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著者	Koike Nobuhiko, Takamura Toshinari, Kaneko Shuichi
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Induction of reactive oxygen species from isolated rat glomeruli by protein kinase C activation and
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Nobuhiko Koike, Toshinari Takamura, Shuich Kaneko

Department of Disease Control and Homeostasis
Kanazawa University Graduate School of Medical Science
13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan.

Corresponding author: Toshinari Takamura, MD, PhD, Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan.

Tel: +81-76-265-2233, Fax: +81-76-234-4250, E-mail: ttakamura@m-kanazawa.jp

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ABSTRACT

Diabetic nephropathy is a major complication of diabetes leading to end-stage renal disease, which requires hemodialysis. Although the mechanism by which it progresses is largely unknown, the role of hyperglycemia-derived oxidative stress has recently been the focus of attention as the cause of diabetic complications. Constituent cells of the renal glomeruli have the capacity to release reactive oxygen species (ROS) upon stimulation of NADPH oxidase activated by protein kinase C (PKC). Hyperglycemia and insulin resistance in the diabetic state are often associated with activation of PKC and tumor necrosis factor (TNF)- α , respectively. The aim of this study is to clarify the signaling pathway leading to ROS production by PKC and TNF- α in rat glomeruli. Isolated rat glomeruli were stimulated with phorbol 12-myristate 13-acetate (PMA) and TNF- α , and the amount of ROS was measured using a chemiluminescence method. Stimulation with PMA (10 ng/ml) generated ROS with a peak value of 136 ± 1.2 cpm/mg-protein (mean $\pm$ SEM). The PKC inhibitor H-7, the NADPH oxidase inhibitor diphenylene iodonium and the phosphatidylinositol-3 (PI-3) kinase inhibitor wortmannin inhibited PMA-induced ROS production by 100%, 100% and 80%, respectively. In addition, TNF- α stimulated ROS production (283 ± 5.8 /mg-protein/20 min). The phosphodiesterase inhibitor cilostazol activates protein kinase A and is reported to improve albuminuria in diabetic rats. Cilostazol (100 μ g/ml) inhibited PMA, and TNF- α -induced ROS production by 78 ± 1.8 , and $19 \pm 2.7\%$, respectively. The effects of cilostazol were not additive with wortmannin. Cilostazol arrests oxidative stress induced by PKC activation by inhibiting the PI-3 kinase-dependent pathway, and may thus prevent the development of diabetic nephropathy.

Introduction

Of the microangiopathies associated with diabetes, the number of patients with diabetic nephropathy continues to increase with the number of patients of diabetes. The onset and progression mechanisms of diabetic nephropathy have not yet been fully clarified. Abnormalities such as increased blood flow in glomerular capillaries, abnormal renal hemodynamics, i.e., excessive filtration (Hostetter et al., 1981), elevated growth factor, including transforming growth factor β 1 (TGF- β 1) (Hayashida and Schnaper, 2004) and advanced glycation endproducts (AGEs) (Vlassara et al., 1994) have been reported in the mesangial cells treated with high doses of glucose. It has also been shown in recent years that oxidative stress is elevated in diabetes (Nishigaki et al., 1981), and more studies suggest a correlation between oxidative stress and chronic diabetic complications (Baynes, 1991; Niedowicz and Daleke, 2005). With regard to diabetic complications, it may be possible to treat diabetic nephropathy and retinopathy by controlling redox (Cojocel et al., 2005; Kowluru, 2005).

Hyperglycemia activates the glycolytic pathway and increases the production of diacylglycerol (DAG) (Wolf et al., 1991). Increases in intracellular DAG levels activate protein kinase C (PKC) (Inoguchi et al., 1992), a serine-threonine kinase, and a study using neutrophils has shown that DAG activates NADPH oxidase via PKC activation (Fujita et al., 1984). In the constituent cells of glomeruli, NADPH oxidase is present, and these cells are capable of producing ROS (Radeke et al., 1991; Greiber et al., 1998). Hence, it is possible that the hyperglycemia associated with diabetes stimulates NADPH oxidase in glomeruli, eliciting oxidative stress, and is thus involved in the onset and progression of diabetic nephropathy.

Furthermore, during the early stages of diabetic nephropathy, it has been shown that macrophages invade the mesangium (Young et al., 1995). Activated macrophages secrete tumor necrosis factor- α (TNF- α) (Beutler et al., 1985), which is involved in the pathology of inflammation and insulin resistance. In inflamed tissue, TNF- α alone facilitates the production of ROS by neutrophils. In addition, a disrupted redox environment may be involved as an onset factor for TNF- α -induced cytotoxicity, and as a study suggested the usefulness of antioxidants (Toborek et al., 1995), it appears that TNF- α is a cytokine that further exacerbates oxidative stress in diabetic nephropathy.

Ieki used rats with streptozotocin (STZ)-induced diabetes and found that cilostazol, a phosphodiesterase (PDE) inhibitor, lowered the excretion rate of urinary albumin, which is an indicator of early-stage diabetic nephropathy, and that this mechanism involved increased Na-K-ATPase activity via elevated cAMP levels in glomeruli (Ieki, 1994). Because cAMP has been reported to suppress ROS production by neutrophils stimulated using formyl-methionyl-leucyl-phenylalanine (fMLP) (Bengis-Garber and Gruener, 1996) or by glomeruli stimulated using phorbol 12-myristate 13-acetate (PMA) (Miyanoshita et al., 1989), cilostazol may improve the redox environment and prevent the onset and progression of diabetic nephropathy by acting on hyperglycemia-induced oxidative stress in glomeruli.

In order to test the above hypothesis, the present study used isolated rat glomeruli to determine whether ROS production is induced by TNF- α , which is elevated by obesity and causes insulin resistance (Hotamisligil et al., 1993), or PMA, which stimulates hyperglycemia-activated PKC. Furthermore, the present study investigated whether cilostazol suppresses excessive

glomerular ROS production, and examined the effects of several signal transduction inhibitors on ROS production and suppression.

Materials and methods

I. Animals

Male Wistar rats weighing about 300 g (Clear Co. Ltd., Tokyo) were fed a solid diet (Oriental Yeast Co., Ltd., Tokyo) and were raised at the Kanazawa University Animal Study Center. All rats were handled in accordance with standards established by the U.S. Animal Welfare Acts set forth in the National Institutes of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins Bloomberg School of Public Health Animal Care and Use.

II. Reagents

Unless otherwise noted, all reagents were purchased from Wako Pure Chemical Industries Ltd., Osaka). Cilostazol powder was donated by Otsuka Pharmaceutical Co., Ltd., Tokyo).

III. Methods

1. Perfusion and isolation of glomeruli

Glomeruli were isolated by the method of Shah (Shah, 1981). Briefly, after administering a lethal dose of ether, laparotomy was immediately performed by placing a median incision and a transverse incision on the costal arch. The abdominal aorta, left and right renal veins and inferior vena cava were detached. A 24-gauge Jelco needle (Johnson & Johnson, Tokyo) was then placed into the abdominal aorta just beyond the bifurcation with the celiac artery toward the periphery, and only the outer casing was left behind. The inferior vena cava was ligated just below the diaphragm,

and the left and right renal veins were severed to release perfusing solution. From the Jelco needle in the abdominal aorta, Hanks' balanced salt solution (HBSS) was injected at a rate of 50 ml/min to perfuse the left and right kidneys for about ten minutes. After perfusion, the kidneys were removed and the renal medullae were cut. Using stainless steel mesh filters (pore size: 63, 150 and 250 μ l, Iida Seisakusyo, Osaka), glomeruli were isolated from the cortex. The resulting glomeruli were stored in cold HBSS until the start of the study.

2. Measurement of glomerular ROS

1) PMA stimulation

After incubating glomeruli at 37°C for 15 min in 2 ml of HBSS, luminol (final concentration: 30 μ M) and PMA (4 μ l, final concentration: 10 μ g/ml, Research Biochemicals International, Natick, USA) were used to stimulate the glomeruli, and chemiluminescence was measured using a luminescence reader (BLR 301, Aloka Inc., Tokyo). For 20 min after adding PMA, chemiluminescence (count per minute: cpm) was measured every 20 s, and the maximum cpm within the 20-min period was determined. The level of ROS production was determined by dividing the maximum cpm by the amount of protein (mg). To investigate glomerular signal transduction pathways, one of the following compounds was added to the glomerulus culture solution at 5 min before PMA stimulation: diphenylene iodonium, an NADPH oxidase-specific inhibitor (DPI, DOJINDO LABORATORIES, Kumamoto); H-7, a PKC inhibitor, [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, BIOMOL, Plymouth Meeting, USA]; wortmannin, a phosphatidylinositol-3 (PI-3) kinase inhibitor, (Sigma, St. Louis, USA); or SB203580, a p38MAPK inhibitor, [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)

imidazole, Calbiochem, La Jolla, USA]. While H-7, wortmannin and SB203580 were added at final concentrations of 100 μ M, DPI was added at a final concentration of 1 μ M. The maximum cpm for each test condition was determined as mentioned above. In addition, using a culture solution in which Ca^{2+} was removed from HBSS, kidneys were perfused to collect glomeruli, and after incubating the glomeruli at 37°C for 15 min using the same culture solution, ROS production was measured. The effects of extracellular Ca^{2+} on the production of PMA-induced ROS and the suppressive effects of cilostazol were thus determined.

2) TNF- α stimulation

Because the results of a preliminary study showed that increases in chemiluminescence due to TNF- α stimulation did not have a clear peak, total chemiluminescence over a 20-min period after TNF- α stimulation was determined. In other words, after adding recombinant TNF- α (final concentration: 10 ng/ml, Pepro Tech, Rocky Hill, USA), chemiluminescence (count) was measured every 20 s for 20 min to determine the total chemiluminescence over the 20-min period. The level of ROS production was determined by dividing the total chemiluminescence over the amount of protein (mg). Next, as was the case with PMA stimulation, the suppressive effects of DPI, H-7, wortmannin and SB203580 were determined.

3) Effects of cilostazol on glomerular ROS production

Glomeruli were pretreated with cilostazol and stimulated using PMA in order to investigate the suppressive effects of cilostazol on ROS production. In other words, as mentioned above, cilostazol (final concentration: 100 μ M) was added to isolated glomeruli before PMA stimulation (final concentration: 10 μ g/ml), and the maximum chemiluminescence over the 20-min

period after PMA stimulation was determined.

Furthermore, in order to compare the suppressive effects of two common scavengers, α -tocopherol and cilostazol, on ROS production, the degree of ROS suppression was measured using 10 nM to 10 μ M of α -tocopherol and cilostazol.

4) Additive effects of cilostazol and wortmannin

After treating freshly cultured glomeruli with 100 μ M of wortmannin for 5 min, cilostazol (100 μ M) was added, and the level of ROS production following PMA or TNF- α stimulation was measured, as mentioned above. In this manner, the additive effects of cilostazol and wortmannin on ROS production were investigated.

Cilostazol powder was dissolved in 100% dimethylsulfoxide (DMSO) and used in the study.

3. Protein determination

Glomerular protein levels were measured by the Lowry method (Lowry, 1951) using bovine serum albumin to produce a standard curve.

IV. Statistical analysis

Results are expressed as mean $\pm$ standard error. An unpaired t-test was used for intergroup comparisons with the level of significance set at $p < 0.05$.

Results

I. Induction of ROS from isolated rat glomeruli

1. PMA stimulation

As shown in Fig. 1a, when luminol was added to a glomerulus suspension solution and PMA (final concentration: 10 $\mu\text{g/ml}$) was added, chemiluminescence increased rapidly. At approximately 15 min after PMA stimulation, the chemiluminescence peaked at 136 ± 1.20 cpm/mg·protein ($n=7$). In order to determine the mechanism of PMA-induced ROS production, the effects of DPI (NADPH oxidase inhibitor), H-7 (PKC inhibitor) and wortmannin (PI-3 kinase inhibitor) on PMA-induced ROS production were investigated. As shown in Fig. 2, DPI and H-7 almost completely suppressed PMA-induced ROS production (both 99%, $n=7$), while wortmannin partially suppressed PMA-induced ROS production (79%, $n=7$). Like H-7, staurosporine (a PKC inhibitor) also almost completely suppressed PMA-induced ROS production. However, the maximum chemiluminescence for SB203580 was 107.0 ± 7.08 cpm/mg·protein ($n=3$), and thus it did not suppress PMA-induced ROS production.

2. TNF- α stimulation

Figure 1b shows the results of glomerular stimulation by TNF- α (final concentration: 10 ng/ml). The rapid increase in chemiluminescence seen after PMA stimulation was not seen with TNF- α stimulation, and low chemiluminescence (below 25 cpm) continued for at least 20 min. As shown in Fig. 3, total chemiluminescence with TNF- α stimulation was 283 ± 6.78 count/mg·protein/20 min. With DPI, H-7, wortmannin and SB203580, the total chemiluminescence was significantly suppressed (18, 32, 75 and 58%, respectively) ($n=4$).

The concentrations obtained by the induction of ROS with regard to PMA and TNF- α were 1 $\mu\text{g/ml}$ or above, and 5 ng/ml or above, respectively.

II. Effects of cilostazol on PMA or TNF- α -induced glomerular ROS production

1. PMA stimulation

As shown in Fig. 4, cilostazol suppressed PMA-induced ROS production in a dose-dependent manner within a concentration range of 10 nM~100 μM : with cilostazol concentrations of 10 nM, 100 nM, 1 μM , 10 μM and 100 μM , PMA-induced ROS production was suppressed $76\pm2\%$, $58\pm2\%$, $52\pm3\%$, $47\pm2\%$ and $22\pm2\%$, respectively. When compared with the same concentrations of α -tocopherol, cilostazol suppressed ROS production significantly greater within a concentration range of 100 nM~100 μM .

2. TNF- α stimulation

As shown in Figure 7, 100 μM of cilostazol suppressed TNF- α -induced ROS production at 228 ± 7.76 count/mg·protein/20min, which was approximately 81% of the TNF- α -induced ROS production without cilostazol.

III. Effects of extracellular Ca^{2+} on glomerular ROS production

Using a culture solution in which Ca^{2+} was removed from HBSS, kidneys were perfused to collect glomeruli, and after incubating the glomeruli at 37°C for 15 min using the same culture solution, ROS production was measured. The effects of extracellular Ca^{2+} on the production of PMA-induced ROS and the suppressive effects of cilostazol were thus determined. As shown in Fig.

5, there were no significant differences in the production of PMA-induced ROS and the suppressive effects of cilostazol with respect to extracellular Ca^{2+} concentration.

IV. Additive effects of cilostazol and wortmannin

The maximum chemiluminescence for PMA-induced ROS production of glomeruli preincubated with wortmannin and then treated with cilostazol was 28.3 ± 3.06 cpm/mg·protein, and there was no significant difference in PMA-induced ROS production with or without wortmannin (Fig. 6).

In the same manner, wortmannin exhibited no additive effects on the suppressive effects of cilostazol on $\text{TNF-}\alpha$ -induced ROS production (Fig. 7).

Discussion

In 1981, Shah clarified that glomeruli produced ROS when stimulated by PMA (Shah, 1981). It has been reported that glomerular ROS production is mediated by NADPH oxidase (Radeke et al., 1991) and that activation of glomerular NADPH oxidase is involved in the pathology of chronic glomerulonephritis (Neale et al., 1993) and diabetic nephropathy (Onozato et al., 2002).

In recent years, studies have found that: there are multiple isoforms of NADPH oxidase due to the polymorphism of gp91-phox, a component of NADPH oxidase, and both gp91-phox and NOX4 are present in renal tissue especially proximal tubules (Shiose et al., 2001). NOX4 is also found in glomeruli, and hyperglycemia upregulates its expression at the mRNA level (Inoguchi et al., 2003).

The hyperglycemia associated with diabetes causes cellular dysfunction through various mechanisms and even induces tissue injury. In recent years, as a factor for cellular injury associated with diabetes, elevated oxidative stress due to hyperglycemia has been examined. Hyperglycemia is known to activate PKC via increased DAG (Wolf, 1991), and it is thought that with PKC activation, hyperglycemia itself activates NADPH oxidase to accelerate oxidative stress. In the present study, we selected PMA and cytokine stimulation, which can cause ROS production using short-term stimulation as an experimental system that reproduces PKC stimulation due to hyperglycemia. It appears that using a response to glomeruli stimulated by elevated glucose levels or DAG as a model for diabetic complications is more appropriate, so we observed ROS production after stimulating glomeruli using high doses of glucose (27.8 mM) and OAG (100 μ M), a synthetic

diacylglycerol, (1-oleoyl-methionyl-leucyl-phenylalanine). However, under these conditions, observations up to 90 minutes did not reveal significant increases in ROS production (data not shown). Lee reported in the past that PKC activation by high doses of glucose requires an incubation time of at least 72 hours (Lee et al., 1989). Unfortunately, unlike a single cell cultivation study, renal glomeruli had a time limit in terms of cellular viability.

The uptake of glucose in constituent cells of glomeruli, except podocyte (Coward et al., 2005), is non-insulin dependent, and in diabetes, glucose flows into the cells in hyperglycemia-dependent manner. Consequently, we assumed that intracellular metabolic deficiencies and PKC activation arise more markedly following hyperglycemia in glomeruli. Moreover, in recent years, since the involvement of NADPH oxidase inhibition has been suggested as an action target shared by drugs such as angiotensin-converting enzyme inhibitor and angiotensin receptor blocker (Onozato et al., 2002), which have been identified as being useful with respect to diabetic nephropathy, we focused on the effect of NADPH oxidase on renal glomeruli. Intracellular signal transduction for NADPH oxidase activation in the constituent cells of glomeruli has not been clarified, but in the present study, wortmannin suppressed PMA-induced glomerular ROS production by approximately 80%, thus suggesting that the ROS production via PMA-activated PKC is almost completely dependent on the signal transduction pathway mediated by PI-3 kinase (Figure 8). Because NADPH oxidase consists of multiple intracellular factors, it is possible that there are multiple signal transduction pathways for each factor. Interestingly, many studies have found that wortmannin does not hinder PMA-induced ROS production by neutrophils (Laudanna et al., 1993; Ding et al., 1995), which contradict the results of the present study using

glomeruli. Therefore, it is possible that even when stimulated by the same compound, different cell types have different signal transduction pathways for NADPH oxidase.

TNF- α has been reported to directly produce ROS in not only neutrophils, but also mesangial cells (Radeke et al., 1990) and fibroblasts (Meier et al., 1989). The mechanism of NADPH oxidase activation by TNF- α has not been fully elucidated. However, studies using neutrophils have shown that while PKC inhibitors do not suppress ROS production, tyrosine kinase inhibitors and wortmannin suppress ROS production (Laudanna et al., 1993). In addition, TNF- α has been shown to activate p47-phox, an NADPH oxidase component, via the phosphorylation of p38MAP (mitogen-activated protein) kinase (Benna et al., 1996). Subsequently, TNF- α appears to activate NADPH oxidase via multiple signal transduction pathways. The results of the present study using isolated glomeruli clarified that TNF- α activates NADPH oxidase via PKC/PI-3 kinase and MAPK pathways (Figure 8).

Phosphodiesterase (PDE) inhibitors express their pharmaceutical effects by suppressing PDE activities and increasing the levels of intracellular cAMP and cGMP, and widely distributed in biological tissues, Eleven PDE families have been identified thus far: PDE1 through PDE11. Cilostazol is a derivative of cilostamide, a selective inhibitor of PDE3. In vivo studies have shown that cilostazol exhibited a potent antiplatelet action and a low positive inotropic action, and as a result, cilostazol is considered more useful than cilostamide and is clinically used in the treatment of arteriosclerosis obliterans. Ieki conducted a study using rats with STZ-induced diabetes and documented that cilostazol suppressed albuminuria caused by diabetes and inhibited the loss of negatively charged particles in the glomerular basement membrane (Ieki, 1994). The involvement

of oxidative stress in the loss of negatively charged particles has been reported (Kashihara, 1992). Hence, the present study investigated the effects of cilostazol on glomerular ROS production mediated by NADPH oxidase. At a concentration of 1 μ M, cilostazol suppressed ROS production by approximately 48%. This suppressive effect on glomerular ROS production is comparable to that achieved by the levels of plasma cilostazol in normal clinical settings (Akiyama et al., 1985).

α -Tocopherol is the main vitamin E analogue that is a fat-soluble antioxidant to be consumed during oxidative modification of low density lipoproteins (Stocker, 1999). It has a chromanic nucleus, with an aliphatic side chain which can protect lipid structures from peroxidation (Munteanu et al, 2004). In addition, it has also been reported that α -tocopherol inhibits NADPH oxidase by suppressing PKC-mediated phosphorylation and translocation of p47-phox (Cachia et al., 1998). Based on a report of the efficacy of a α -tocopherol against diabetic nephropathy (Koya et al., 1997), we directly compared the efficacy of cilostazol and α -tocopherol in inhibiting ROS. The results showed that cilostazol has a strong efficacy in inhibiting ROS at clinical levels (100 nM~100 μ M, Figure 4) than α -tocopherol at physiologic plasma levels (about 25 μ M) (Munteanu et al, 2004).

PKC is a Ca^{2+} dependent enzyme, and Ca^{2+} is required for activation. On the other hand, an investigation using mesangial cells revealed a decrease in cytoplasmic Ca^{2+} and changes in dynamics due to PKC activation (Mene et al., 1997; Rong et al., 2001). Furthermore, interaction between cAMP and Ca^{2+} in various cells has been reported (Ahmed et al., 1995; Landa et al, 2005). From these findings, we inferred that Ca^{2+} is involved in NADPH oxidase activation. In this study, significant changes were not identified in the quantity of ROS produced by PMA stimulation or in

the effects of cilostazol on ROS inhibition, regardless of the presence or absence of Ca^{2+} in extracellular fluid (Figure 5). Based on this, as the mechanism of ROS inhibition by cilostazol, changes in Ca^{2+} following an increase in cAMP and the direct inhibition of PKC by cilostazol were negative.

Studies have been conducted using neutrophils to determine the mechanism of the suppressive effects of cAMP on NADPH oxidase, and Quilliam et al. reported that when rap1a, a GTP-binding protein, was phosphorylated by PKA, its affinity for cytochrome b558 lowered, thus inhibiting cytochrome b558 activation (Quilliam et al., 1991). In addition, Ahmed et al. reported that PKA suppressed PI-3 kinase activation (Ahmed et al., 1995). Using isolated glomeruli, we investigated the inhibition site due to cilostazol on NADPH oxidase suppression. The ROS inhibition effect of cilostazol is about 80%, while the inhibition rate of wortmannin is also about 80%. Further, neither of the inhibitors had an additive inhibition effect. The above results suggest the involvement of PI-3 kinase in the activation of NADPH oxidase in renal glomeruli, thus we inferred that the inhibition levels of wortmannin and cilostazol are extremely close further downstream than PKC (Figure 8). In other words, the results of the present study support the mechanism proposed by Ahmed et al. As is the case with neutrophils, the involvement of a PI-3 kinase-dependent signal transduction pathway with NADPH oxidase is suggested for glomerular ROS production, and PKA may suppress glomerular NADPH oxidase by hindering PI-3 kinase activation. However, cilostazol suppressed ROS production by approximately 20%, thus suggesting a signal transduction pathway that is independent of PI-3 kinase activation.

Hence, the suppressive effects of cilostazol on glomerular ROS production originating

from NADPH oxidase were comparable to those of wortmannin, a PI-3 kinase inhibitor. Because cilostazol is already being used as a therapeutic agent for arteriosclerosis obliterans, it appears to be a promising drug for suppressing cellular injuries caused by oxidative stress in diabetes patients in everyday clinical practice.

These findings suggest that PKC activation stimulates NADPH oxidase via a PI-3 kinase-dependent pathway (80%) and a PI-3 kinase independent pathway (20%) to induce ROS production; and that cilostazol inhibits PKC activity at the PI-3 kinase level to suppress NADPH oxidase activation in the presence of PMA or TNF- α . Therefore, PDE3 inhibitors may not only suppress platelet aggregation, hinder vascular smooth muscle cell proliferation and induce vasodilatation through PKA activation, but also suppress acceleration of oxidative stress in glomeruli by controlling the PI-3 kinase-dependent pathway of PKC, which is activated in the diabetic state, thus blocking the onset and progression of diabetic nephropathy and arteriosclerosis.

In conclusion, cilostazol arrests oxidative stress in glomeruli induced by PKC activation by inhibiting the PI-3 kinase-dependent pathway, and may thus prevent the development of diabetic nephropathy.

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Figure Legends

Fig 1. Typical chemiluminescence responses of isolated glomeruli by PMA (a) or TNF- α (b), and control (open circle). Glomeruli were preincubated for 15 min at 37°C before PMA (10 μ g/ml) or TNF- α (10 ng/ml) was added. (a) After addition of PMA, a rapid increase in chemiluminescence was observed. Peak chemiluminescence was measured. (b) After addition of TNF- α , low and continuous chemiluminescence response was observed. Chemiluminescence measurement was continued at 20 seconds intervals for 20 minutes.

Fig 2. Effects of DPI, H-7, wortmannin and SB203580 on PMA-stimulated glomerous ROS production. Glomeruli were preincubated for 15 min at 37°C before DPI (1 μ M), H-7 (100 μ M), wortmannin (100 μ M) or SB203580p38 (100 μ M) was added. PMA (10 μ g/ml) was added after a further 5 min. Peak chemiluminescence was determined. Data are expressed as mean $\pm$ SEM (N=7, * $p < 0.001$, ✕ $p < 0.01$ vs. PMA alone).

Fig 3. Effects of DPI, H-7, wortmannin and SB203580 on TNF- α -stimulated glomerous ROS production. Glomeruli were preincubated for 15 min at 37°C before DPI (1 μ M), H-7 (100 μ M), wortmannin (100 μ M) or SB203580 (100 μ M) was added. PMA (10 μ g/ml) was added after a further 5 min. Total chemiluminescence over 20 min was determined. Data are expressed as mean $\pm$ SEM (N=4, * $p < 0.01$, ✕ $p < 0.05$ vs. TNF- α alone).

Fig 4. Dose-dependent suppressive effects of cilostazol (closed circle) and α -tocopherol (open circle) on PMA-stimulated glomerous ROS production. Glomeruli were preincubated for 15 min at 37°C before cilostazol or α -tocopherol was added at a concentration range of 10 nM to 100 μ M. PMA (10 μ g/ml) was added after a further 5 min. Peak glomerous ROS production on stimulation by PMA (10 μ g/ml) was taken 100%. Data are expressed as mean $\pm$ SEM (N=7, *p<0.01 vs. α -tocopherol).

Fig 5. PMA-induced ROS generation and suppressive effects of cilostazol on ROS in the presence (open bar) or absence (hatched bar) of extracellular Ca^{2+} . Glomeruli were preincubated for 15 min at 37°C in HBSS or HBSS lacking Ca^{2+} , before cilostazol (100 μ M) was added. PMA (10 μ g/ml) was added after a further 5 min. Peak chemiluminescence was determined. Data are expressed as mean $\pm$ SEM (N=5).

Fig 6. Effects of cilostazol on PMA-stimulated glomerous ROS production. Glomeruli were preincubated for 15 min at 37°C before cilostazol (100 μ M), wortmannin (100 μ M) and/or cilostazol (100 μ M) was added. PMA (10 μ g/ml) was added after a further 5 min. Peak chemiluminescence was determined. Data are expressed as mean $\pm$ SEM (N=7, *p<0.01 vs. PMA alone).

Fig 7. Effects of cilostazol on TNF- α -stimulated glomerous ROS production. Glomeruli were preincubated for 15 min at 37°C before cilostazol (100 μ M), wortmannin (100 μ M) and/or

cilostazol (100 μ M) was added. TNF- α (10 ng/ml) was added after a further 5 min. Total chemiluminescence over 20 min was determined. Data are expressed as mean $\pm$ SEM (N=4, * $p < 0.05$ vs. TNF- α alone).

Fig 8. Possible signal transduction pathway to activate renal glomerular NADPH oxidase. PMA-induced activation of PKC activates NADPH oxidase mainly via a PI-3 kinase pathway and partly via a PI-3 kinase-independent pathway. TNF- α activates NADPH oxidase via both PKC- and MAPK-pathways. Cilostazol inhibits PMA- and TNF- α -induced generation of superoxide, probably by inhibiting the PI-3 kinase pathway. Abbreviations used are: GTP, guanosine 5'-triphosphate; MAP, mitogen-activated protein; phox, phagocyte oxidase; PMA, phorbol 12-myristate 13-acetate.