Epac-Rap Signaling Reduces Oxidative Stress in the Tubular Epithelium

Geurt Stokman,* Yu Qin,* Tijmen H. Booij,* Sreenivasa Ramaiahgari,* Marie Lacombe,† M. Emmy M. Dolman,‡ Kim M.A. van Dorenmalen,‡ Gwendoline J.D. Teske,§ Sandrine Florquin,§ Frank Schwede,‖ Bob van de Water,* Robbert J. Kok,‡ and Leo S. Price*¶

*Division of Toxicology, Leiden Academic Centre for Drug Research, Leiden, The Netherlands; †LinXis B.V., Amsterdam, The Netherlands; ‡Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands; §Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands; ‖BIOLOG Life Science Institute, Bremen, Germany; and ¶OcellO BV, Leiden, The Netherlands


Renal ischemia-reperfusion (IR) injury is an important cause of AKI1 and a significant risk factor for the development of renal dysfunction after kidney transplantation.2 During IR injury, morphologic and functional alterations of the proximal tubular epithelium occur that are linked to the development of renal failure and activation of immune cells via release of proinflammatory cytokines.3

Exchange protein activated by cAMP (Epac) is a guanine nucleotide exchange factor for the small GTPase Rap1.4 Activation of Epac by cAMP or by the Epac-selective cAMP analog 8-pCPT-2′-O-Me-cAMP (also referred to as 007) induces functional activation of Rap1.5 Initial studies showed that Epac-Rap signaling enhances cell adhesion by supporting maturation of cell-cell junctions6-7 and promoting integrin-mediated cell-matrix adhesion.8,9 In line with these studies, we recently demonstrated that selective activation of Epac reduces proximal tubular epithelial cell (PTEC) detachment during IR injury using in vitro and in vivo models.10 Activation of Epac-Rap was associated with reduced expression of markers for cellular stress in PTECs. In addition,
in vitro cisplatin-induced apoptosis of PTECs could be significantly reduced by activation of Epac and this was also associated with improved adhesion of cells. On the basis of these findings, we hypothesized that activation of Epac-Rap signaling may protect against a common cytotoxic event in these injury models.

Unbalanced and uncontrolled production of reactive oxygen species (ROS) is an important mediator of cell injury and occurs during cisplatin nephrotoxicity, IR injury, and renal fibrosis. In renal pathology, intracellular ROS can be produced enzymatically such as by NADPH oxidase (NOX) complexes or derive from dysfunctional mitochondrial activity. Mitochondrial ROS production appears to be the driving force behind hypoxia-reoxygenation cell injury and cisplatin cytotoxicity.

Here we studied the role of specific proximal tubular activation of Epac and how this protects against renal injury in both in vitro and in vivo models for IR injury. We found that ROS production during reoxygenation after hypoxia was decreased by activation of Epac. Selective proximal tubular activation of Epac by renal targeting of 8-pCPT-2′-O-Me-cAMP conjugated to lysozyme (LZM-007) reduced oxidative stress in an in vivo model for IR injury and significantly decreased IR injury–associated renal failure and tubular damage. Our data show that Epac activation reduces ROS-mediated cellular injury in renal disease and may be a therapeutic strategy for modulation of oxidative stress.

RESULTS

Activation of Epac by 8-pCPT-2′-O-Me-cAMP Reduces Production of ROS after Hypoxic Cell Stress

We first examined a possible role for Epac-Rap signaling in controlling ROS production in vitro using the conditionally immortalized (IM) PTEC line IM-PTECs, which expresses endogenous Epac1 and shows robust activation of Rap1 upon stimulation with 8-pCPT-2′-O-Me-cAMP-AM, a membrane-permeable prodrug of 8-pCPT-2′-O-Me-cAMP, or forskolin. IM-PTECs were preloaded with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), which is converted into fluorescent DCF via oxidation by radicals. After hypoxia, reoxygenation was induced by addition of fresh medium to the cells. Reoxygenation induced a rapid increase in probe conversion (Figure 1A), indicating fast accumulation of cytosolic ROS. We did not observe ROS production during hypoxia and in cells that were maintained in medium with a layer of paraffin oil on top (data not shown), demonstrating that ROS production only occurs in cells that undergo reoxygenation after a hypoxic event. Addition of 5 μM 8-pCPT-2′-O-Me-cAMP-AM or 10 μM forskolin during reoxygenation significantly reduced ROS production (Figure 1A). The role of ROS production in this assay was confirmed by exposure to antioxidants, 5 mM N-acetyl-cysteine (NAC) and 100 μM resveratrol, which significantly reduced the conversion of carboxy-DCF-DA (Figure 1A). The increase in ROS production was apparent up to approximately 60 minutes after the start of reoxygenation, indicating that ROS production is a rapid response of cells that are subjected to reoxygenation after hypoxia. At 60 minutes of reoxygenation, 8-pCPT-2′-O-Me-cAMP-AM and forskolin significantly reduced ROS production by 50% compared with controls (Figure 1B). These results show that Epac activation affects accumulation of cytosolic ROS during reoxygenation after hypoxia. Epac activation by

Figure 1. Activation of Epac reduces ROS production after hypoxia. ROS production is determined during reoxygenation (recovery) after 60 minutes of hypoxia by measuring carboxy-DCF-DA activation using an automated fluorescence plate reader at 5 minutes after the start of reoxygenation. (A) Cells maintained in medium under normoxic conditions show no significant increase in ROS production, whereas cells that are subjected to 60 minutes of hypoxia show rapid production of ROS during reoxygenation. Addition of 5 μM 8-pCPT-2′-O-Me-cAMP-AM (007-AM), 10 μM forskolin, or the antioxidants NAC (5 mM) and resveratrol (100 μM) reduces ROS production during reoxygenation. Data are expressed as the mean±SEM. *P<0.05; **P<0.01. N, normoxic conditions; R, reoxygenation.
8-pCPT-2′-O-Me-cAMP has previously been implicated in NOX inhibition. The NOX inhibitor diphenyleneiodonium (10 µM) did not result in decreased ROS production (data not shown), suggesting that Epac activation by 8-pCPT-2′-O-Me-cAMP in our model does not involve inhibition of NOX.

**Hypoxia-Induced Mitochondrial ROS Production Is Decreased by Exposure to 8-pCPT-2′-O-Me-cAMP**

Mitochondrial generation of superoxide and other ROS is considered the principal mechanism underlying initiation of cellular oxidative stress in IR injury. We therefore examined whether mitochondrial ROS production was involved by detecting oxide radical formation using the superoxide activated probe MitoSOX Red. As a positive control, cells were incubated with 25 µM antimycin A, a mitochondrial complex III inhibitor, which induced significant mitochondrial ROS production (Figure 2A). Cells subjected to hypoxia followed by reoxygenation for 30 minutes showed increased mitochondrial ROS production compared with normoxic controls (Figure 2, B and C). Addition of 5 µM 8-pCPT-2′-O-Me-cAMP-AM or 10 µM forskolin reduced MitoSOX Red staining during reoxygenation indicating a reduction in mitochondrial ROS production (Figure 2, B and C). Similarly, addition of 5 mM NAC and 100 µM resveratrol prevented mitochondrial ROS formation (Figure 2, B and C).

To examine the role of functional Epac-Rap signaling on mitochondrial ROS production, cells were subjected to hypoxia and exposed to the pharmacologic Epac1/2 inhibitors HJC0197 (21 or 10 µM) during reoxygenation (Figure 2D). Both Epac inhibitors increased MitoSOX Red fluorescence intensity and resulted in detachment of cells during reoxygenation. Stimulation with 8-pCPT-2′-O-Me-cAMP-AM did not reduce MitoSOX Red staining or cell detachment during reoxygenation and exposure to HJC0197 or ESI-09. On the basis of these results, we propose that activation of Epac-Rap signaling dampens oxidative stress by reducing mitochondrial ROS.

**Oxidative Stress–Induced Tubular Disintegration Can Be Prevented by 8-pCPT-2′-O-Me-cAMP-AM**

Because hypoxia-reoxygenation results in an acute increase in ROS production, we also investigated the effect of Epac activation in a model for progressive, long-term oxidative stress. For this purpose, IM-PTECs were cultured for 5 days in Matrigel–collagen gels. During this period, tubulogenic outgrowth of IM-PTECs resulted in the formation of a branched tubular network (Figure 3B). Overnight exposure to diethyly maleate (DEM), a compound known to provoke oxidative stress by depletion of glutathione, had clear morphologic effects on the integrity of the network. DEM induced mitochondrial ROS accumulation (Figure 3A), decreased the size of tubule-forming objects (Figure 3C), and reduced the total length of tubules formed per object (Figure 3D) compared with controls. Coexposure to the antioxidant NAC did not result in complete protection against tubular damage (Figure 3, B–D). Addition of 8-pCPT-2′-O-Me-cAMP-AM reduced DEM-induced tubular damage (Figure 3, B–D), demonstrating that activation of Epac provides protection against morphologic alterations in oxidative stress–induced cell injury. Interestingly, exposure to forskolin, a nonselective activator of Epac, decreased object size and tubule length under control conditions and did not provide any beneficial effect on DEM-induced damage (Figure 3, B–D). These findings show that selective Epac activation is cytoprotective against both acute and chronic exposure to oxidative stress.

**Selective and Prolonged Rap1 Activation in the Kidney after Systemic Administration of 8-pCPT-2′-O-Me-cAMP Conjugated to Lysozyme**

Although local intrarenal administration of 8-pCPT-2′-O-Me-cAMP is a feasible experimental approach to activate tubular Epac-Rap signaling, systemic administration and renal targeting is preferable in the context of a renal ischemia model. To this end, an 8-pCPT-2′-O-Me-cAMP-lysozyme conjugate was generated (Figure 4A). Conjugation of bioactive compounds to the low molecular mass protein lysozyme (14 kD) leads to efficient and selective proximal tubular uptake of the compound via megalin-dependent endocytosis of the drug-protein conjugate from the ultrafiltrate.

Systemic administration of nonconjugated 8-pCPT-2′-O-Me-cAMP (20 or 50 mg/kg bodyweight) or LZM-007 conjugate (40 mg/kg bodyweight) resulted in increased Rap1 activity compared with vehicle-treated controls within 2 hours after intravenous injection (Figure 4, B and C). Because the lysozyme conjugate contains 40 µg of 8-pCPT-2′-O-Me-cAMP per milligram of conjugate, a 12.5-fold reduction in total 8-pCPT-2′-O-Me-cAMP could be achieved to induce Rap1 activation by 40 mg/kg LZM-007 compared with 20 mg/kg free nonconjugated 8-pCPT-2′-O-Me-cAMP.

In addition, kidneys were collected at 3, 6, 12, and 24 hours after administration of either 20 mg/kg 8-pCPT-2′-O-Me-cAMP or 40 mg/kg LZM-007. Renal Rap1 activation peaked at 3 hours after 8-pCPT-2′-O-Me-cAMP administration but returned to control levels at later time points. In contrast, Rap1 activity was significantly increased for up to 24 hours in kidneys of animals that received LZM-007 (Figure 5, A and B), which is in agreement with the prolonged renal residence of this type of LZM-drug conjugate. No significant Rap1 activation was detected in liver, heart, or lung tissue after LZM-007 administration (data not shown).

Biodistribution of the LZM-007 conjugate was determined by immunostaining for chicken lysozyme. As previously demonstrated, we detected accumulation of this lysozyme ortholog after administration of the conjugate in most proximal tubules located in the cortex and corticomedullary area of the kidney (Figure 5C). Lung, brain, liver, and heart tissue obtained from the same animals did not stain positive for lysozyme (Figure 5D). These findings suggest that LZM-007 after systemic administration is filtered by the glomerulus and taken up by the proximal tubular epithelium, leading to prolonged activation of Rap1 compared with nonconjugated 8-pCPT-2′-O-Me-cAMP.
Differential Antioxidant Response in Proximal Tubules during Early Reperfusion after Epac Activation

In vivo ROS production resulting from mitochondrial dysfunction occurs rapidly during reperfusion. To examine the effect of Lzm-007 treatment on cellular responses to oxidative stress in a model for bilateral renal IR injury, we treated animals with 40 mg/kg Lzm-007 or vehicle 3 hours before bilateral clamping of the renal pedicles. Animals were euthanized after 1 hour of reperfusion.

Nuclear factor erythroid 2–related factor-2 (Nrf2) controls expression of cytoprotective proteins such as heme oxygenase-1 (HO-1) and a variety of enzymes involved in the antioxidant response, including γ-glutamylcysteine synthetase (γ-GCS). Staining for Nrf2 showed a decreased number of positive nuclei during IR injury in tubules in the corticomedullary area of Lzm-007–treated animals compared with controls (Figure 6A). The enzyme γ-GCS is involved in glutathione synthesis. In sham-operated kidneys, γ-GCS was predominantly localized in the brush border of proximal tubules in both vehicle and Lzm-007–treated animals (Figure 6B, upper panel) and glomeruli. IR injury led to decreased cytoplasmic staining intensity and loss of brush border–associated γ-GCS in control kidneys. Both staining intensity and localization were protected in Lzm-007–treated animals (Figure 6B, lower panel). The expression of HO-1 was not yet detectable at this time point in both groups (data not shown). These data suggest that ROS-induced activation of the Nrf2 pathway is decreased after Epac activation and loss of tubular γ-GCS is prevented.

Proximal Tubule–Specific Activation of Epac Reduces Renal Failure and Tissue Injury during IR Injury

To examine the effect of proximal tubular Epac-Rap activation on IR-induced renal failure and tissue damage, we injected animals with Lzm-007 3 hours before induction of bilateral IR injury and euthanized animals after 1 or 3 days. Compared with vehicle-treated controls, mice treated with Lzm-007 showed a significant reduction in plasma urea and a trend toward reduced plasma creatinine levels at day 1 after ischemia (Figure 7, A and B). Tubular injury, determined by staining...
for clusterin-α, was significantly reduced in kidneys of mice treated with LZM-007 (Figure 7, C and D). Histopathology was scored using a semiquantitative grading system and confirmed a decrease in tubular injury during IR injury in mice treated with LZM-007 compared with controls (Figure 7E).

To rule out any effect of lysozyme itself on renal function and tubular injury during IR injury, we administered 40 mg/kg lysozyme to mice. We found that plasma urea, plasma creatinine, and histopathologic scoring of tubular injury were not significantly different between vehicle- and lysozyme-treated animals (Figure 7F).

HO-1 expression was used to evaluate the Nrf2-mediated antioxidant response in proximal tubules. Treatment with LZM-007 significantly reduced HO-1 expression by proximal tubules compared with that in control animals (Figure 8A), suggesting that, in combination with improved renal function, activation of Epac-Rap signaling decreased the degree of oxidative stress during IR injury. In addition, we detected significantly fewer apoptotic tubular epithelial cells during IR injury in LZM-007–treated animals (Figure 8B). Consistent with a decrease in tubular injury after treatment with LZM-007, the number of granulocytes that infiltrated the tubulointerstitium was significantly lower compared with controls (Figure 8C).

These findings suggest that renal targeting of Epac decreases oxidative stress during IR injury and is associated with improved renal function and prevention of tubular injury.

**DISCUSSION**

In our previous studies, we demonstrated that pharmacologic activation of Epac-Rap signaling provides protection against cell detachment during IR injury and apoptosis during exposure to cisplatin. Because excessive generation of ROS by mitochondria is a common feature in a diverse array of renal pathologies including IR injury and cisplatin nephrotoxicity, we hypothesized that Epac-Rap signaling influences mitochondrial function during injury.

Although antioxidant therapy has been successfully applied in experimental models for renal injury and the clinical effects in patients with kidney disease are less well characterized and do not show the same potential as observed in experimental models.
studies. For example, studies concerning administration of the antioxidant NAC showed responses varying from slightly beneficial to responses without any significant clinical effect.\textsuperscript{30,31} Although NAC does reduce oxidative stress, presumably by replenishment of glutathione, it does not act on the source of ROS production, being mitochondrial dysfunction. Regulation of pathways that mediate mitochondrial function or dysfunction may provide better protection against oxidative stress.

A possible link between Epac and regulation of mitochondrial metabolism was previously suggested\textsuperscript{32} and may be reflected by the mitochondrial localization of Epac\textsuperscript{33} and the mitochondrial localization of Epac.\textsuperscript{33} A recent study by Park et al.\textsuperscript{34} demonstrated that inhibition of cAMP phosphodiesterases by resveratrol promotes mitochondrial function in skeletal muscle cells via Epac1 through an indirect interaction with AMP-activated protein kinase and peroxisome proliferator-activated receptor γ coactivator-1α. Mukai et al.\textsuperscript{35} reported that high glucose that results in mitochondrial ROS production in pancreatic β cells\textsuperscript{36} was decreased by stimulation to the glucagon-like peptide-1 receptor ligand and exendin-4 in an Epac-dependent fashion. A similar effect was obtained using 8-pCPT-2′-O-Me-cAMP. Although their results did not imply Epac, Reedquist et al.\textsuperscript{37} demonstrated that Rap1 signaling was required for Ras-induced ROS in T lymphocytes. These studies, in combination with our results, suggest an unexpected role for Epac-Rap signaling in mediating mitochondrial function and ROS production upon cellular injury or stress.

Whether Epac-Rap signaling prevents mitochondrial ROS production during IR injury directly or indirectly via its effect on improved cell adhesion remains to be investigated.

Using acute \textit{in vitro} models for IR injury, we found that ROS production occurs rapidly during recovery from hypoxia and is associated with increased superoxide accumulation in mitochondria. To examine the effect of Epac activation during long-term exposure to ROS and to study its effects on morphologic alterations, we developed a three-dimensional (3D) culture assay combined with DEM-induced oxidative stress. DEM is conjugated to glutathione by glutathione S-transferase, resulting in loss of the cellular antioxidant capacity. Although forskolin significantly decreased ROS formation in monolayer assays, we found that forskolin did not protect against disruption of tubular network during exposure to DEM. We previously established that 8-pCPT-2′-O-Me-cAMP-AM selectively activates Epac, whereas forskolin induces activation of Epac as well as protein kinase A (PKA).\textsuperscript{10,11} The effect of long-term activation of PKA by forskolin may antagonize the protective outcome we observed in the acute exposures in monolayer assays and reflect PKA-mediated differences in gene expression.

In this study, we used ESI-09\textsuperscript{22} and HJC0197,\textsuperscript{21} two novel identified Epac1/2 inhibitors. A recent publication shows that ESI-09 and HJC0197 do not act as selective inhibitors for both Epac isoforms, but might also inhibit RapGEF6 activity.\textsuperscript{38} In addition, both compounds appear to have protein denaturing properties but only at higher concentrations than those used in our study (10 μM for both compounds).
We determined the effect of Epac activation on in vivo ROS production and cell stress activity by examining established markers for ROS-induced pathway activation. Nrf2 protein levels increase in the presence of ROS, whereas treatment with NAC reduces Nrf2 expression after hypoxia. In line with this, we found that treatment with LZM-007 decreased nuclear Nrf2 translocation during IR injury compared with control-treated animals. The expression of HO-1 is dependent on Nrf2-mediated transcriptional activation; the number of HO-1–expressing tubular epithelial cells in the corticomedullary area was significantly decreased in LZM-007–treated animals, reflecting a reduction in Nrf2-mediated transcription upon activation of Epac. In addition, LZM-007 treatment prevented redistribution of γ-GCS from the apical membrane and decreased staining intensity in proximal tubules during IR injury, suggesting that Epac activation may also act on maintaining glutathione synthesis.

The proximal tubular epithelium plays an important role in the pathogenesis of IR injury. In the tubular compartment, it is the proximal tubules that are most affected by IR injury and are crucially involved in activation of the inflammatory response. In our previous study, we used intrarenal administration of 8-pCPT-2′-O-Me-cAMP to activate Epac. Epac is highly expressed in the kidney but expression is also described in endothelial cells and other organs. To limit Epac activation to proximal tubules in combination with systemic administration, we used a protein-based carrier system in which 8-pCPT-2′-O-Me-cAMP was conjugated to chicken egg white lysozyme via a platinum (II)–based linker molecule (Lx). This approach is dependent on glomerular filtration of the low molecular weight protein lysozyme followed by proximal tubular uptake by megalin.24 Lysozyme-drug complexes have high selectivity for proximal tubular uptake; in accordance with previous studies, we were unable to find any significant labeling for chicken lysozyme in heart, lung, liver, and brain tissue, whereas proximal tubules stained positive in kidney tissue (Figure 5, C and D). Lysozyme itself also has no significant effect on the outcome of IR injury (Figure 7F). Renal targeting of 8-pCPT-2′-O-Me-cAMP therefore results in prolonged activation of the Epac target, allowing a reduced systemic dose and reduced bioavailability in nonrenal tissues.

In this study, we found a clear and unexpected role for Epac-Rap signaling in controlling oxidative stress in PTECs during IR injury. Specific immunostainings were performed for detection of chicken lysozyme as an indication for uptake of LZM-007. Proximal tubules showed specific staining (brown) at the apical membrane (indicated by arrows) or a distinct cytosolic staining pattern (boxed area, indicated by arrow head) suggesting endocytic uptake. (D) Lung, brain, liver, and heart tissue sections from vehicle and LZM-007–treated mice do not stain positive for lysozyme. Nuclei are counterstained with hematoxylin (blue). Data are expressed as the mean ± SEM. *P<0.05; **P<0.01. a.u., arbitrary unit. Original magnification, ×20 in D.
IR injury, most likely by reducing mitochondrial ROS production as found in our in vitro assays. Therapeutic activation of Epac-Rap signaling may thus improve the clinical outcome of renal disease associated with oxidative stress.

**CONCISE METHODS**

**Reagents**

Diethylmaleate, antimycin A, diphenylene iodonium, Hoechst 33258 and 33342, forskolin, NAC, and resveratrol were purchased from Sigma-Aldrich (St. Louis, MO). 8-pCPT-2′-O-Me-cAMP, 8-pCPT-2′-O-Me-cAMP-AM, HJC0197, and ESI-09 were from Biolog (Bremen, Germany). Paraffinum liquidum (110–230 mPa) was from Spruyt Hillen (IJsselstein, The Netherlands). Matrigel was from BD Biosciences (Erembodegem, Belgium). All cell culture reagents and rat tail collagen were from Invitrogen (Bleiswijk, The Netherlands). Buffered formaldehyde solutions were from Added Pharma (Oss, The Netherlands). LZM-007 was coupled to chicken egg white lysozyme (Sigma-Aldrich) via the Lx linker (LinXis B.V., Breda, The Netherlands) as described previously for other conjugates, resulting in a LZM-drug conjugate with a 1:1 drug/LZM molar ratio.

Rabbit anti-Rap1 (clone 121), goat anti-clusterin (M-18), rabbit anti-Nrf2 (C-20), rabbit anti-γ-GCS modifier subunit, and mouse anti-β-actin-IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-cleaved caspase-3 was from Cell Signaling Technology (Danvers, MA). The rabbit anti-HO-1 (SPA-895) antibody was purchased from Enzo Lifesciences (Zandhoven, Belgium). The rat anti-Gr-1 was from BD Biosciences. Texas Red-labeled donkey anti-goat is from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies are from DAKO (Glostrup, Denmark).

**Cell Culture and 3D Assay**

Conditionally immortalized IM-PTECs were cultured as previously described. All experiments were performed using cells that had been cultured under restrictive conditions for at least 7 days. For 3D culture assays, IM-PTECs were cultured under restrictive conditions for 2 days in normal culture dishes prior to “in gel” 3D culture in 96 wells μClear imaging plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) using a Matrigel-collagen gel.
mix as previously described. To prevent adhesion and monolayer formation to the plate bottom, a 35-μl base gel layer was created in each well, on top of which a 30-μl cell-gel suspension was added. After 24 hours, cells were stimulated with hepatocyte growth factor and TGF-β1 (R&D Systems, Abingdon, UK). Cells were cultured for an additional 4 days.

**Cellular ROS Production**

To detect ROS production cells were cultured in black 96-well Clear plates (Greiner Bio-One) and loaded with 40 μM DCF-DA (Invitrogen) in phenol red–free culture medium for 1 hour. After washing in PBS, cells were maintained in serum-free medium without phenol red or under paraffin oil for 60 minutes. Serum-free medium without phenol red and supplemented with compounds (as indicated in the Results) was added to the wells to induce reoxygenation. Fluorescence intensity (480 nm excitation, 520 nm emission) was measured on an automated fluorescence plate reader (FLUOstar OPTIMA; BMG Labtech, Ortenberg, Germany) at 37°C at 2-minute intervals for a total of 2 hours. Data were normalized by plotting values against the starting value.

Mitochondrial dysfunction and superoxide production were determined by loading cells with MitoSOX Red (Invitrogen). Cells cultured on glass coverslips were subjected to hypoxia as described above and 1 mM MitoSOX Red was added to the medium during reoxygenation for 30 minutes. Cells were washed with PBS and fixed in 4% formaldehyde. Cells were then washed with PBS and fixed in 4% formaldehyde. Cells were then washed in PBS with 0.1% (v/v) Triton X-100, counterstained with 2 mg/ml Hoechst 33258, and mounted with Aqua-Polymount (Polysciences, Eppelheim, Germany). Stainings were imaged using a Nikon E600 epi-fluorescence microscope (Nikon Instruments Europe, Amstelveen, The Netherlands) and a Coolsnap CCD camera (Roper Scientific, Ottobrunn, Germany). Images were analyzed using Image-Pro Plus software (version 6.2; Media Cybernetics, Marlow, UK).

**Chronic Oxidative Stress Assay and 3D Imaging**

IM-PTECs were cultured in 3D gel matrices for 5 days as described above. After establishment of tubular structures, cells were exposed to 0.15 mM diethylmaleate in culture medium supplemented with 8-pCPT-2‘-O- Me-cAMP-AM, forskolin, NAC, or appropriate vehicle solutions. After 24 hours, cells were fixed in 4% formaldehyde

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**Figure 7.** LZM-007 prevents renal failure and tubular injury during IR injury. Renal function of animals treated with vehicle (white bars) or LZM-007 (black bars) is determined by measuring plasma urea (A) and plasma creatinine (B). (C and D) Tubular injury is determined by staining for marker expression of clusterin-α. (C) Clusterin-α stainings on kidney sections from animals treated with vehicle (white bars) or LZM-007 (black bars) are quantified using digital image analysis and expressed as percentage of positive staining per field. (D) Representative images of immunostainings used for clusterin-α quantification. Clusterin-α is stained in red, and nuclei are counterstained in blue. (E) Representative examples of periodic acid-Schiff reagent–stained tissue sections (left) and histopathology scoring of tubular injury of mice treated with vehicle (white bars) or LZM-007 (black bars) (right). (F) Renal function of animals treated with vehicle (white bars) or lysozyme (gray bars) is determined by measuring plasma urea (left) or plasma creatinine (middle). Histopathology scoring of tubular injury (right) of animals treated with vehicle (white bars) or lysozyme (gray bars). Data are expressed as mean±SEM. *P<0.05. Original magnification, ×10 in C and D.
Mice and IR Injury

Eight-week-old wild-type male C57BL/6 mice were purchased from Janvier SAS (Le Genest Saint Isle, France). 8-pCPT-2'-O-Me-cAMP, LZM-007, or lysozyme were dissolved in sterile PBS and administered at the times indicated in the text by tail vein injection in a total volume 100 μl, control animals were injected with PBS only. Kidneys were collected at the indicated time points. Mice (n=10 per group) were anesthetized using Dormicure (Roche, Woerden, The Netherlands) and Hypnorm (Vetapharma, Leeds, UK). Bilateral renal ischemia was induced by clamping of the renal pedicles for 35 minutes using B-2 vascular clamps (S&T AG, Neuhausen, Switzerland) during which the animals were kept at 32°C in a temperature-controlled environment. All animals received one postoperative dose of buprenorphine (subcutaneous, 0.15 mg/kg; Schering-Plough, Brussels, Belgium). Shams (n=6 per group) received identical treatment without clamping of the renal arteries. The lysozyme control experiment used sham animals (n=3 per group) and mice with IR injury (n=6 per group). Animals were allowed to recover overnight and temperature was maintained at 28°C. Animals were sacrificed at 1 hour, day 1, or day 3 after ischemia. Blood samples were collected by heart puncture and transferred to heparin-coated containers containing separation gels (BD Biosciences). Both kidneys were removed and fixed in 4% formaldehyde or snap-frozen in liquid nitrogen. For intrarenal administration, animals were anesthetized as described above. We injected 30 or 60μg 8-pCPT-2’-O-Me-cAMP in saline under the capsule into both renal poles (20 μl per injection) during clamping of the renal pedicle. Kidneys were collected after 30 minutes. All experimental procedures were approved by the Animal Care and Use Committee of Leiden University.

Immunofluorescence and Immunohistochemistry

For immunofluorescence stainings, cryosections (10-μm thick) were cut and briefly fixed with 4% paraformaldehyde in PBS (clusterin-α) or ice-cold acetone (Nrf2) for 10 minutes. Sections were permeabilized with 0.2% Triton X-100 for 15 minutes and blocked with Ultra V Block (Labvision, Fremont, CA) for 30 minutes. Sections were counterstained with 2 μg/ml Hoechst 33342 in PBS for 15 minutes and mounted with Aqua-Poly/Mount (Brunschwig). Formaldehyde-fixed kidneys were embedded in paraffin in a routine fashion at the Leiden University Medical Center Department of Pathology. Sections (4-μm thick) were used for all stainings. After dewaxing, sections were treated with 0.3% hydrogen peroxide in methanol for 15 minutes. For cleaved caspase-3 and HO-1 stainings, sections were boiled for 10 minutes in a citrate buffer (pH 6.0), for the Gr-1 staining incubated in a pepsin buffer (0.01 U/ml) at 37°C for 15 minutes. Sections were blocked with 5% normal goat serum for 30 minutes and incubated with primary antibodies overnight at 4°C. Sections were incubated with horseradish peroxidase-conjugated secondary antibodies for 30 minutes. Antibodies and serum were diluted in PBS. Sections were stained with 3,3-diaminobenzidine and analysis software (OcellO, Leiden, The Netherlands) and KNIME and expressed as the mean sum of the total tubule lengths per object.

Figure 8. Oxidative stress, apoptosis, and inflammatory cell influx during IR injury are decreased after LZM-007 treatment. Scoring and representative examples of immunostainings for HO-1 (A), cleaved caspase-3 (B), and the granulocytic Gr-1 epitope (C). Images are from kidney sections collected at day 1 after ischemia. Positive cells are counted per HPF in the corticomedullary area of kidneys from mice treated with vehicle (white bars) or LZM-007 (black bars). Data are expressed as the mean±SEM. *P<0.05; **P<0.01. TEC, tubular epithelial cell.

Containing rhodamine-phalloidin and Hoechst 33258. To demonstrate mitochondrial ROS production, cells were labeled with 1 μM MitoSOX Red for 30 minutes and fixed in 4% formaldehyde containing Hoechst 33258. Plates were imaged using a BD Pathway 855 high content bioimager (BD Biosciences) using a long-working distance objective lens (×4 magnification) and Z-stacks were compressed as single collapsed images composed of ×3 image montages. Alternatively, whole gels were mounted on glass slides using Aqua-PolyMount (Brunschwig, Amsterdam, The Netherlands) and imaged by epifluorescence microscopy. Tubule length was calculated after image processing of the F-actin stain using watershed masked clustering segmentation44 in Konstanz Information Miner Open Source Software (KNIME.com AG, Zürich, Switzerland). Quantification of tubule length was performed by OcellO using 3D image
alternatively nuclei were counterstained with hematoxylin or methylene green.

Pull-Down Analyses and Western Blotting
To determine in vivo renal Rap1 activation, 10 cryosections (10-μm thick) per sample were used for analysis. Sections were incubated with a lysis buffer containing 10% glycerol, 1% Nonidet P40, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5 mM MgCl2 supplemented with 1 μM aprotinin, and 2 μM leupeptidase for 30 minutes at 4°C. Lysates were centrifuged and the supernatants were incubated with Glutathione Sepharose 4B (Roche) beads coated with RaGDS-RBD fusion protein as previously described.43 A 6× Laemml sample buffer containing β-mercaptoethanol solution was added to the samples before boiling for 5 minutes followed by protein separation and blotting on Immobilon-P (Millipore, Amsterdam, The Netherlands). Immunoblots were blocked in Tris-buffered saline with 5% (w/v) BSA and incubated overnight with primary antibodies. For detection, 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).

Renal Function, Immunostaining, and Histopathology Scoring
Plasma urea and creatinine were measured using CREA plus (Roche Diagnostics, Almere, The Netherlands) at the Laboratory for Clinical Chemistry (Amsterdam, The Netherlands) following standard protocols. Clusterin-α staining was measured using Image-Pro Plus software. The area of specific staining was determined per field (×10 magnification) in the corticomedullary region of the kidney. Cells positive for HO-1, caspase-3, and Gr-1 were counted per high power field (HPF) (×40 magnification) of the corticomedullary region of the kidney. γ-GCS-positive tubules were counted and expressed as the percentage of all tubules present per HPF in the corticomedullary area. For the Nr2f2 staining, positive nuclei were counted per HPF. For histopathology scoring, sections were stained with periodic acid–Schiff reagents after diastase digestion. Injury to tubuli was determined by quantifying the percentage of affected tubules per 10 HPF in the corticomedullary region according to the following criteria: tubular dilation, epithelial necrosis, cast deposition, and loss of brush border. Injury was graded on a scale from 0 to 5: 0, 0%; 1, 10%–25%; 2, 25%–50%; 3, 50%–75%; and 5, >75%.

Statistical Analyses
Results are expressed as the mean ±SEM. Data were tested for normality using the Kolmogorov-Smirnov test and analyzed using an unpaired t test. P values ≤0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA).

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DISCLOSURES
None.

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