TER is reduced, but we cannot rule out that this effect on TER may be influenced by Epac-mediated cell spreading affecting the intercellular space in an adhesion-independent fashion. Here we demonstrated that hypoxia-induced loss of focal adhesions is reduced by activation of Epac using paxillin as a functional marker (Figure 5). In addition, we found that Epac activation induced focal adhesion dynamics by promoting phosphorylation of Tyr118 on paxillin during hypoxia (data not shown).

Loss of cell-cell adhesion has been linked to renal failure in patients undergoing allograft transplantation. Furthermore, the loss of matrix adhesion is thought to lead to exfoliation of viable tubular epithelial cells in the urine and has been described in patients with acute renal failure and animal models. In this study, we have not examined whether this process occurs in our animals but did find decreased intraluminal obstruction in 8-pCPT-2′-O-Me-cAMP-treated kidneys after ischemia (Figure 7, F and G). Our in vitro data suggest that Epac-mediated cell junction and focal adhesion stabilization underlie these findings; however, direct evidence of this proposed mechanism will require additional experiments.

Addition of the AM ester to 8-pCPT-2′-O-Me-cAMP increases its biologic activity by facilitating cellular uptake. The stability of 8-pCPT-2′-O-Me-cAMP-AM is, however, dependent on the absence of extracellular esterases, which would remove the AM ester from the molecule. Because of high levels of esterases in serum, we chose to use 8-pCPT-2′-O-Me-cAMP injected intrareally in our in vivo experiments. It is likely that Epac-specific analogs that have improved renal uptake can widen the therapeutic window of this prototype drug in follow-up studies. In conclusion, we propose that enhancement of tubular epithelial cell adhesion in general and specifically activation of the Epac-Rap signaling pathway represent a novel therapeutic strategy for reducing renal failure during early IR injury.

CONCISE METHODS

Antibodies and Reagents

Epac antibodies were generated in the laboratory of J. L. Bos (Utrecht, The Netherlands). For Epac1 detection, the mouse monoclonal SD3 was used for Western blot analysis and the rabbit polyclonal 2293 for immunostainings. Rabbit anti-Rap1 (121), goat anti-clusterin (M-18), and mouse anti-β-actin-IgG were purchased from Santa Cruz Biotech (Santa Cruz, CA). Rabbit anti-ZO-1-IgG was from Zymed (Burlington, NC), the anti-pY118-paxillin and anti-cleaved caspase 3 were from Cell Signaling (Danvers, MA), and the anti-β-catenin was from BD Biosciences (San Jose, CA). The mouse anti-tubulin antibodies was purchased from Sigma (St. Louis, MO). The mouse anti-HIF-1α and anti-SV40 antibodies were from Abcam (Cambridge, UK). Secondary antibodies conjugated to horseradish peroxidase were from Jackson Immunoresearch (Newmarket, UK); antibodies conjugated to Alexa-488 and Cy3 and rhodamine-conjugated phallolidin were from Invitrogen (Breda, The Netherlands). Forskolin was purchased from Calbiochem (Nottingham, UK); PO4-AM, 8-pCPT-2′-O-Me-cAMP, 8-pCPT-2′-O-Me-cAMP-AM, N6-Bnz-cAMP, and Rp-8-Br-cAMPS were from BIOLOG (Bremen, Germany). The rabbit anti-HO-1 (SPA-895) antibody was purchased from Enzo Lifesciences (Zandhoven, Belgium).

Animals and Experimental IR Model

Eight-week-old wild-type male C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands). The mice were anesthetized using Dormicum (Roche, Woerden, The Netherlands) and Hypnorn (Vetapharma, Leeds, UK). Both renal pedicles were clamped for 25 minutes using B-2 vascular clamps (S&T AG, Neuhausen, Switzerland). Intrarenal treatment with 8-pCPT-2′-O-Me-cAMP was performed directly after placement of clamps, by a double 20-μl administration containing 1.45 mM 8-pCPT-2′-O-Me-cAMP or vehicle (saline) in both renal poles of each kidney during ischemia (n = 10/group). All of the animals received one postoperative dose of buprenorfineline (subcutaneous, 0.15 mg/kg; Schering-Plough, Brussels, Belgium). Shams (n = 6/group) received identical treatment without clamping of the renal arteries. The animals were sacrificed at 24, 48, or 72 hours after ischemia (sham-operated animals after 24 hours only). The blood samples were collected by heart puncture and transferred to heparin-coated containers containing separation gels (BD, Alphen a/d Rijn, The Netherlands). Both kidneys were removed and fixed in 4% formaldehyde or snap-frozen in liquid nitrogen. All of the experimental procedures were approved by the Animal Care and Use Committee of Leiden University (Leiden, The Netherlands).

Histology, Renal Function, and Immunohistochemistry

Renal tissue was fixed in 4% formaldehyde for 24 hours and embedded in paraffin in a routine fashion. Four-micrometer-thick sections were cut and used for all stainings. To determine tubular damage, the sections were pretreated with α-amylase (Sigma), stained with PAS/D, and counterstained using hematoxylin. Plasma-urea and creatinine concentrations were measured in a routine fashion using the autoanalyzer facility at the clinical diagnostics department of the Academic Medical Center (Amsterdam, The Netherlands). Tubular damage was scored semiquantitatively on 10 nonoverlapping fields in the corticomedullary area as described previously using the criteria tubular dilation, brush border shedding, cast deposition, and epithelial necrosis on a scale from 0 to 5 on the basis of the percentage of tubules involved: 0 = no tubular damage; 1 = ≤10%; 2 = 11 to 25%; 3 = 26 to 50%; 4 = 51 to 75%; and 5 = 76 to 100% of tubules. Intraluminal obstruction was scored by counting the number of tubules located in the inner stripe of the medulla that displayed intraluminal cellular debris. For all of the immunostainings, the tissue sections were dewaxed and treated with 0.37% H2O2 in methanol for 15 minutes. For the HO-1 staining, the sections were boiled (10’) in a 10
Figure 7. Epac activation reduces renal failure and tubular epithelial cell stress during IR injury. (A) Plasma samples were collected 24 hours after surgery from sham-operated animals and after 24, 48, and 72 hours for IR animals. Plasma urea (upper graph) and creatinine (lower graph) were measured. Treatment with 8-pCPT-2’-O-Me-cAMP(007) significantly reduced plasma urea at day 1 after ischemia compared with saline-treated controls. The data are expressed as the means ± SEM. *, P = 0.026. (B) Clusterin-α expression in kidney sections was analyzed using immunostainings and image analysis software. Representative images of each group are shown. Clusterin-α expression in saline-treated animals was increased at day 1 after ischemia, compared with sham-operated animals. The image details
mM citrate buffer (pH 6.0) before blocking with normal goat serum (Jackson). Primary antibodies were labeled with horseradish peroxidase-conjugated secondary antibody. Visualization was performed using 3,3'-diaminobenzidine, and sections were counterstained with hematoxylin. The sections were imaged using a Leica DM6000B light microscope (Rijswijk, The Netherlands).

**Reverse Transcription-PCR**

Total RNA was isolated from cells using an RNeasy® Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol. cDNA was synthesized using SuperScript® III reverse transcriptase (Invitrogen). Oligonucleotide primers are compiled in Table 1. For amplification REDTaq DNA polymerase (Sigma) was used. For analysis of Epac1, Epac2, and megalin transcription, an annealing temperature of 58°C was used, and for SGLT1 and SGLT2, 62°C was used. PCR products were analyzed on a standard 3% agarose gel and visualized with a BioCapt gel imager (Vilber Lourmat, Torcy, France).

**Rap1-GTP Pull-Down Assay on Cell and Tissue Lysates**

To determine in vitro Rap1 activation, the cells were lysed for 15 minutes in a lysis buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 2.5 mM MgCl2 supplemented with 1 μM aprotonin and 2 μM leupeptidase. To determine in vivo renal Rap1 activation, ten 10-μm-thick cryosections/sample were used for analysis. The sections were incubated with lysis buffer for 30 minutes at 4°C. The lysates were centrifuged, and the supernatants were incubated with glutathione-Sepharose 4B (Roche) beads coated with RaGDS-RBD fusion protein as described previously.37 The samples were then used for Rap1 immunoblotting as described below.

**Immunoblotting**

The cells were lysed in the above mentioned lysis buffer supplemented with protease inhibitor cocktail II (Sigma-Aldrich), sodium fluoride, and vanadate. After centrifugation, the supernatants were boiled for 5 minutes in Laemmli sample buffer containing β-mercaptoethanol, then subjected to protein separation, and blotted on Immobilon-P (Millipore, Amsterdam, The Netherlands). Immunoblots were blocked in Tris-buffered saline with 5% (w/v) bovine serum albumin and incubated overnight with primary antibodies. For detection, the immunoblots were incubated with peroxidase-conjugated secondary antibodies, and the presence of proteins was visualized using ECL+ (Amersham, Little Chalfont, UK) on a Typhoon imager (GE Healthcare, Diegem, Belgium).

**Tissue Homogenates and ELISA**

Frozen kidney samples were thawed in PBS containing 1% (vol/vol) Triton X-100 (Sigma-Aldrich) and 1 mM EDTA supplemented with protease inhibitors. Renal homogenates were made using a Potter tissue grinder. Homogenates were centrifuged, and the supernatant was stored at −80°C. A Duoset ELISA kit specific for detection of mouse KC (R&D Systems, Abingdon, UK) was performed according to the supplied protocol using 3,3′,5′-tetramethylbenzidine (Sigma-Aldrich) as a substrate. Renal KC levels were corrected for total protein contents, measured using the Bradford technique (Bio-Rad, Veenendaal, The Netherlands).

Table 1. Primers used for reverse transcription-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
<tbody>
<tr>
<td>Mouse Epac1</td>
<td>5′-GCCTGCTGTCTTACAGGCTA-3′</td>
<td>5′-CTCTGAAATCCAGGAGGTCTT-3′</td>
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<tr>
<td>Mouse Epac2</td>
<td>5′-CCCGAAAAAGAATCAGCTA-3′</td>
<td>5′-CAGACATTAAACATCAAAGACC-3′</td>
</tr>
<tr>
<td>Mouse megalin</td>
<td>5′-ACAGGTGTGGCATGTCAGT-3′</td>
<td>5′-GTCGCCGTTGACCAAAGTAAG-3′</td>
</tr>
<tr>
<td>Mouse TBP</td>
<td>5′-ACAGGGCCAGAGTGAAAGAC-3′</td>
<td>5′-GGGA AAAA ATCTGGCTCAGTAC-3′</td>
</tr>
<tr>
<td>Mouse peptidylpropyl isomerase B</td>
<td>5′-ACAGCTCAACCGGGAGATG-3′</td>
<td>5′-GGTGTCTTTGCTGCATTG-3′</td>
</tr>
<tr>
<td>Mouse SGLT1</td>
<td>5′-GAATGGAACGCCCTTGGTCTT-3′</td>
<td>5′-AGATACTCCGGCATGTACGAC-3′</td>
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<tr>
<td>Mouse SGLT2</td>
<td>5′-GGCAGCTGTGGGTGCTCCTT-3′</td>
<td>5′-AGAGGCCATCCACTCAA-3′</td>
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</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
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</thead>
<tbody>
<tr>
<td>Mouse clusterin</td>
<td>5′-ACGGACTGCGGTCTTGTCCTT-3′</td>
<td>5′-GCTGTCTTTGCTGCATTG-3′</td>
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<td>Mouse peptidylpropyl isomerase B</td>
<td>5′-ACAGCTCAACCGGGAGATG-3′</td>
<td>5′-GGTGTCTTTGCTGCATTG-3′</td>
</tr>
<tr>
<td>Mouse SGLT1</td>
<td>5′-GAATGGAACGCCCTTGGTCTT-3′</td>
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<tr>
<td>Mouse SGLT2</td>
<td>5′-GGCAGCTGTGGGTGCTCCTT-3′</td>
<td>5′-AGAGGCCATCCACTCAA-3′</td>
</tr>
</tbody>
</table>

On the bottom row are magnifications of the boxed areas. Original magnification, 200×. (C) Representative tissue immunostainings for HO-1 on kidney sections from sham-operated animals and animals sacrificed at day 1 after ischemia. The white arrows indicate examples of positive cells. Original magnification, 200×. (D) Paraffin-embedded tissue sections from animals sacrificed at day 1 after ischemia were stained for β-catenin (blue) and counterstained with Hoechst 33258 (blue). β-Catenin is localized at the cell membrane in tissue from sham-operated animals. During IR injury, β-catenin shows an irregular, more pronounced staining pattern in saline-treated control animals, whereas its expression in 8-pCPT-2′-O-Me-cAMP-treated (007) animals shows an expression pattern that is more equal to that found in shams. Original magnification, 400×. (E) Analysis of HO-1 and clusterin-α tissue stainings. (Left panel) HO-1-positive cells located in the corticomedullary area were counted. Significantly more HO-1-positive cells were found in ischemic control-treated kidneys (white bars) compared with kidneys treated with 8-pCPT-2′-O-Me-cAMP (007, black bars). (Right panel) Digital-image analysis was used for signal quantification of clusterin-α staining. Treatment with 8-pCPT-2′-O-Me-cAMP (007, black bars) significantly reduced clusterin-α expression during IR injury compared with saline-treated controls (white bars). The data are expressed as the means ± SEM. *, P = 0.005; **, P = 0.04. (F, left panel) Incidence of intraluminal obstruction with cellular debris of tubules located in the inner stripe of the outer medulla was examined in PAS/D stainings on kidney sections from control and 8-pCPT-2′-O-Me-cAMP (007)-treated animals. (Right panel) Magnifications of boxed areas are magnified. Black arrows indicate the presence of nuclei Original magnification, 400×. (G) Quantification of tubule obstruction shows a significant decrease in the number of obstructed tubules in the inner stripe of the outer medulla at day 1 after ischemia after 8-pCPT-2′-O-Me-cAMP(007) treatment (black bars) compared with saline controls (white bars). The data are expressed as the means ± SEM. *, P = 0.05.
Cells and in Vitro Hypoxia

IM-PTEC were isolated from Immorto mice as described previously\(^\text{21}\) labeled with antibodies to nephrilin/C1D0 and aquaporin 4 combined, as markers for proximal tubular epithelium\(^{38,39}\) and sorted by flow cytometry on a FACS Aria cell sorter (BD Biosciences). The cells were grown in HK-2 medium (DMEM/F12 medium (Invitrogen) with 10% fetal bovine serum (HyClone, Etten-Leur, The Netherlands), 5 µg/ml insulin and transferrin, 5 ng/ml sodium selenite (Roche), 20 ng/ml triiodo-thyronine (Sigma-Aldrich), 50 ng/ml hydrocortisone (Sigma-Aldrich), and 5 ng/ml prostat glandatin E1 (Sigma-Aldrich) with L-glutamine and antibiotics (both from Invitrogen) and mouse IFN-γ (1 ng/ml; R&D)) at 33°C in 5% CO\(_2\) and 95% air. From this cell population, monoclonal cell lines were generated by limiting dilution and examined for downregulation of SV40 activity during restrictive conditions (culture temperature at 37°C in the absence of IFN-γ) by Western blotting (Figure 1F) with recurrence of the cobble stone-like morphology (data not shown) and megalin transcription (Figure 1G). One clone was used for all experiments described below and referred to as IM-PTEC throughout the paper.

IM-PTEC were transfected retrovirally with a LZRS vector encoding GFP-tagged paxillin,\(^\text{40}\) and a single cell clone (IM-PTEC/GFP-pax) was used for analysis of paxillin localization.

The cells were grown in flasks at restrictive conditions for 7 days, passed to the appropriate assay plates at high density, and cultured for an additional 2 days. The cells were briefly serum-starved in DMEM/F12 for 2 hours. Before being subjected to hypoxia, the cells were pretreated with 50 µM 8-pCPT-2′′-O-Me-cAMP, 2.5 µM 8-pCPT-2′′-O-Me-cAMP-AM and 10 µM forskolin or vehicle for 30 minutes. Hypoxia was induced by submersion of the monolayer in paraffin oil (Bufa, Uitgeest, The Netherlands) for 60 minutes as described previously.\(^\text{21}\) The cells on coverslips were fixed directly by adding 4% formaldehyde solution under the oil layer. The cells were washed with PBS after 10 minutes and processed for staining.

siRNA- and shRNA-mediated Epac1 Knockdown

Mouse-specific siGENOME SMARTpool siRNA to Epac1 and siRNA to GFP were purchased from Dharmacon (Lafayette, CO). Knockdown of target proteins by siRNA was performed by reverse transfection after 5 days of culture on restrictive conditions. For transfections, 100 nM siRNA were diluted in INTERFERin (Polyplus, New York, NY) reagent and added to cell suspensions. Mock-treated cells were exposed to transfection reagent only. The cells were plated in 24-well glass-bottomed SensoPlates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), and medium was changed after 24 hours. The cells were cultured under normal conditions for an additional 2 days before the experiment.

Lentiviral shRNA constructs (TRC1 Library, Mission™ shRNA; Sigma-Aldrich) were provided by M. Rabelink, Leiden University Medical Center, Leiden, The Netherlands. Lentiviral particles were generated via transfection of HEK293T packaging cells with five different mouse Epac1 specific shRNA constructs. Lentiviral transduced IM-PTEC were cultured under permissive conditions and puromycin selection. Epac1 protein expression in puromycin-resistant cell pools was determined by Western blotting after restrictive culture conditions. The cell assays were performed in the absence of puromycin.

Cell and Cytoscore Immunofluorescence Staining

Formaldehyde-fixed cells on glass slides were permeabilized and blocked in PBS containing 0.05% (vol/vol) Triton X-100 and 0.5% (w/v) bovine serum albumin (TBP). All of the antibodies were diluted in TBP. Ten-micrometer cryosections were used for immunostainings. Sections were air dried and fixed in 4% buffered formaldehyde. Sections were permeabilized in 0.2% Triton X-100 in PBS and blocked with 5% normal horse serum (Jackson) in PBS with 0.05% Triton X-100. All of the antibodies were diluted in PBS with 0.05% Triton X-100. The cells and sections were counterstained with Hoechst 33258 dye. For the immunofluorescence staining of β-catenin, paraffin-embedded tissue sections were obtained and treated as described above. Autofluorescence was quenched by incubating slides in 0.1 M glycine-PBS (pH 7.4) for 20 minutes after epitope retrieval followed by a PBS wash. Blocking and antibody labeling were performed similarly as above. A secondary antibody labeled with Cy3 and 1 µg/ml Hoechst 33258 in PBS was used to label the anti-β-catenin and nuclei, respectively. The coverslips and sections were mounted with Aqua-poly/mount (Polysciences, Eppelheim, Germany). The stainings were imaged using a Nikon Ef600 epifluorescence microscope (Nikon, Tokyo, Japan), except paxillin stainings on a Bio-Rad Radiance 2100 confocal laser scanning microscope (Bio-Rad, Hercules, CA) using a 60× Plan Apo NA1.4 objective lens (Nikon).

Monolayer Analysis, Image Analysis, and Signal Quantification

IM-PTEC were cultured in 24-well plates, exposed to 8-pCPT-2′′-O-Me-cAMP, 8-pCPT-2′′-O-Me-cAMP-AM, and forskolin and subjected to hypoxia in dupe as described above. At 15-minute intervals, the cells were fixed and stained with rhodamine-phalloidin to visualize F-actin. The plates were imaged using a BD Pathway 855 high-content bioimager (BD Biosciences) using a long-working distance objective lens (magnification, 20×). Six images per well were made. Phalloidin staining was analyzed using Image-Pro Plus v6.2 analysis software (MediaCybernetics, Gießen, Germany). Monolayer disruption was scored by quantifying the area of gaps in phalloidin staining caused by cell-cell detachment. A score of 1 represents maximal disruption, which was caused by hypoxia treatment of control cells (with no further treatments), whereas a fully intact monolayer (control cells no hypoxia) was ascribed a score of 0 for disruption. The values from stimulated cells were expressed accordingly.

β-Catenin localization was analyzed using 20 separate 600× magnified fields per treatment using Image-Pro Plus v6.2 analysis software. Stainings were analyzed by measuring cell-junction area, junction length, and mean pixel intensity of junctions. For knockdown experiments, the cells were stained “in plate” for β-catenin as described above and imaged using the BD Pathway high-content bioimager with a low magnification (10×) long-working distance lens. Junction β-catenin signal was selected and quantified using Image-Pro Plus v6.2 analysis software by determining the percentage of area containing specific staining per whole well. Focal ad-
hensions were identified by expression of GFP and phospho-paxillin and analyzed by determining the total area of all focal adhesions per field using Image-Pro Plus v6.2. All of the experiments were repeated three times.

Clusterin expression was quantified by using five 20× magnifications per stained cryosection. Clusterin expression was expressed as the percentage of the area with positive signal per total field in the corticomedullary region of the kidney using Image-Pro Plus v6.2 analysis software. HO-1-expressing cells were counted in the corticomedullary area of the kidney and expressed as the number of cells per high-power field using a 40× objective.

Epithelial Barrier Function Measurement
Epithelial barrier function was determined using the ECIS method on an ECIS 1600R using 8W10E electrode array slides (Applied Biophysics, Troy, NY). All of the measurements were performed using 400 Hz frequency. The cells were subjected to prestimulation and hypoxia as described above. After 60 minutes, an equal volume of DMEM/F12 medium was added to the cells submerged in paraffin oil, enabling recontinuation of the ECIS measurement. Barrier function was determined before and during pretreatment with 8-pCPT-2′-O-Me-cAMP-AM and directly after recovery from hypoxia.

Statistical Analyses
The results are expressed as the means ± SEM. The data were tested for normality using the Kolmogorov-Smirnov test and analyzed using an unpaired t test. Tubular injury scores were analyzed using the nonparametric Mann-Whitney U Test. Values of $P \leq 0.05$ were considered statistically significant. All of the statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

ACKNOWLEDGMENTS
This study was supported by a grant from the Dutch Kidney Foundation (G.S.) and by the Netherlands Toxicogenomics Center/The Netherlands Genomics Initiative (L.S.P.).

The authors wish to thank Hans de Bont for help with image analysis and Jantine van Dijk for assistance with the TER measurements. H. G. Genieser owns BIOLOG Life Science Institute, which sells OMe-cAMP for research purposes.

DISCLOSURES
None.

REFERENCES


