

Reactive Oxygen Species-Regulated Signaling Pathways in Diabetic Nephropathy

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Abstract. Diabetic nephropathy is characterized by excessive deposition of extracellular matrix (ECM) in the kidney. TGF- β 1 has been identified as the key mediator of ECM accumulation in diabetic kidney. High glucose induces TGF- β 1 in glomerular mesangial and tubular epithelial cells and in diabetic kidney. Antioxidants inhibit high glucose-induced TGF- β 1 and ECM expression in glomerular mesangial and tubular epithelial cells and ameliorate features of diabetic nephropathy, suggesting that oxidative stress plays an important role in diabetic renal injury. High glucose induces intracellular reactive oxygen species (ROS) in mesangial and tubular epithelial cells. High glucose-induced ROS in mesangial cells can be effectively blocked by inhibition of protein kinase C (PKC), NADPH oxidase, and mitochondrial electron transfer chain complex I, suggesting that PKC, NADPH oxidase, and

mitochondrial metabolism all play a role in high glucose-induced ROS generation. Advanced glycation end products, TGF- β 1, and angiotensin II can also induce ROS generation and may amplify high glucose-activated signaling in diabetic kidney. Both high glucose and ROS activate signal transduction cascade (PKC, mitogen-activated protein kinases, and janus kinase/signal transducers and activators of transcription) and transcription factors (nuclear factor- κ B, activated protein-1, and specificity protein 1) and upregulate TGF- β 1 and ECM genes and proteins. These observations suggest that ROS act as intracellular messengers and integral glucose signaling molecules in diabetic kidney. Future studies elucidating various other target molecules activated by ROS in renal cells cultured under high glucose or in diabetic kidney will allow a better understanding of the final cellular responses to high glucose.

Diabetic nephropathy is characterized by excessive deposition of extracellular matrix (ECM) in the kidney, leading to glomerular mesangial expansion and tubulointerstitial fibrosis. Clinical studies (1,2) have demonstrated that high blood glucose is the main determinant of initiation and progression of diabetic vascular complications including nephropathy. High glucose induces reactive oxygen species (ROS) (3,4) and upregulates TGF- β 1 (5,6) and ECM (5–7) expression in the glomerular mesangial cells. Exogenous hydrogen peroxide (H_2O_2) or H_2O_2 continuously generated by glucose oxidase (GO) also upregulates TGF- β 1 and fibronectin expression in mesangial cells (4,8) and fibronectin in tubular epithelial cells (9). Finally, antioxidants effectively inhibit high glucose- and H_2O_2 -induced TGF- β 1 and fibronectin upregulation (4,8,9–11), thus providing evidence that ROS play an important role in high glucose-induced renal injury. ROS-regulated signaling pathways leading to final renal cellular responses in diabetic kidney are not entirely clear, however.

In this article, we review the mechanisms of high glucose-induced ROS generation and ROS-induced activation of signal

transduction cascade and transcription factors and overexpression of genes and proteins in glomerular mesangial and tubular epithelial cells leading to ECM accumulation in diabetic kidney. We also briefly comment on the mechanisms of ROS generation by advanced glycation end products (AGE), TGF- β 1, and angiotensin II (Ang II), which may amplify high glucose-activated signaling in diabetic kidney.

Mechanisms of High Glucose-Induced ROS Generation

We have demonstrated that high glucose induces dichlorofluorescein (DCF)-sensitive ROS in the glomerular mesangial (3) and tubular epithelial (12) cells in a time-dependent manner. 3-O-methyl-D-glucose or L-glucose in the same concentrations as D-glucose failed to induce intracellular ROS in mesangial cells, and cytochalasin B, an inhibitor of glucose transporter, completely inhibited D-glucose-induced intracellular ROS, suggesting that high glucose-induced intracellular ROS depends on glucose uptake and metabolism in mesangial cells (3).

High glucose-induced ROS generation in mesangial cells is effectively blocked by calphostin C, a protein kinase C (PKC) inhibitor, or by depletion of intracellular PKC by preincubating cells with PMA for 24 h (3), suggesting that high glucose-induced ROS generation in mesangial cells is PKC dependent as in aortic smooth muscle and endothelial cells (13). PKC has been shown to play an important role in the activation of both phagocytic and nonphagocytic NADPH oxidase (13–15). NADPH oxidase is accepted as the most important mechanism for receptor-stimulated ROS generation in both phagocytic and

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nonphagocytic cells (16,17). However, Nishikawa *et al.* (18) demonstrated that high glucose generates superoxide anion in bovine aortic endothelial cells through mitochondrial metabolism. By normalizing mitochondrial superoxide overproduction, they were able to inhibit the activation of PKC, AGE formation, and increased aldose reductase pathway, suggesting that ROS produced by mitochondria play a critical role in high glucose-induced diabetic vascular complications (18). We observed that NADPH oxidase inhibitors apocynin and diphenylene iodonium (DPI), and an inhibitor of mitochondrial electron transfer chain complex I, rotenone, effectively block high glucose-induced ROS generation in mesangial cells (Figure 1A) and high glucose-induced fibronectin secretion by tubular epithelial cells (Figure 1B), suggesting that both NADPH oxidase system and mitochondrial metabolism are involved. The relative contribution of PKC, NADPH oxidase, and mitochondrial metabolism to high glucose-induced ROS is unknown.

High glucose also induces intracellular ROS indirectly through AGE and cytokines, including TGF- β 1 and Ang II. AGE increase intracellular ROS in neonatal mesangial (19) and

proximal tubular epithelial cells (20). In human umbilical vein endothelial cells, AGE/receptor for AGE-mediated generation of ROS is NADPH oxidase dependent (21). AGE-induced activation of nuclear factor- κ B (NF- κ B), PKC, and TGF- β 1 in mesangial cells is effectively inhibited by antioxidants (22). TGF- β 1 increases intracellular ROS in mesangial (Figure 2A) and tubular epithelial (9) cells. TGF- β 1 upregulates the expression of p22^{phox}, p47^{phox}, p67^{phox}, and gp91^{phox} in rat mesangial cells (Figure 2B) and p22^{phox} mRNA in tubular epithelial cells (Figure 2C), suggesting that TGF- β 1-induced ROS may be NADPH oxidase dependent. Ang II also induces ROS in glomerular mesangial (23) and tubular epithelial (12) cells. Ang II-induced ROS in vascular smooth muscle cells were shown to be derived from NADPH oxidase (24). ROS induce AGE (18) and upregulate TGF- β 1 (4,8), both of which, in turn, induce ROS generation in mesangial cells, thus providing an amplification loop in high glucose signaling in diabetic kidney (Figure 3).

ROS Activate Signal Transduction Cascade

Studer *et al.* (10) showed that high glucose-induced PKC activation in mesangial cells is effectively blocked by antioxidants vitamin E, N-acetyl cysteine (NAC), and taurine, suggesting that ROS mediate high glucose-induced PKC activation. We (25) demonstrated that antioxidant taurine effectively inhibits membrane translocation of PKC δ and PKC ϵ in streptozotocin-induced diabetic rat glomeruli, suggesting that ROS activate PKC in diabetic kidney as well.

Dunlop *et al.* (26) demonstrated significant activation of p38 mitogen-activated protein kinase (MAPK) in streptozotocin-induced diabetic rat glomeruli, whereas, in control glomeruli, H₂O₂ activated p38 MAPK. Wilmer *et al.* (27) showed that high glucose-induced activation of P38 MAPK in mesangial cells is effectively blocked by NAC or DPI. These studies suggest ROS-dependent activation of p38 MAPK in mesangial cells cultured under high glucose and in diabetic kidney. We observed that antioxidant lithospermate B (28,29) significantly inhibits p42/p44 MAPK and P38 MAPK activation in streptozotocin-induced diabetic rat renal cortex.

Wang *et al.* (30) observed that high glucose activates Janus kinase/signal transducers and activators of transcription (JAK/STAT) in mesangial cells and that high glucose-induced TGF- β 1 and fibronectin secretion is effectively inhibited by inhibition of JAK2 and STAT1. Although ROS have been shown to regulate JAK/STAT in both fibroblasts and A-431 carcinoma cells (31), it is not known whether ROS regulate JAK/STAT in mesangial cells or in diabetic kidney. These observations suggest that ROS mediate high glucose-induced activation of PKC, MAPK, and possibly JAK/STAT in diabetic kidney.

ROS Activate Transcription Factors

NF- κ B and activated protein-1 (AP-1) are ubiquitous transcription factors that are activated by ROS (32). We have demonstrated that high glucose and exogenous H₂O₂ activate NF- κ B (3) and AP-1 (4) in mesangial cells. NF- κ B activation is required to upregulate monocyte chemotactic protein-1 ex-

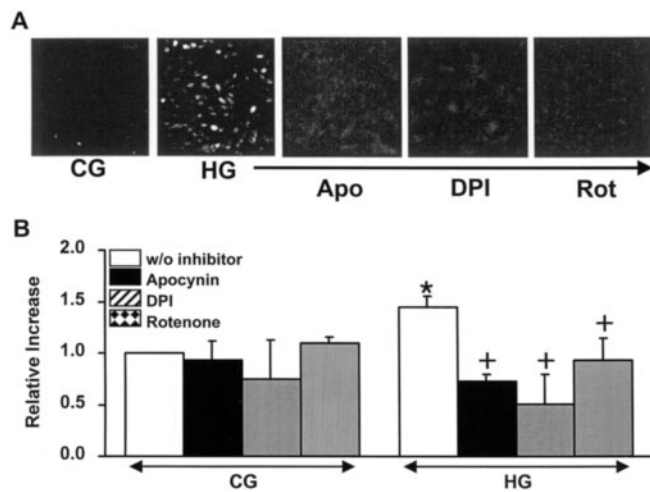


Figure 1. NADPH oxidase inhibitors apocynin and DPI and an inhibitor of mitochondrial electron transfer chain complex I, rotenone, block high glucose-induced dichlorofluorescein-sensitive ROS in mesangial cells (A) and high glucose-induced fibronectin secretion by tubular epithelial cells (B). (A) Synchronized quiescent mesangial cells grown on cover glass were incubated with 5.6 (control glucose [CG]) or 30 mM glucose (high glucose [HG]) for 1 hr in the presence or absence of apocynin (100 μ M), DPI (100 nM), and rotenone (10 nM), washed with Dulbecco's PBS and incubated in the dark for 20 min containing 5 mM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate. Culture dishes were transferred to Leica DM IRB/E inverted microscope, equipped with 20 \times Fluotar objective and Leica TCS NT confocal attachment and the ROS generation was visualized. (B) Synchronized quiescent LLC-PK1 cells were incubated with CG or HG for 48 h in the presence or absence of apocynin (100 μ M), DPI (100 nM), and rotenone (10 nM). Fibronectin secreted into the media was measured by Western blot analysis after electrophoresis under reducing condition. * $P < 0.05$ versus control glucose without inhibitor; + $P < 0.05$ versus high glucose without inhibitor.

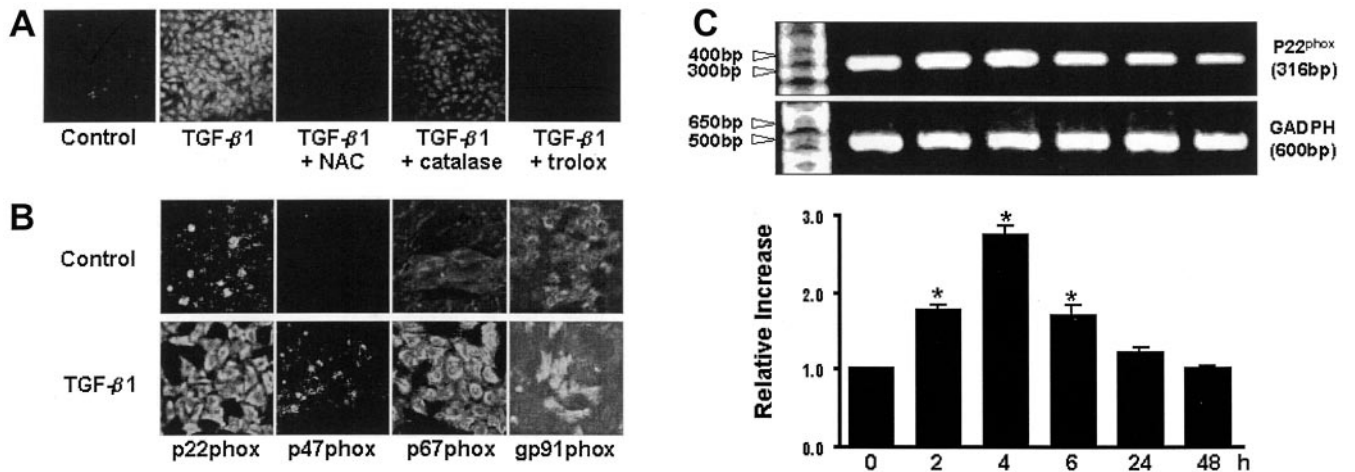


Figure 2. TGF- β 1 induces dichlorofluorescein-sensitive ROS in mesangial cells (A) and upregulates NADPH oxidase subunit expression in mesangial (B) and tubular epithelial cells (C). (A) Intracellular ROS were measured as described in Figure 1, after incubating synchronized quiescent mesangial cells with 2 ng TGF- β 1/ml in the presence or absence of NAC (5 mM), catalase (500 U/ml), and trolox (500 μ M) for 5 min. (B) NADPH oxidase subunits were visualized with immunocytochemistry after incubating synchronized quiescent mesangial cells with 2 ng TGF- β 1/ml for 48 h. (C) p22^{phox} mRNA expression in LLC-PK1 cells was measured by RT-PCR after incubating synchronized quiescent LLC-PK1 cells with 2 ng TGF- β 1/ml for up to 48 h. Forward and reverse primers specific for p22^{phox} (forward, 5'-GTTTGTGTGCCTGCTGGAGT-3'; reverse, 5'-TGGGCGGCTGCTTGATGGT-3') or GAPDH (forward, 5'-CCACCCATGGCAAATTCATGGCA-3'; reverse, 5'-TCTAGACGGCAGGTCAGTCCACC-3') were used.

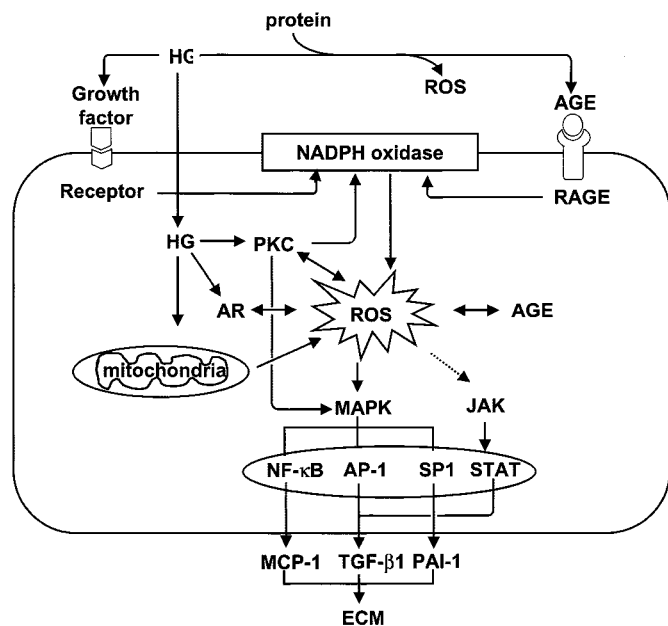


Figure 3. ROS-regulated signaling in renal cells cultured under high glucose. Mitochondrial electron transfer chain and receptor-stimulated NADPH oxidases are important sources of intracellular ROS generation under high glucose condition. High glucose-induced ROS activate signal transduction cascade and transcription factors, leading to upregulation of genes and proteins involved in ECM remodeling in the kidney. HG, high glucose; MCP-1, monocyte chemoattractant protein-1; RAGE, receptors for advanced glycation end products; Sp1, specificity protein 1.

pression in mesangial cells cultured under high glucose (3). Given that inflammatory events initiated by monocyte infiltration are involved in the pathogenesis of diabetic glomerular

(33) as well as tubular injury (34), ROS-mediated NF- κ B activation may play an important role in the pathogenesis of diabetic nephropathy. AP-1 mediates high glucose-induced activation of human TGF- β 1 promoter in mesangial cells (35). Mutation in either one or both AP-1 binding sites or the addition of curcumin, an AP-1 inhibitor, abolishes high glucose-induced TGF- β 1 promoter activity (35). We observed that high glucose-induced fibronectin secretion by NF- κ B dominant negative as well as control mesangial cells was effectively inhibited by curcumin (Figure 4). These observations suggest that ROS-induced AP-1 may play an important role in high glucose-induced TGF- β 1 and fibronectin expression in mesangial cells. Du *et al.* (36) showed that high glucose-induced mitochondrial superoxide generation induces activation of specificity protein 1 and plasminogen activator inhibitor-1 (PAI-1) expression in bovine aortic endothelial cells. The exact role of AP-1 and specificity protein 1 in high glucose-induced TGF- β 1, PAI-1, and fibronectin upregulation in mesangial cells and in diabetic kidney remains to be elucidated.

ROS Upregulate ECM Expression

TGF- β 1 is the key regulator of ECM remodeling in the mesangium leading to mesangial expansion (37) and of tubular epithelial-mesenchymal transition (EMT) (38) leading to tubulointerstitial fibrosis. TGF- β 1 upregulates not only ECM mRNA and proteins but also PAI-1 mRNA and protein expression and downregulates genes encoding proteases (39). PAI-1 induced by TGF- β 1 in mesangial cells inhibits plasminogen activator, decreases plasmin, and decreases ECM degradation (39). The observations that exogenous H₂O₂ or H₂O₂ continuously generated by GO upregulates the expression of mRNA

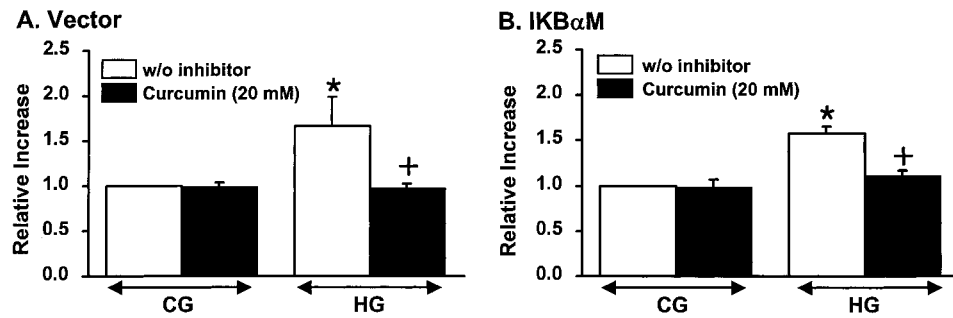


Figure 4. Curcumin, an inhibitor of AP-1, inhibits high glucose–induced fibronectin secretion in mesangial cells. Synchronized quiescent rat mesangial cells transfected with control vector (A) or I κ B α M (B), as characterized previously (3), were incubated with 5.6 (control glucose [CG]) or 30 mM glucose (high glucose [HG]) in the presence or absence of curcumin 20 mM for 48 h. Fibronectin secreted into the media was measured by Western blot analysis after electrophoresis under reducing condition. * $P < 0.05$ versus control glucose without curcumin; † $P < 0.05$ versus high glucose without curcumin.

and proteins of TGF- β 1 (4,8), PAI-1 (40), and fibronectin (4,8) in mesangial cells and that antioxidants effectively inhibit TGF- β 1, PAI-1, and fibronectin upregulation in mesangial cells cultured under high glucose (8,10,11,28,40) and in diabetic kidneys (11,28,41,42) suggest that ROS mediate ECM accumulation in diabetic glomeruli. We (9) recently demonstrated that exogenous H₂O₂ and TGF- β 1 upregulate PAI-1 and fibronectin expression and induce EMT in tubular epithelial cells as characterized by decrease in E-cadherin and increase in α -smooth muscle actin expression. Antioxidants NAC and catalase effectively inhibit TGF- β 1–induced EMT (9). Both NADPH oxidase inhibitors apocynin and DPI and an inhibitor of mitochondrial metabolism, rotenone, block high glucose–induced fibronectin upregulation in tubular epithelial cells (Figure 1B), suggesting that ROS may also play an important role in tubulointerstitial fibrosis in diabetic kidney.

Conclusion

ROS are induced in glomerular mesangial and tubular epithelial cells by high glucose, AGE, and cytokines. PKC, NADPH oxidase, and mitochondrial metabolism all seem to play a role in ROS generation. ROS activate signal transduction cascade and transcription factors, leading to upregulation of genes and proteins involved in glomerular mesangial expansion and tubulointerstitial fibrosis. Future studies to elucidate various other target molecules activated by ROS in renal cells cultured under high glucose and in diabetic kidney will allow a better understanding of the final cellular responses to high glucose.

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