Gq-Dependent Signaling Upregulates COX2 in Glomerular Podocytes

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ABSTRACT

Accumulating evidence suggests that upregulation of cyclooxygenase 2 (COX2) in glomerular podocytes promotes podocyte injury. Because Gq signaling activates calcineurin and calcineurin-dependent mechanisms are known to mediate COX2 expression, this study investigated the role of Gqα in promoting COX2 expression in podocytes. A constitutively active Gqα subunit tagged with the TAT HIV protein sequence was introduced into an immortalized podocyte cell line by protein transduction. This stimulated inositol trisphosphate production, activated a nuclear factor of activated T cells–responsive reporter construct, and enhanced levels of both COX2 mRNA and protein compared with cells treated with a Gq protein lacking the TAT sequence. Induction of COX2 was associated with increased prostaglandin E2 production and podocyte death, both of which were attenuated by selective COX2 inhibition. In vivo, levels of COX2 mRNA and protein were significantly enhanced in podocytes from transgenic mice that expressed podocyte-targeted constitutively active Gqα compared with nontransgenic littermates. These data suggest that Gq-dependent signaling cascades stimulate calcineurin and, in turn, upregulate COX2 mRNA and protein, increase eicosanoid production, and cause podocyte injury.


Glomerular podocytes are highly differentiated cells that play a key role in maintaining glomerular permselectivity. In glomerular diseases, podocyte damage causes proteinuria and progressive loss of kidney function. The underlying mechanisms that predispose podocytes to injury, however, are incompletely understood. Recent studies suggested that upregulation of cyclooxygenase 2 (COX2) in glomerular podocytes promotes podocyte damage. In this regard, COX2 expression in podocytes is increased in diabetic kidney disease, subtotal nephrectomy, Thy-1 glomerulonephritis, and human renal allograft rejection, and selective COX2 inhibition reduces renal injury and proteinuria in animal models of diabetes, renal ablation, and salt-sensitive hypertension. More recently, Kennedy et al. demonstrated that mechanical stress induces COX2 expression in cultured podocytes. Moreover, overexpression of COX2 specifically in glomerular podocytes enhances albuminuria and foot process effacement and reduces glomerular nephrin expression in adriamycin nephropathy. These data suggest that COX2 may be an important mediator of renal injury in glomerular disease processes.

COX2 expression is stimulated, in part, by activation of the serine/threonine phosphatase calcineurin, through activation of nuclear factor of activated T cells (NFAT)-responsive elements in the COX2 promoter. NFAT transcription factors were originally thought to be expressed only in cells of the lymphoid lineage, but abundant evidence now indicates that...
NFAT isoforms are expressed in nonimmune cells with some family members expressed ubiquitously. In unstimulated cells, NFAT transcription factors are located in the cytoplasm and are highly phosphorylated. Enhanced intracellular calcium activates calcineurin, which, in turn, dephosphorylates NFAT family members and permits their translocation to the nucleus and stimulation of gene transcription.

An important signaling pathway linked to increases in intracellular calcium is activation of Gqα subunits. This signaling pathway stimulates phospholipase Cβ (PLCβ) activity and generates the second messengers diacyl glycerol (DAG) and inositol phosphates (IP). DAG is a potent activator of protein kinase C (PKC), and IP mobilizes calcium from intracellular stores. Calcium, in turn, can activate additional signaling molecules, including calcineurin. In the heart, Gq-dependent signaling cascades are potent activators of calcineurin, and this Gq-dependent calcineurin activation promotes cardiac myocyte hypertrophic responses and cardiac hypertrophy. We, therefore, determined whether Gq-dependent signaling cascades activate calcineurin and upregulate COX2 in glomerular podocytes using both cultured podocytes and a transgenic (TG) mouse model in which a constitutively active Gqα subunit (GqQL) was expressed specifically in podocytes in an inducible manner. We found that Gq-dependent signaling cascades potently upregulated COX2 expression at the mRNA and protein level both in vitro and in vivo. We speculate that upregulation of COX2 by Gq-dependent signaling cascades may be an important mechanism for promoting podocyte injury.

RESULTS

GqQ>L Stimulates IP3 Generation and Activates an NFAT Reporter Construct

We used protein transduction to introduce GqQ>L into an immortalized podocyte cell line by tagging GqQ>L with the Tat HIV protein sequence [GqQ>L(+)]. A GqQ>L protein lacking the Tat sequence was used as a control [GqQ>L(−)]. For the experiments, cells were incubated with GqQ>L(+) or GqQ>L(−) for 30 min before measurement of IP3 generation. As shown in Figure 1A, IP3 generation was significantly enhanced in podocytes treated with the GqQ>L(+) compared with cells treated with the GqQ>L(−). The inset shows that GqQ>L(+) proteins were readily taken up by cultured cells. In contrast, GqQ>L(−) proteins were difficult to detect in podocytes by immunoblotting even after a long exposure.

For determination of whether GqQ>L(+) activated an NFAT reporter construct, podocytes were treated overnight with GqQ>L(+) or GqQ>L(−) in the presence or absence of protein inhibitor of calcineurin myocyte-enriched calcineurin-interacting protein 1 (MCIP1). We used MCIP1 because pharmacologic inhibition of calcineurin potently upregulated COX2 expression in studies described in the next paragraph. For the experiments, MCIP1 was tagged with the TAT HIV protein sequence [MCIP(+)] to permit uptake of MCIP1 by cultured podocytes. An MCIP1 protein lacking the Tat protein sequence, [MCIP(−)] was used as a control. As shown in Figure 1B, GqQ>L(+) significantly enhanced luciferase activity compared with cells treated with GqQ>L(−). The increase in luciferase activity induced by GqQ>L(+) was blocked by MCIP(+) but not the control MCIP(−). All data are expressed as the percentage of the response in DMEM-treated control cells. Data are the results of four to six separate experiments. §P < 0.025 versus GqQ>L(−) or GqQ>L(−); †P < 0.01 versus GqQ>L(−) or GqQ>L(−) treated with MCIP(−).

GqQ>L Enhances COX2 mRNA and Protein Levels and Increases Prostaglandin E2 Generation

Podocytes were treated overnight with GqQ>L(+) or GqQ>L(−), and then COX2 mRNA was measured by quantitative reverse transcriptase–PCR (Q-RT-PCR). As shown in Figure 2A, GqQ>L(+) significantly enhanced COX2 mRNA levels compared with cells treated with GqQ>L(−). Wilms’ tumor–associated antigen levels were not changed by GqQ>L(+) treatment. We next determined whether inhibi-
GqQ>L(+) enhances COX mRNA levels through calcineurin-dependent mechanisms. (A) Podocytes were treated overnight with GqQ>L(+) or GqQ>L(−), and then either COX2 or Wilms’ tumor–associated antigen (WT1) mRNA levels were measured by Q-RT-PCR. GqQ>L(+) significantly enhanced COX2 mRNA levels compared with cells treated with GqQ>L(−). WT1 levels were not changed by GqQ>L(+) treatment. (B) Podocytes were incubated overnight with GqQ>L(+) or GqQ>L(−) in the presence or absence of the calcineurin inhibitor MCIP1(+). COX2 mRNA levels were then measured by Q-RT-PCR. In the presence of MCIP1(−), GqQ>L(+) significantly enhanced COX2 mRNA levels compared with cells treated with GqQ>L(−). All data are expressed as expression relative to DMEM-control treated cells, and data are the results of four to six separate experiments. *P < 0.01 versus GqQ>L(−); †P < 0.01 versus GqQ>L(−) treated with MCIP1(−); **P < 0.005 versus GqQ>L(+) treated with MCIP1(−).

We next investigated the effect of GqQ>L(TAT) proteins on COX2 protein levels. As shown in Figure 3A and in the representative immunoblot shown in the inset, there was little change in COX2 expression in cells treated with both GqQ>L(−) and MCIP1(−) compared with vehicle-treated (DMEM) control cells. In the presence of MCIP1(−), treatment with GqQ>L(+) significantly increased COX2 protein levels compared with cells treated with GqQ>L(−). In contrast, the calcineurin inhibitor MCIP1(+) significantly inhibited induction of COX2 protein by GqQ>L(+) in cells treated with GqQ>L(−). Treatment with MCIP1(+) tended to reduce COX2 protein levels compared with cells treated with GqQ>L(+) and GqQ>L(−). As shown in Figure 3A, treatment with GqQ>L(+) or GqQ>L(−) overnight in the presence or absence of the specific COX2 inhibitor CAY10404, and then PGE2 generation was measured as described in the Concise Methods section. In the presence of vehicle, GqQ>L(+) significantly enhanced PGE2 generation compared with cells treated with GqQ>L(−). In contrast, the COX2 inhibitor CAY10404 significantly inhibited PGE2 generation by both GqQ>L(+) and GqQ>L(−)-treated cells. Data are the results of four to six separate experiments. *P < 0.01 versus MCIP1(−); †P < 0.01 versus GqQ>L(+) treated with MCIP1(−); **P < 0.005 versus GqQ>L(−); fP < 0.005 versus vehicle treated GqQ>L(−).

Induction of COX2 by GqQ>L Promotes Podocyte Injury
Podocytes were treated overnight with GqQ>L(+) or GqQ>L(−) in the presence or absence of CAY10404. Cell death was then quantified by trypan blue exclusion. As shown in Figure 2, we calculated the percentage of cells that were trypan blue positive in each treatment condition. As shown in Figure 3B, treatment with GqQ>L(+) or GqQ>L(−) overnight in the presence or absence of vehicle significantly increased trypan blue positivity compared with cells treated with GqQ>L(−). In contrast, the COX2 inhibitor CAY10404 significantly inhibited PGE2 generation by both GqQ>L(+) and GqQ>L(−)-treated cells. Data are the results of four to six separate experiments. *P < 0.01 versus vehicle treated GqQ>L(−); **P < 0.005 versus GqQ>L(−); †P < 0.01 versus vehicle treated GqQ>L(−); fP < 0.005 versus vehicle-treated GqQ>L(+) and GqQ>L(−).
in Table 1, treatment with GqQ>L(+) enhanced cell death compared with cells treated with Gq(Q>L(−)). Selective COX2 inhibition with CAY1040424 attenuated cell death induced by GqQ>L(+).

Creation of a GqQ>L TG Mice

In previous studies, we expressed GqQ>L in glomerular podocytes using the mouse nephrin promoter. These TG mice develop albuminuria and glomerular histologic abnormalities as well as have a reduction in kidney mass. Because reduced renal mass may also cause proteinuria and renal histologic abnormalities, it is difficult to determine the relative roles of reduced kidney mass versus activation of Gq-linked signaling cascades in the renal phenotype. We, therefore, developed a TG model that permits inducible expression of GqQ>L in a podocyte-specific manner. For these studies, we used the Tet-On system, which has been successfully used by other investigators for inducible transgene expression. For a detailed description of the Tet-On system, the reader is referred to the Concise Methods section, but, briefly, the system requires two TG mice for podocyte-specific expression. As shown in Figure 4A, the first TG animal expresses the reverse tetracycline–controlled transcriptional activator (rtTA) under the control of the human podocin (NPHS2) promoter. This TG mouse was created by Kopp and colleagues and demonstrates podocyte-specific expression. The second TG mouse expresses GqQ>L under the control of tet operator sequence (tetO) and a minimal cytomegalovirus (CMV) promoter (PminCMV). By breeding the two TG mice, animals that express both transgenes are obtained. In these “double” TG mice, treatment with doxycycline induces GqQ>L expression.

For the experiments, two independent GqQ>L TG lines that expressed the transgene in an inducible manner were established. Experimental results were similar using the progeny from these independent lines. Figure 4B shows inducible expression of the HA-tagged GqQ>L transgene by immunoblotting for the HA epitope in GqQ>L mice in the absence of doxycycline treatment. As shown in Figure 4B, top, in the presence of doxycycline, GqQ>L protein was not detectable in non-TG controls, GqQ>L TG mice, or rtTA TG mice (single TG mice) or combined rtTA and GqQ>L TG mice (double-TG mice). In contrast, in the presence of doxycycline, GqQ>L was detectable by immunoblotting in double-TG mice but not in single-TG mice or non-TG controls.

Figure 4C shows tissue-specific expression of the transgene by RT-PCR in a double-TG mouse after treatment with doxycycline using transgene-specific primers. The GqQ>L RT-PCR product was detected in both kidney cortex and isolated glomerular preparations from double-TG mice (top). No GqQ>L RT-PCR products were detected in other tissues from the double-TG mice (top), and the glyceraldehyde-3-phosphate dehydrogenase control confirmed that the RT reaction was successful in the tissues examined (bottom). B, brain; H, heart; Li, liver, Lu, lung; S, spleen; M, muscle (skeletal); G, glomeruli; K, kidney cortex.

Table 1. GqQ>L(+)) induces podocyte death

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<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>CAY10404</th>
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<td>GqQ&gt;L(+))</td>
<td>12.10 ± 0.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.36 ± 1.06&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>GqQ&gt;L(−))</td>
<td>2.39 ± 1.22</td>
<td>2.33 ± 1.35</td>
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<sup>a</sup>Data are results of five experiments.  
<sup>b</sup>P < 0.005 versus vehicle-treated GqQ>L(−).  
<sup>c</sup>P < 0.01 versus vehicle-treated GqQ>L(+).
doxycycline treatment in double-TG mice at the number of PCR cycles used for the experiments.

We next determined cell-specific expression of the transgene using immunohistochemical techniques. For these studies, tissue sections were stained for expression of the HA-tagged GqQ>L transgene and the podocyte marker synaptopodin. As shown in Figure 5A, only synaptopodin was detected in this single-TG GqQ>L mouse treated with doxycycline. Similar results were seen in rtTA single-TG mice and non-TG controls in the presence of doxycycline as well as in double-TG mice in the absence of doxycycline. In contrast, Figure 5B shows that both the HA epitope and synaptopodin were detected in this double-TG mouse treated with doxycycline. Merging the two images suggested that the HA epitope and synaptopodin shared a similar cellular distribution in this double-TG mouse.

Expression of COX2 mRNA and COX2 Protein in GqQ>L TG Mice

Figure 6 shows that treatment with doxycycline significantly enhanced COX2 mRNA levels in double-TG mice compared with either single-TG mice or non-TG controls by Q-RT-PCR. COX2 mRNA levels were also significantly enhanced in double-TG mice treated with doxycycline compared with double-TG mice not receiving doxycycline treatment (1.27 ± 0.32 [no doxycycline] versus 2.32 ± 0.38 [doxycycline]; \( P = 0.0359 \)). We next assessed COX2 expression at the protein level by immunohistochemistry in doxycycline-treated animals. For these studies, tissue sections were stained for expression of COX2 and the podocyte marker synaptopodin. As shown in Figure 7A, COX2 was difficult to detect in this GqQ>L single-TG mouse treated with doxycycline. In contrast, synaptopodin was readily detected in this single-TG mouse. Similar results were seen in rtTA single-TG mice and non-TG controls.
in the presence of doxycycline as well as in double-TG mice in the absence of doxycycline. In double-TG mice treated with doxycycline (Figure 7B), both COX2 and synaptopodin expressions were detectable. Merging the two images suggested that COX2 and synaptopodin had a similar cellular distribution in this double-TG mouse.

For determination of whether induction of GqQL in vivo caused podocyte injury, mice were treated for 3 wk with doxycycline, and then urine was collected for measurement of proteinuria. Mice were then killed, and kidneys were harvested for light microscopic examination. Proteinuria was similar in single-TG mice and non-TG mice. These data, therefore, were combined as a control group. After 3 wk of doxycycline treatment, proteinuria in double-TG mice was not statistically different from that in control mice (0.46 ± 0.13 [controls] versus 0.67 ± 0.06 [TG]; NS). Light microscopic examination of formalin-fixed kidneys was also similar in double-TG mice and the control group. These data suggest that short-term induction of the GqQL transgene did not significantly alter renal histology or glomerular filtration barrier function.

**DISCUSSION**

In these studies, we found that Gq-linked signaling cascades upregulate COX2 expression at both the mRNA and the protein level in cultured podocytes through calcineurin-dependent mechanisms and, in turn, stimulate prostaglandin production and promote podocyte death. Moreover, activation of Gq in vivo enhanced COX2 mRNA and COX2 protein levels. These findings may be relevant to glomerular disease processes because podocyte expression of COX2 is upregulated in human and animal glomerular diseases, and overexpression of COX2 specifically in glomerular podocytes enhances podocyte injury induced by adriamycin. Moreover, COX2 inhibitors decrease proteinuria and attenuate glomerular damage in animal models of renal ablation, diabetic nephropathy, and salt-sensitive hypertension. Although additional studies will be necessary to determine whether enhanced COX2 expression in our TG model predisposes to podocyte injury in vivo, we speculate that Gq-dependent signaling cascades may play a key role in glomerular disease processes by upregulating COX2 and, in turn, promoting glomerular injury.

The mechanisms that predispose podocytes to injury after COX2 induction are not known with certainty. As mentioned, however, prostaglandin and thromboxane receptors are expressed by podocytes; therefore, podocyte eicosanoid generation could act in an autocrine manner to promote podocyte damage. Indeed, our data suggest that podocytes are capable of generating E-series prostaglandins, which, in turn, could act on Gq-coupled EP1 receptors in podocytes both to stimulate further calcineurin activity and to upregulate COX2. It is possible that this positive feedback loop for stimulating calcineurin activity might have additional adverse consequences, such as promoting podocyte apoptosis by dephosphorylating the proapoptotic protein BAD. Indeed, we found that the Gq-coupled angiotensin II (AngII) receptor induced apoptosis of glomerular podocytes by calcineurin-dependent mechanisms. Alternatively, increased COX2 catalytic activity could lead to increased generation of reactive oxygen species, which might injure podocytes by inducing DNA damage and lipid peroxidation. Thus, multiple potential mechanisms may play a role in promoting COX2-dependent podocyte injury.

Multiple G protein-coupled receptors (GPCR) that have been implicated in the pathogenesis of glomerular disease processes are coupled to Gq activation, including receptors for AngII (AT1 receptor), thromboxane, E-series prostaglandins (EP1 receptor), endothelin 1 (ETA receptor), and cysteinyl-leukotrienes. These injury-promoting GPCR systems are found in podocytes and, thus, may contribute to podocyte injury in disease states, perhaps by upregulating COX2 expression. In this regard, endothelin 1 induces COX2 expression in glomerular mesangial cells by activating NFAT transcription factors. Moreover, AngII has been shown to enhance COX2 expression in cultured mesangial cells and upregulate glomerular COX2 expression in vivo through activation of the Gq-coupled AT1 receptor. In this last study, COX2 inhibitors as well as specific blockade of the E-series prostaglandin EP1 receptor prevented mesangial cell hypertrophy, suggesting that enhanced eicosanoid generation may act in an autocrine manner and have adverse consequences as suggested for podocyte PGE2 generation.

Given that induction of COX2 by Gq is calcineurin dependent, a logical therapeutic strategy for preventing COX2 upregulation would be treatment with calcineurin inhibitors such as FK506. Indeed, these agents are already in clinical use for the treatment of glomerular diseases. Unfortunately, we found FK506 potently enhanced COX2 expression. It is therefore of interest that enhanced eicosanoid production may play a role in nephrotoxicity induced by FK506. Although additional studies will be required to determine the mechanisms of FK506-induced COX2 expression, these data are consistent with the notion that induction of COX2 may play a role in renal injury mediated by calcineurin inhibitors.

Last, expression of COX2 was detected in cultured podocytes in the absence of TAT protein treatment (Figure 3A, inset) as reported by other investigators. Moreover, the COX2 inhibitor significantly reduced PGE2 production in GqQL(-)–treated cells (Figure 3B). These data suggest that COX2 is expressed under basal conditions in cultured podocytes and results in COX2-dependent prostaglandin generation. In contrast, glomerular expression of COX2 was not detectable in vivo in the absence of GqQL induction (Figure 7) as reported by other investigators. Although several explanations are possible, one potential explanation is that standard conditions for culturing podocytes induce COX2 expression compared with the in vivo situation.

In summary, we found that Gq activation increased COX2 mRNA and protein levels in a calcineurin-dependent manner.
in cultured podocytes and in vivo. Enhanced COX2 expression was associated with increased PGE₂ generation by cultured podocytes and enhanced cell death, both of which were mediated by COX2-dependent mechanisms. Because COX2 is upregulated in human and experimental glomerular diseases⁴⁻¹¹ and promotes podocyte injury,¹⁵ we speculate that activation of Gq-dependent signaling cascades and, in turn, enhanced COX2 expression may play an important role in the pathogenesis of glomerular disease processes.

**CONCISE METHODS**

**Culture of SV40 Transformed Mouse Glomerular Epithelial Cells**
The immortalized mouse podocyte cell line was a gift of Dr. Paul E. Klotman (Mount Sinai Medical Center, New York, NY) and was maintained in culture as described previously.⁵⁷ Cells were discarded after a maximum of 10 passages.

**DNA Constructs**
The constitutively active Gq construct (GqQ+L) was obtained from Dr. J. Silvio Gutkind (National Institute of Dental Health, Bethesda, MD).⁴⁶ The human MCIP1²³ construct was a gift of Dr. Paul Rosenberg and R. Sanders Williams (Duke University Medical Center, Durham, NC). The β-globin polyadenylation signal⁴⁹ was provided by Dr. Robert Lefkowitz (Duke University Medical Center, Durham, NC).

**Creation of GqQ+L and MCIP1 TAT Proteins**
Protein transduction was used to introduce GqQ+L into cultured podocytes by tagging GqQ+L with the HIV TAT sequence [GqQ+L(+) using methods adapted from Becker-Hapak et al.⁵⁰ For creation of the HA-tagged GqQ+L(+), GqQ+L was first subcloned into the BamH I sites of the vector pBK-CMV (Stratagene, La Jolla, CA). The resulting construct was then cut with BamHI followed by a partial fill-in of the 5′ overhangs by treating with TaqDNA polymerase and dTTP. The GqQ+L fragment was gel-purified and ligated into pTAT-HA²⁵ into the BamH I sites of the vector pBK-CMV (Stratagene, La Jolla, CA). The resulting construct was then cut with BamHI, followed by partial fill-in of the 5′ overhangs by treating with Pfu DNA polymerase (Stratagene) in the presence of 2.5 mM dGTP, dATP, and dTTP. For creation of the HA-tagged GqQ+L(+), GqQ+L was first subcloned into the BamH I sites of the vector pBK-CMV (Stratagene, La Jolla, CA). The resulting construct was then cut with BamHI followed by a partial fill-in of the 5′ overhangs by treating with TaqDNA polymerase and dTTP. For creation of GqQ+L lacking the TAT protein sequence [GqQ+L(−)], the GqQ+L (+) construct was cut with BamHI and religated, which removes only the TAT sequence, leaving the remainder of the construct intact.⁵⁰ All constructs were sequenced at the DNA Analysis Facility at Duke University Medical Center.

MCIP1 binds directly to the catalytic subunit of calcineurin and selectively inhibits calcineurin activity.²¹ To cell a pericyte protein inhibitor of calcineurin, we tagged the MCIP1 cDNA with the TAT protein sequence. For these experiments, the cDNA was amplified from the MCIP construct by PCR using TaqDNA polymerase and the following primer pairs: AGCAGAATGCATTTAGGGAC and CTCAGTCTGCGGGCGCGTGTTCA. The resulting PCR product was ligated into the vector pCR2.1 (Invitrogen, Carlsbad, CA). The resulting construct was then cut with the restriction enzymes Acc65I and XhoI. The MCIP1 fragment was then gel-purified and ligated into the Acc65I and XhoI sites of pTAT-HA². For creation of MCIP1 construct lacking the TAT sequence, the resulting construct was cut with BamHI and religated, which removes only the TAT sequence, leaving the remainder of the construct intact.⁵⁰ All constructs were sequenced at the DNA Analysis Facility at Duke University Medical Center.

**Measurement of [¹³⁵]IP₃ Generation**
Podocytes were plated in six-well tissue culture clusters (Evergreen Scientific, Los Angeles, CA) and then differentiated for 5 to 7 d as described previously⁴⁷ before treatment overnight with 100 to 200 nM GqQ+L-TAT proteins [GqQ+L(+)] or an identical amount of GqQ+L-TAT proteins lacking the TAT sequence [GqQ+L(−)] in the presence or absence of additional agents as indicated. Pilot experiments suggested that this concentration of TAT protein optimized uptake of GqQ+L(+) by podocytes with maximal uptake after 10 to 15 min. After the overnight incubation, cells were harvested and then (1) total cellular RNA was isolated using the Trizol reagent (Invitrogen) according to the directions of the manufacturer, (2) microsomal proteins were prepared as described in the next paragraph, or (3) cell supernatants were harvested for measurement of PGE₂ generation as described in the next paragraph. Supernatants, microsomal proteins, and RNA samples were saved at −70°C before study.

**Luciferase Reporter Assays**
Podocytes were plated in 12-well tissue culture clusters (Evergreen Scientific, Los Angeles, CA) and then differentiated for 5 to 7 d before study. After differentiation, cells were equilibrated for 24 h in DMEM low-inositol medium (Chemicon, Temecula, CA) with 10% dialyzed FCS (Invitrogen) containing 5 μCi/ml myo-[¹³⁵]inositol (General Electric Health Care). The following day, cells were treated for 30 min with the indicated TAT proteins. IP₃ was then measured by anion exchange chromatography as described previously.⁵¹ Data are expressed as a percentage of the response in vehicle-treated (DMEM) control cells.
cells were also transfected with pRL-TK (0.25 µg/well; Promega, Madison, WI) in which the thymidine kinase promoter drives expression of Renilla luciferase. Two days after transfection, the cells were harvested using the Promega Dual Luciferase Reporter Assay System, and both firefly and Renilla luciferase intensity were measured with a luminometer (MGM Instruments, Hamden, CT) according to the directions of the manufacturer (Promega). For correction for transfection efficiency, the firefly luciferase values were divided by the Renilla luciferase values after subtracting the background light intensity. Data are expressed as a percentage of the response in vehicle-treated (DMEM) control cells.

**Immunoblotting for COX2 and HA-Tagged TAT Proteins**

For assessment of uptake of TAT proteins by podocytes, cells were washed extensively in Dulbecco’s PBS (D-PBS; Invitrogen) and then were solubilized in 50 mM Tris-HCl, 150 mM sodium chloride, 2 mM EDTA, 0.2% SDS, and 0.2% Triton X-100 (pH 7.4). Expression of the HA-tagged TAT proteins was then assessed using a rabbit anti-HA polyclonal antibody (UpState Biotechnology, Lake Placid, NY). For COX2, podocytes were scraped into 30 mM Tris-HCl (pH 8.0) with 100 µM PMSF. After centrifugation at 10,000 × g for 10 min, the supernatant was centrifuged for 60 min at 110,000 × g to prepare microsomes. The microsomal pellet was solubilized in 50 mM Tris-HCl, 150 mM sodium chloride, 2 mM EDTA, 0.2% SDS, and 0.2% Triton X-100 (pH 7.4). COX2 expression was then assessed by immunoblotting using a rabbit polyclonal anti-COX2 antibody (Cayman Chemical, Ann Arbor, MI). For immunoblotting, proteins were separated on 4 to 12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes using methods adapted from previous studies. Proteins were detected by enhanced chemiluminescence according to the directions of the manufacturer (General Electric Health Care). For the COX2 studies, immunoblots were stripped according to the directions of the manufacturer (General Electric Health Care), and immunoblotting was performed using a mouse mAb to β-actin (Chemicon Int.) at a concentration of 1 µg/ml. For densitometric quantification of COX2 proteins, the immunoblots were converted into a digital format using an Epson Perfection scanner 1670 (Seiko Epsom Corp., Nagano, Japan) and analyzed using ScanAnalysis 2.5 software (Biosoft, Ferguson, MO). All densitometric values were normalized to an actin loading control, and data are presented as relative expression by dividing individual values by the average value for vehicle-treated (DMEM) controls.

**Measurement of PGE2 Generation**

For measurement of PGE2 generation, podocytes were plated and treated with TAT proteins as described before stimulation with 10 µM ionomycin in serum-free DMEM for 10 min in the presence or absence of the specific COX2 inhibitor CAY1040424 (Cayman Chemical). Supernatants were then harvested for measurement of PGE2 levels using an ELISA kit according to the directions of the manufacturer (Cayman Chemical).

**Creation of an Inducible GqQ>L Transgene**

To create an inducible GqQ>L transgene, we used the Tet-On strategy from BD Biosciences (Palo Alto, CA). This system uses regulatory elements derived from the *Escherichia coli* tetracycline resistance gene including (1) the rtTA, which is a chimeric protein composed of a mutant tetracycline repressor and the VP16 transcription activator domain, and (2) a tetracycline-responsive element (TRE) composed of the 42-bp tet operator sequence (tetO) and a minimal CMV promoter (PminCMV). For inducible expression, the TRE sequence is located 5’ to the target gene and drives gene expression in the presence of the rtTA protein and tetracycline. More recent versions of this system (pTr2) have less background expression in the absence of doxycycline, are more stable in eukaryotic cells, and function at lower concentrations of doxycycline. To ensure podocyte-specific expression, we obtained from Dr. Jeffery Kopp (National Institutes of Health, Bethesda, MD) TG mice in which the podocyte-specific human podocin promoter (NPHS2) drives expression of rtTA. To create the inducible GqQ>L transgene, we first subcloned the human β-globin polyadenylation signal into the Scal/NotI sites of the vector pBK-CMV (Stratagene). The resulting construct was cut with Asel followed by treatment with Pfu DNA polymerase (Stratagene) to create a blunt end and then digested with BssHII. The pTr2 construct (BD Biosciences) was cut with Asel followed by treatment with Pfu DNA polymerase (Stratagene) to create a blunt end and then digested with MluI. The restriction digests were then gel-purified, and the appropriate fragments were ligated together. The resulting construct was cut with BamHI and Xhol and ligated to an Xhol/BamHI fragment of GqQ>L+(+) containing approximately 1000 bp of the 5’ end to GqQ>L and an N-terminal HA epitope. The full-length GqQ>L was reconstituted by cutting the resulting construct with BglIII and Xhol and ligating to a BglIII/Xhol fragment from the GqQ>L in pBK-CMV containing the 3’ end of GqQ>L. The DNA sequences and orientation of the constructs were verified by sequencing at the Duke DNA sequencing facility.

**Creation of GqQ>L TG Mice and Induction of Transgene Expression**

The transgene was linearized by cutting with the restriction enzymes ApaLI/ClaI and then separated from vector sequences on a 0.8% agarose gel and extracted from the gel using the QIAquick gel extraction kit (Qiagen). For removal of endotoxins, the transgene was further purified by treatment with the EndoFree Kit (Qiagen). TG mice were created at the Duke University TG facility using an inbred mouse strain (FVB/NJ) and standard techniques. For transgene expression, rTαA TG mice (background FVB/NJ) were bred with GqQ>L TG mice to create non-TG controls, TG mice with either the rtTA or GqQ>L transgene (single-TG mice), or TG mice with both transgenes (double-TG mice). For induction of transgene expression, mice were treated for 1 wk before study with 2 mg/ml doxycycline in drinking water with 5% sucrose to enhance palatability. All animal procedures were approved by the Animal Care and Use Committee of Duke University Medical Center.

**Screening for TG Mice by PCR**

PCR was performed using TaqDNA polymerase (Promega), and 100 to 200 ng of DNA was prepared from mouse tails using the DNeasy Tissue Kit (Qiagen). The PCR reaction was performed for 30 cycles using the primer pairs encompassing nucleotides 958 through 979.
(TTCATCCTGAAATGTTCGTG) of the mouse Gq<sup>48</sup> and nucleotides 469 through 450 (GAAATTGGACAGCAAGAAAG) in the 3′ untranslated region of the human β-globulin mRNA<sup>49</sup> with the thermal cycler set at 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min.

**Transgene mRNA Expression by RT-PCR**

Total cellular RNA was prepared from mouse tissues with the Trizol reagent (Invitrogen). The RNA was treated with RNase-free DNase (Invitrogen) and then reverse-transcribed before performing RT-PCR. The reverse transcription reaction was performed with Superscript reverse transcriptase (Invitrogen) and oligo (dT) primers using 2 μg of total cellular RNA. PCR was performed for 30 cycles using TaqDNA polymerase (Promega) and primer pairs described previously with the thermal cycler set at 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min. Control PCR reactions were performed for 30 cycles using glyceraldehyde-3-phosphate dehydrogenase primers (Clontech) with the thermal cycler set at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min. PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide.

**Expression of COX2 mRNA Using Q-RT-PCR**

Total cellular RNA was isolated from enriched glomerular preparations, mouse tissues, or cultured podocytes using the Trizol reagent (Invitrogen) according to the directions of the manufacturer. RNA was frozen at −70°C before study. For Q-RT-PCR, RNA was treated with RNase-free DNase as described already, and then the reverse transcription reaction was performed with Superscript reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed using the ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems Division) and the universal SYBR Green PCR master Mix Kit (Perkin-Elmer Applied Biosystems Division, Wellesley, MA). For the studies, the thermal cycler was set at 50°C for 2 min and 95°C for 10 min before performing 40 cycles of PCR with the cycler set at 95°C for 15 s and then 60°C for 1 min in a total reaction volume of 25 μl that included 300 nM of both primers. The amplification signals were analyzed with Perkin-Elmer ABI Prism 7700 Sequence detection software and were normalized to the endogenous cyclophilin A mRNA level. Data are presented as relative expression by dividing individual values by the average value for vehicle-treated (DMEM) control cells. The following sequences were used for the primers: COX2 TGCAGAATTGAAAGCCCTCT and CCCCCAGATAGCATCTGGA, Wilms’ tumor–associated antigen AGGTTT-
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DISCLOSURES

None.

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