Inhibition of NADPH Oxidase Prevents Advanced Glycation End Product–Mediated Damage in Diabetic Nephropathy Through a Protein Kinase C-α–Dependent Pathway

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OBJECTIVE—Excessive production of reactive oxygen species (ROS) via NADPH oxidase has been implicated in the pathogenesis of diabetic nephropathy. Since NADPH oxidase activation is closely linked to other putative pathways, its interaction with changes in protein kinase C (PKC) and increased advanced glycation was examined.

RESEARCH DESIGN AND METHODS—Streptozotocin-induced diabetic or nondiabetic Sprague Dawley rats were followed for 32 weeks, with groups randomized to no treatment or the NADPH oxidase assembly inhibitor apocynin (15 mg · kg⁻¹ · day⁻¹; weeks 16–32). Complementary in vitro studies were performed in which primary rat mesangial cells, in the presence and absence of advanced glycation end products (AGEs)-BSA, were treated with either apocynin or the PKC-α inhibitor Ro-32-0432.

RESULTS—Apocynin attenuated diabetes-associated increases in albuminuria and glomerulosclerosis. Circulating, renal cytosolic, and skin collagen–associated AGE levels in diabetic rats were not reduced by apocynin. Diabetes-induced translocation of PKC, specifically PKC-α to renal membranes, was associated with increased NADPH-dependent superoxide production and elevated renal, serum, and urinary vascular endothelial growth factor (VEGF) concentrations. In both diabetic rodents and in AGE-treated mesangial cells, blockade of NADPH oxidase or PKC-α attenuated cytosolic superoxide and PKC activation and increased VEGF. Finally, renal extracellular matrix accumulation of fibronectin and collagen IV was decreased by apocynin.

CONCLUSIONS—in the context of these and previous findings by our group, we conclude that activation of NADPH oxidase via phosphorylation of PKC-α is downstream of the AGE–receptor for AGE interaction in diabetic renal disease and may provide a novel therapeutic target for diabetic nephropathy. Diabetes 57: 460–469, 2008

Diabetic nephropathy, as with other diabetic vascular complications, appears to be multifactorial in origin, involving a number of key pathways, including advanced glycation, activation of intracellular signaling molecules such as protein kinase C (PKC), and increased generation of reactive oxygen species (ROS) (1). However, the nature of the interactions among these various pathways remains uncertain.

While mitochondria are the predominant source of “accidental” oxidative stress in nondisease states during oxidative phosphorylation, cells deliberately use other sources of ROS, from the cytosol for host defense against pathogens or to induce cell signaling. This cytosolic ROS production can result from the activation of various enzymes, including NADPH oxidase, nitric oxide (NO) synthase, and myeloperoxidase, with increasing evidence that NADPH oxidase appears to be the major cytosolic source of ROS generation in diabetes (2). Classically, NADPH oxidase is composed of two membrane-associated components, p22phox and gp91phox and four major cytosolic components, p47phox, p40phox, p67phox, and rac-1/2. In addition, gp91phox has other homologues that are present within the kidney, namely nox-3, seen in fetal kidney, and nox-4, which is predominately expressed in the renal cortex (3). The receptor for advanced glycation end products (RAGE) is a pattern recognition inflammatory receptor, which engages a number of ligands, including advanced glycation end products (AGEs). AGEs are formed when certain, free amino groups on proteins become permanently modified by reducing glucose, facilitated in diabetes by hyperglycemia and oxidative stress (4). Both the accumulation of AGEs (5–7) and RAGE (8,9) expression play important roles in the pathogenesis of diabetic nephropathy. In addition, engagement of RAGE by AGEs induces signal transduction and generates cellular oxidative stress via activation of NADPH oxidase in endothelial cells (10). Specific upregulation of the subunits of NADPH oxidase, including p47phox (11), nox-4 (12), and p22phox (13) have been demonstrated in diabetes within the kidney. In addition, the inhibitor of NADPH oxidase, apocynin, improves renal function in streptozotocin-induced diabetic rats, albeit over the relatively short period of 4 weeks (3). This agent’s postulated mode of action is to impede the
recruitment of the p47phox and p67phox subunits to the NADPH oxidase complex (14,15).

The cytosolic generation of ROS has been demonstrated in vitro through activation of RAGE in both proximal tubular and mesangial cells in association with specific activation of NADPH oxidase (16,17). Furthermore, the AGE-RAGE interaction enhances production of the cyto-
kine vascular endothelial growth factor (VEGF) (9), which is postulated to be directly induced by NADPH oxidase and is linked to the pathogenesis of albuminuria in diabe-
tes (18).

Therefore, this study initially aimed to test the utility of the NADPH oxidase inhibitor apocynin in established experimental diabetic nephropathy. This series of experi-
ments addressed AGE-RAGE–induced activation of NADPH oxidase via PKC-α phosphorylation and its down-
stream signaling events, including generation of cytosolic ROS, induction of VEGF, and accumulation of basement membrane proteins. Based on these in vivo findings, a series of complementary in vitro studies were performed that further examined the sequence of intracellular events resulting from the AGE-RAGE interaction.

RESEARCH DESIGN AND METHODS

Experimental animal model. Experimental diabetes was induced in male Sprague Dawley rats (200–250 g) by injection of the β-cell toxin streptozocin (50 mg/kg) following an overnight fast. Animals with plasma glucose concentrations in excess of 15 mmol/l, 1 week postinduction of diabetes, were included in the study. Sham-injected control animals (sodium citrate buffer, pH 4.5) were followed concurrently. Diabetic and control animals were randomized into groups (12) that received NADPH oxidase inhibitor apocynin (15 mg/kg 4′-hydroxy-3′-
methoxy-acetophenone; Merck, Whitehouse Station, NJ) by oral gavage from week 16 to 32 (groups C/Apo and D/Apo) as a late intervention. Delayed intervention was used (19) to assess the role of such a therapeutic approach with animals that already had evidence of renal disease, albeit not very advanced. It was considered that this strategy was analogous to the human context, in which treatment is generally started in diabetic subjects once they already have some features of diabetic nephropathy such as microalbumi-
numia. Two to three units of Ultralente insulin (Ultratard HM; Novo Industries, Bagsvaerd, Denmark) were administered daily to diabetic animals to prevent ketoacidosis and improve survival. Body weight, mean systolic blood pressure (13), mean glucose (25 mmol/l) concentrations in 2% fetal bovine serum with appropriate treatments and incubated for 7 days. AGE-modified bovine serum albumin (AGE-BSA) was added to some groups at 100 μg/ml as previously described (24). Endotoxin was removed from AGE-BSA preparations by Detoxigel columns (Pierce Biotechnology, Rockford, IL). Endotoxin content of subse-
quent eluates was assessed via Limulus amoebocyte lysate validity testing (AMS Laboratories, Sydney, Australia). There was no detectable endotoxin within the filtered samples. Cells were harvested as outlined below. Ro-32-0432 (Calbiochem, Darmstadt, Germany) is a selective cell permeable PKC inhibitor, which displays a 10-fold greater selectivity for PKC-α over other PKC isoforms and was used at a concentration of 10 mmol/l. Apocynin was used at a concentration of 1 mmol/l glucose (25 mmol/l) was used as an osmotic control.

Histological assessment of kidney injury. Kidney sections were stained with periodic acid Schiff’s stain for quantitation of glomerulosclerosis. The degree of glomerulosclerosis, which was defined as thickening of the base-
membrane and mesangial expansion, was evaluated by a semiquantitative method as described previously (19). In brief, 2 μm kidney sections were stained with periodic acid Schiff’s stain and observed under a light microscope in a masked fashion at a magnification of ×400 using the Analysis Imaging System (Imaging Research, St. Catharines, ON, Canada). Forty glomeruli in each kidney were graded in accordance with their severity of glomerular damage (0, normal; 1, slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion involving <25% of the glomerulus; 2, sclerosis of 26–50%; 3, sclerosis of 51–75% and 4, sclerosis of >75% of the glomerulus). The glomerulosclerotic indexes were calculated using the following formula: glomerulosclerotic index = (1 × n1) + (2 × n2) + (3 × n3) + (4 × n4) / n, where n is number of glomeruli in each grade of glomerulosclerosis (19,25).

TABLE 1

Physiological and structural parameters for all rodents at week 32

|                          | Control (n = 10/group) | Diabetic (n = 10/group) |
|--------------------------|------------------------|-------------------------|
|                          | Untreated              | Apocynin                | Untreated              | Apocynin                |
| Plasma glucose mmol/l    | 6.9 ± 0.9              | 6.2 ± 0.5               | 33.2 ± 2.7*            | 32.6 ± 3.2*             |
| Glycated hemoglobin (%)  | 5.6 ± 0.6              | 4.9 ± 0.8               | 18.1 ± 2.6*            | 16.7 ± 1.8*             |
| Body weight (g)          | 707 ± 67               | 725 ± 54                | 421 ± 32*              | 424 ± 33*               |
| Albumin excretion rate (mg/24 h) (geometric mean ×/± tolerance factor) | 11.9 ×/± 1.4 | 20.7 ×/± 1.1 | 70.3 ×/± 1.3* | 32.8 ×/± 1.4† |
| Mean systolic blood pressure (mmHg) | 114 ± 8               | 119 ± 9                 | 134 ± 13*              | 124 ± 7                 |
| Glomerular filtration rate (ml · min⁻¹ · g body wt⁻¹) | 6.1 ± 1.1              | 7.1 ± 1.3               | 11.2 ± 1.4*            | 12.8 ± 1.0*             |
| Glomerulosclerotic index | 1.2 ± 0.1               | 1.1 ± 0.1               | 1.9 ± 0.3*             | 1.5 ± 0.1†              |
| Tubulointerstitial area (%) | 4.3 ± 1.1              | 5.5 ± 1.4               | 14.8 ± 2.0*            | 7.0 ± 2.2†              |

Data are means ± SD, unless otherwise indicated. *P < 0.001 vs. control group; †P < 0.001 vs. diabetic group.
phosphatase inhibitors (Sigma, St. Louis, MO). The homogenates were centrifuged at 1,000g for 10 min. These supernatants were then centrifuged at 10,000g at 4°C for 20 min. The supernatants were then ultracentrifuged at 100,000g for 1 h at 4°C. This supernatant was then resuspended as the cytosolic fraction, and the pellet was resuspended in buffer B (buffer A with 1% Triton X-100) and ultracentrifuged at 100,000g for 1 h at 4°C. This supernatant was then resuspended as the membranous fraction (28). Membrane and cytosolic fractions were assayed for PKC activity using the StressXpress PKC Kinase Activity Assay Kit (EKS-420A, Stressgen Bioreagents, Victoria, BC, Canada) as described previously (27).

Western immunoblotting. Membrane fractions prepared for the PKC activity assay above were used for Western immunoblotting. Mesangial cells were sonicated and spun at 400g for 5 min. The supernatant was collected and the protein content was measured using the BCA protein assay (Perbio Science UK, Cheshire, U.K.).

For Western blotting, 5 μg of membranous samples and 15 μg of mesangial cell supernatants were incubated at 95°C for 3 min and separated on an 8% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Non-specific binding sites were blocked for 1 h with 5% (wt/vol) nonfat milk powder in TBS followed by overnight incubation of primary antibodies, rabbit anti-PKCα (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho PKCα (1:1,000; Upstate, Lake Placid, NY), and mouse monoclonal β-actin (1:5,000; Abcam, Cambridge, U.K.). Bound antibodies were amplified and detected by reaction with an Amersham ECL kit (Amersham Biosciences, Piscataway, NJ). The band intensity was quantified using a microcomputer imaging device and expressed as (density times area) minus background.

VEGF ELISA. The QuantiKine Mouse ELISA kit (R&D Systems, Minneapolis, MN) was used to measure VEGF, in an aliquot of urine from the 24-h collection and serum. The cytosolic and membranous kidney samples obtained for the PKC activity assay described above were also used for the measurement of VEGF, as was the media collected from the culture of mesangial cells, which were concentrated before quantitation. The values of urinary and serum VEGF are expressed as picograms per millimole of creatinine as determined by an autoanalyzer (Alfred Hospital, Melbourne, Australia) (27).

Immunohistochemistry. Immunohistochemistry was performed as previously described (19). In brief, slides for fibronectin and collagen IV were digested with 0.4% pepsin for 4 min at 37°C. Slides for p47phox required no pretreatment. The primary antibodies used were rabbit anti-human fibronectin (1:4,000; DakoCytomation, Carpentaria, CA), goat anti-human collagen IV (1:500; Southern Biotech, Birmingham, AL), and rabbit anti-p47phox (1:4,000; Upstate). Quantitation of renal cortical immunohistochemistry was performed by computer-aided densitometry (Optimus 6.5; Media Cybernetics, Silver Spring, MD), as previously described (19).

ROS production in primary mesangial cells. Hydrogen peroxide production in primary rat mesangial cells was determined using CM-DH2DCFDA (5',6'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) (Molecular Probes, Eugene, OR). Mesangial cells were seeded onto 12-well plates at a concentration of 5 × 10^5 per well in 10% serum media and exposed to 10 mg/ml AGE-BSA or BSA with or without 1 μmol/l apocynin. After incubation for 3 days, serum-starved cells were exposed to 5 μmol/l CM-DH2DCFDA for 10 min at 37°C. After 10 min, the cells were washed twice with serum-free media and images were collected using a Zeiss LSM 510 META confocal microscope (23).

Flow cytometric analysis of surface RAGE expression on primary mesangial cells. Primary mesangial cells were grown for 7 days as outlined above in the cell culture section. Mesangial cells were washed in sterile PBS and centrifuged 1,000 rpm for 5 min and counted in a hemocytometer. A total of 5 × 10^5 cells were stained in each tube with 15 μl of the primary antibody, goat anti-RAGE (Santa Cruz Biotechnology), in the dark for 30 min. Cells were then washed with excess buffer (PBS with 2.5% fetal bovine serum and 0.01% NaN3), and fluorescein isothiocyanate–conjugated rabbit anti-goat IgG, F(ab')2 (1:100, Chemicon International, Temecula, CA), was added as the secondary antibody. Following a second 30-min incubation in the dark, the cells were washed again in excess buffer. A minimum of 10,000 cells were collected on FACScalibur (BD Biosciences, Lane Cove, NSW, Australia) and the data analyzed using WinMDI 2.8. RAGE-positive cells were identified relative to the appropriate isotopic controls.

Statistical analysis. Results are expressed as means ± SD, unless otherwise specified. Data for albuminuria were not normally distributed and were therefore analyzed following logarithmic transformation and are expressed as geometric mean ± tolerance factors. Analyses were performed by ANOVA followed by post hoc analysis using Tukey’s test. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Physiological and structural parameters. Diabetic rodents demonstrated a significant elevation in plasma glucose concentrations and glycated hemoglobin, which was unaffected by apocynin treatment (Table 1). Diabetes was also associated with decreased body weight. The 24-h albumin excretion rate was increased with diabetes and reduced significantly by apocynin therapy (Table 1). Although diabetes induced increases in albuminuria at week 16 (C group 1.7 ± 1.5 mg/24 h; D group 14.1 ± 2.3 mg/24 h; P < 0.01 with geometric mean ± tolerance factors shown), there were no differences seen between the untreated D and DApo groups at that time (DApo group, 13.1 ± 2.1 mg/24 h; P > 0.05 vs. D group). Thus
there were no differences in albumin excretion rate among the diabetic groups before the commencement of treatment at week 16. Mean systolic blood pressure was increased with diabetes, but no statistically significant change was noted with apocynin treatment. Glomerular filtration rate corrected for body weight was increased with diabetes, which was not attenuated with apocynin treatment (Table 1). The glomerulosclerotic index and tubulointerstitial area were increased with diabetes, and both markers of renal structural injury were attenuated by apocynin treatment (Table 1).

**The accumulation of tissue and circulating AGEs are unaffected by NADPH oxidase.** Circulating levels of the nonfluorescent AGE and CML were increased significantly with diabetes but were not affected by apocynin treatment (Fig. 1A). In addition, cytosolic CML levels in renal cortices from diabetic rats were also elevated (Fig. 1B), but no changes were seen with apocynin therapy (Fig. 1B). Furthermore, diabetic rats had increased concentrations of skin collagen–associated CML, and, again, this parameter was not affected by apocynin treatment (Fig. 1C).

**Renal cytosolic superoxide generation and activation of PKC-α are prevented by blockade of NADPH oxidase.** Lucigenin-enhanced superoxide generation by NADPH oxidase in the cytosol of renal cortices was increased by 300% in diabetic rats and totally prevented by apocynin treatment (Fig. 2A). Immunohistochemical staining of the NADPH oxidase isoform, p47phox, in kidney glomeruli of 32-week-old rats (D–F). A and D represent control kidney; B and E represent diabetic kidney; D and F represent diabetic plus apocynin-treated kidney (×400 magnification).
There was no change in the activity of PKC within the cytosol among the various groups (Fig. 3B). The ratio of phosphorylated PKC-α to total PKC-α was also increased in renal cortical membranes from diabetic rats (Fig. 3C and D), and this was normalized in the apocynin group. The PKC-α-dependent cytokine VEGF is decreased following therapy to reduce NADPH oxidase activity. Diabetes was associated with significant elevations in urinary (Fig. 4A) and serum (Fig. 4B) VEGF concentrations after 32 weeks. These diabetes-induced increases in urine and serum VEGF were ameliorated by apocynin therapy. Membrane, but not cytosolic, fractions from diabetic renal cortices also demonstrated elevations in VEGF (Fig. 4C and D) when compared with the appropriate control groups. Apocynin therapy attenuated the increase in membranous VEGF seen in diabetic kidneys. Diabetes-induced increases in extracellular matrix proteins are not seen in diabetic rats treated with an NADPH oxidase inhibitor. The expression of renal cortical fibronectin was significantly increased in diabetic kidneys and reduced by apocynin (Fig. 5A and C [A–C]). In addition, renal cortical expression of collagen IV was increased almost twofold with diabetes and was attenuated by apocynin (Fig. 5B and C [D–F]).

Primary mesangial cells exposed to AGEs generate cytosolic ROS via an NADPH oxidase and PKC-α-dependent pathway. Previous studies by our group showed that AGE blockade reduced VEGF expression via PKC-α (6), and in this study NADPH blockade also appeared to affect this signaling molecule in a similar manner. However, since NADPH oxidase blockade does not influence AGEs it was postulated that NADPH oxidase activation, as had been suggested in other studies, is downstream of the AGE/RAGE interaction. Therefore, in vitro studies with mesangial cells were performed to further characterize this pathway.

The ratio of phosphorylated PKC-α to total PKC-α in primary mesangial cells exposed to high glucose in the presence of AGE-BSA was elevated compared with controls (Fig. 6A) and BSA exposed cells (data not shown) ($P = 0.006$). Both apocynin and Ro-32-0432 significantly reduced this AGE-induced elevation in the phosphorylated PKC-α-to–PKC-α ratio. These results are consistent with those seen with respect to activity of PKC. Specifically, PKC activity was increased by concomitant AGE exposure and attenuated by both apocynin and Ro-32-0432 (Fig. 6B).

The concentration of VEGF secreted into the cell culture media was increased by incubation with AGE-BSA when compared with controls (Fig. 6C). Furthermore, these AGE-induced elevations were ameliorated by treatment with either apocynin or Ro-32-0432 (Fig. 6C). Cell surface expression of RAGE in cells exposed to high glucose in the presence of AGE-BSA was increased when compared with control cells. This increase in RAGE expression was ameliorated by treatment with either apocynin or Ro-32-0432 (Fig. 6D). Cytosolic ROS was higher in primary mesangial cells that were treated with AGE-BSA for 7 days when compared with treatment with BSA alone.
Both blockade of NADPH oxidase with apocynin or inhibition of PKC-α activity with Ro-32-0432 ameliorated the elevations in cytosolic ROS generated by AGE exposure (Fig. 6E).

DISCUSSION

This study extends the therapeutic potential of blockade of NADPH oxidase in experimental diabetic nephropathy. Previously, Asaba et al. (29) have shown that apocynin reduced proteinuria and retarded mesangial expansion in an experimental study over the period of 4 and 8 weeks. In the present study, more prolonged apocynin administration was commenced (from week 16) once early renal changes, such as microalbuminuria, were already evident. This was associated with similar renal functional and structural benefits (Table 1, Fig. 2 [A–C]), with apocynin specifically reducing diabetes-associated albuminuria, glomerulosclerosis, and tubulointerstitial injury. Blockade of NADPH oxidase to prevent progressive diabetic nephropathy, as assessed in the current study design, has important clinical implications, since renoprotective therapies in humans are not generally administered at the time of diagnosis of diabetes but usually after some renal dysfunction, such as microalbuminuria, is already evident. Although apocynin is not suitable for human use, the present study suggests that blockade of NADPH oxidase is a valid intervention to consider for combating established diabetic nephropathy. Furthermore, to our knowledge this is the first study to look at the long-term effect of apocynin administration, since previous studies have only been performed for up to 8 weeks’ duration. This therapeutic strategy warrants further investigation, since other novel compounds other than apocynin that block NADPH oxidase with as-yet unknown toxicity profiles in humans are currently in clinical development and could ultimately have a role in human diabetic nephropathy. Furthermore, with increasing availability of animals where Nox isoforms (30) have been specifically targeted, it is now feasible to consider mechanistic studies in such models.

In the present in vivo study, we have shown an increase in renal cytosolic generation of superoxide, which is ameliorated by apocynin, indicating a role for NADPH oxidase. This was seen in the context of increased glomerular expression of the NADPH oxidase subunit p47phox in diabetic kidneys, which was ameliorated by treatment with apocynin as well as translocation of phosphorylated PKC-α to diabetic renal membranes in association with PKC activation. Indeed, previous studies in rodent aortas have shown that vascular superoxide is normalized by a PKC inhibitor and that the major source of superoxide was indeed NADPH oxidase (31). Interestingly, our results indicate that accumulation of both tissue and circulating AGEs are unaffected by NADPH oxidase in this model, as apocynin did not attenuate the diabetes-induced increases in these parameters. The expression of RAGE, although increased by experimental diabetes or AGE-BSA in vitro, was ameliorated by blockade of NADPH oxidase or PKC-α. Furthermore, our in vitro studies in primary mesangial cells showed that cellular ROS generation was induced by exposure to AGE-BSA and was ameliorated with apocynin or blockade of PKC-α.

Previously, it has been suggested that phosphorylation of the NADPH oxidase subunit p47phox is required for assembly of this complex. In the present study, there is increased expression of renal glomerular p47phox seen with diabetes, which was ameliorated with apocynin. In neutrophils, NADPH oxidase is activated via PKC-mediated phosphorylation of the p47phox subunit (32). In vivo, renal translocation and activation of a number of PKC isoforms, including PKC-α, have been reported in various
models of experimental diabetic nephropathy (33–35). In addition, mice with a genetic deletion of PKC-α are protected against the development of albuminuria and overt diabetic nephropathy (36). We have previously shown that AGE-RAGE interactions induce PKC-α in diabetic rats (6). In addition, in the present study, PKC-α inhibition normalized AGE-induced cellular ROS generation. However, it should be noted that increases in the ratio of phosphorylated to total PKC-α were ameliorated by NADPH oxidase inhibition in vivo, and these findings were confirmed in vitro with NADPH oxidase inhibition attenuating mesangial cell increases in PKC-α phosphorylation induced by AGEs. It is important to note that sustained activation of PKC is also mediated by ROS (37). Therefore, the effects of apocynin on PKC activity and phosphorylated PKC-α expression may be via the inhibition of renal cellular ROS generation, in an ongoing feedback loop involving phosphorylation of p47^phox.

PKC-α is a key regulator of VEGF expression, particularly in the setting of hyperglycemia, since diabetes in PKC-α–deficient mice does not induce increased renal VEGF expression (36). Furthermore, our own group has shown that decreasing PKC-α activation with the AGE-lowering therapy alagebrium (ALT-711) results in decreased VEGF expression (6). Indeed, we postulated in that study and confirmed in vascular smooth muscle cells in vitro that alagebrium is, in fact, a direct inhibitor of the activity of PKC-α. Therefore, it is not surprising in the present study that accumulation of AGEs appears to be upstream of PKC activation. This is further supported by our in vitro studies in which blockade of either NADPH oxidase or PKC-α resulted in less secreted VEGF in the

FIG. 5. Morphometric determination of immunohistochemistry of extracellular matrix proteins (n = 10/group). A: Computer-aided analysis of renal glomerular fibronectin at 32 weeks expressed as percent area. B: Computer-aided analysis of renal glomerular collagen IV at 32 weeks expressed as percent area. C: Representative photomicrographs of fibronectin (A–C) and collagen IV (D–F) immunostaining. A and B represent control kidney; B and E represent diabetic kidney; C and F represent diabetic plus apocynin kidney (×200 magnification). *P < 0.001 vs. control group; †P < 0.01 vs. diabetic group; ‡P < 0.001 vs. diabetic group. (A high-quality digital representation of this image can be found at http://dx.doi.org/10.2337/db07-1119.)
cell culture medium of AGE-treated primary mesangial cells. Furthermore, these studies confirm that the significant increases in VEGF in urine, serum, and renal membranes of diabetic rats are ameliorated by NADPH oxidase blockade. Although albuminuria in diabetes is likely to occur as a result of a number of pathways, VEGF is increasingly considered to play an important role. Indeed, neutralizing antibodies to VEGF have been shown to decrease albuminuria in both short- and long-term diabetes (8,38), and, more recently, a tyrosine kinase inhibitor that targets the VEGF receptor has been shown to confer similar benefits on albuminuria (39). Therefore, it is possible that the antialbuminuric effect of apocynin is related, at least in part, to reduced renal VEGF expression.

A number of studies have implicated AGE accumulation and PKC activation in diabetes complications (35) by identifying a link between these two pathways as well as being critical in downstream expression of extracellular matrix proteins. Indeed, fibronectin deposition is known to result from activation of either pathway (6,40,41). Within the current study, inhibition of NADPH oxidase with subsequent reductions in cytosolic superoxide and PKC-α activation ameliorated the diabetes-associated increases in renal fibronectin expression. Collagen IV is another extracellular matrix protein influenced by PKC-dependent pathways (42,43) and is also linked to increased ROS generation (44,45). Furthermore, Xia et al. (46) reported that mesangial cell ROS generation by

FIG. 6. AGE-mediated effects on cultured primary mesangial cells (n = 6/group). Experiments were performed for 7 days in 25 mmol/l glucose in the presence and absence of AGE-BSA (100 μg/ml). APO, apocynin (NADPH oxidase inhibitor 1 μmol/l; Ro-32-0432 [PKC-α inhibitor 10 nmol/l]); HG, high glucose. A: Phosphorylated PKC-α-to–total PKC-α ratio. B: Cellular PKC activity by ELISA. C: Secreted VEGF in concentrated cell culture media. D: Flow cytometric analysis of cell surface RAGE. E: Cytosolic ROS generation with the fluorophore CM-H_2DCFDA. *P < 0.05 vs. HG; †P < 0.001 vs. HG + AGE; ‡P < 0.01 vs. HG; §P < 0.01 vs. HG + AGE; ††P < 0.001 vs. HG. **P < 0.001 vs. HG + AGE; ††P < 0.001 vs. HG.
HYPERGLYCAEMIA

? AGEs

PKC α

? ROS

NADPH oxidase

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FIG. 7. Speculative schema for the involvement of NADPH oxidase in progressive diabetic nephropathy.

NADPH oxidase in high glucose is dependent on conventional PKC-α and -β, with these isoforms also required for the increased expression of collagen IV (9,46). In the present study, the increased expression of collagen IV, as well as expansion of the tubulointerstitium in diabetic kidneys, was also reduced by blockade of NADPH oxidase, an effect that has been previously reported by our group following AGE-lowering therapy with aminoguanidine (6) and aminoguanidine (47). In addition, previous in vitro studies (40,48) demonstrated that blockade of NADPH oxidase in various renal cell types leads to reduced expression of extracellular matrix proteins such as fibronectin.

This series of experiments has investigated the efficacy of therapies that interfere with NADPH oxidase-dependent ROS generation, which is thought to result from AGE-RAGE interactions in the diabetic kidney. Although the accumulation of AGEs remained unaffected by NAPDH oxidase blockade, important downstream pathways implicated in the pathogenesis of diabetic nephropathy, namely RAGE expression, cytosolic ROS generation, PKC activation, VEGF secretion, and extracellular matrix accumulation, were normalized by this intervention (Fig. 7). Nevertheless, one cannot exclude that other effects of Nox inhibition, including systemic effects such as improvement in endothelial function, may also participate in conferring end-organ protection (49). With increasing elucidation of the key interactions between NADPH oxidase and the critical pathways implicated in the progression of diabetic nephropathy, it appears likely that this enzyme is an appropriate therapeutic target to investigate further in human diabetic nephropathy.
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