Hyperglycemia Promotes Oxidative Stress through Inhibition of Thioredoxin Function by Thioredoxin-interacting Protein*

P. Christian Schulze‡, Jun Yoshioka‡, Tomosaburo Takahashi‡, Zhiheng He§, George L. King§, and Richard T. Lee¶

From the ¤Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School and §Vascular Cell Biology & Complications, Joslin Diabetes Center and Harvard Medical School, Boston, Massachusetts 02139

Increased intracellular reactive oxygen species (ROS) contribute to vascular disease and pro-atherosclerotic effects of diabetes mellitus may be mediated by oxidative stress. Several ROS-scavenging systems tightly control cellular redox balance; however, their role in hyperglycemia-induced oxidative stress is unclear. A ubiquitous antioxidative mechanism for regulating cellular redox balance is thioredoxin, a highly conserved thiol reductase that interacts with an endogenous inhibitor, thioredoxin-interacting protein (Txnip). Here we show that hyperglycemia inhibits thioredoxin ROS-scavenging function through p38 MAPK-mediated induction of Txnip. Overexpression of Txnip increased oxidative stress, while Txnip gene silencing restored thioredoxin activity in hyperglycemia. Diabetic animals exhibited increased vascular expression of Txnip and reduced thioredoxin activity, which normalized with insulin treatment. These results provide evidence for the impairment of a major ROS-scavenging system in hyperglycemia. These studies implicate reduced thioredoxin activity through interaction with Txnip as an important mechanism for vascular oxidative stress in diabetes mellitus.

Vascular complications such as coronary disease, peripheral artery disease, and stroke are major determinants of morbidity and mortality of patients with diabetes mellitus (1). Atherosclerosis accelerates in the diabetic state (1–3). Increased formation of reactive oxygen species (ROS) contributes to endothelial dysfunction, vessel wall thickening, and lesion formation, thereby playing a crucial role in the progressive deterioration of vascular function and structure (4, 5). While low levels of ROS participate in important cellular signaling mechanisms (6), increased formation of ROS results in cytotoxic oxidative stress (5). Several antioxidative systems tightly regulate cellular redox balance and control formation and reduction of ROS (5, 7, 8). Previously, oxidative stress in diabetes mellitus has been linked to enhanced production of superoxide anion by mitochondria (9) and through protein kinase C-dependent activation of membranous NADPH oxidase (10). However, the intracellular redox balance is maintained by ROS-scavenging systems, and the two major intracellular thiol-reducing mechanisms are the interacting glutathione and thioredoxin systems (8, 11, 12). Thioredoxin reduces ROS through reversible oxidation of thioredoxin at two cysteine residues (Cys-32 and Cys-35); thioredoxin is then reduced by thioredoxin reductase and NAPDH (11). Thioredoxin-interacting protein (Txnip), the endogenous inhibitor of thioredoxin also known as vitamin D3 up-regulated protein-1 (VDUP-1) (13, 14) or thioredoxin-binding protein-2 (TBP-2) (15), inhibits thioredoxin antioxidative function by binding to its redox-active cysteine residues (15, 16).

We show here the inhibition of the thioredoxin ROS-scavenging system through induction of Txnip in hyperglycemia. In contrast to previous reports describing an increased formation of reactive oxygen species in hyperglycemia, our study demonstrates the functional inhibition of a major cytoplasmic antioxidant system in hyperglycemia. Moreover, the inhibition of thioredoxin function in hyperglycemia through increased interaction with its inhibitor Txnip forms the molecular basis of increased levels of the freely diffusible molecule hydrogen peroxide contributing to oxidative stress. This molecular interaction might play a central role in increasing levels of reactive oxygen species by reduced flow through the enzymatic pathways of ROS scavenging.

EXPERIMENTAL PROCEDURES

Cell Culture—Human aortic smooth muscle cells (SMCs) were isolated from surgical specimens and cultured in Dulbecco’s modified essential medium with 10% fetal calf serum. Cells were starved for 48 h in insulin-transferrin medium prior to experiments. Stimulation experiments were performed using glucose (Sigma), insulin (100 nm, Sigma), insulin-like growth factor-1 (IGF-1) (100 ng/ml, Upstate Biotechnology), PDGF-BB (4 ng/ml, Upstate Biotechnology). The following inhibitors were used: wortmannin (100 nm, Sigma), PD169316 (100 nm, Calbiochem), U0126 (10 μM, Calbiochem), GF109203X (5 μM, Calbiochem), and pertussis toxin (500 ng/ml, Sigma).

Northern and Western Analyses—For the detection of mRNA transcripts by Northern analysis, specific cDNA probes were synthesized using the following oligonucleotides: thioredoxin, 5’-AGACGCCAAGATGGTGAAAGCAGA-3’ and 5’-GCTCCAGAAATTCCACCCAC-3’; Txnip, 5’-TCTGCAAAAAAAGGAGAAAGAAG-3’ and 5’-GGCTGACATAAAGATGGCGT-3’. Total RNA was isolated and loaded to a 1% agarose formaldehyde gel. After transfer, the membranes were incubated with radioactive labeled probes, and specific binding was visualized with autoradiography. Western analysis was performed using a specific monoclonal anti-Txnip antibody that was raised against full-
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RESULTS

Induction of Txinp in Hyperglycemia Enhances Oxidative Stress—Since increased oxidative stress contributes to the development of vascular complications in diabetes, we investigated the regulation of thioredoxin antioxidant function in hyperglycemia. Human aortic SMCs incubated with increasing concentrations of glucose showed a concentration-dependent reduction of thioredoxin activity (25 mM glucose: −55 ± 19% versus base line, p < 0.01; Fig. 1A). Thioredoxin can be oxidized through interaction with hydrogen peroxide (11), which can be measured by the cell-permeable and redox-sensitive compoundDCFDA (6). Incubation of SMCs with glucose increased intracellular levels of ROS as assessed by DCFDA fluorescence in a concentration-dependent manner (10 mM glucose: +40.2 ± 8.5%; p < 0.01 versus 5.6 mM glucose and 25 mM glucose: +76.3 ± 2.7%; p < 0.001 versus 5.6 mM glucose). Glucose incubation did not change the expression of thioredoxin mRNA or protein levels (Fig. 1B). However, incubation with glucose markedly induced expression of Txinp (18.6 ± 6.4-fold versus base line; p < 0.05; Fig. 1B) both in cells stimulated with 25 mM glucose after being cultured for 48 h with 5.6 mM glucose (340 ± 64%) and cells cultured in glucose-free medium (440 ± 112% versus base line; p < 0.05). Co-incubation with insulin had no effect on the regulation of Txinp suggesting that glucose directly and independently regulates Txinp expression (Fig. 1C). Co-immunoprecipitation studies showed that the hyperglycemia-induced increase in Txinp protein levels enhanced the thioredoxin/Txinp protein interaction (Fig. 1D). The induction of Txinp by glucose was also found in several primary and non-primary cells such as neonatal rat cardiomyocytes (17.0 ± 5.1-fold; p < 0.01), human endothelial cells (8.9 ± 2.3-fold; p < 0.01), HeLa cells (4.71 ± 2.6-fold; p < 0.05), SOL myoblasts (+24.0 ± 13.0-fold; p < 0.001) and NIH 3T3 fibroblasts (+9.6 ± 4.6-fold; p < 0.05; data not shown). Thus, glucose-mediated regulation of thioredoxin through Txinp appears to be a ubiquitous mechanism.

Overexpression of Txinp using adenoviral gene transfer resulted in increased cellular levels of ROS at 10 mM glucose (+68.8 ± 32.9% versus AdGFP-infected cells; p < 0.01) and at 25 mM glucose (+95.8 ± 15.1% versus AdGFP-infected cells; p < 0.001). In contrast, overexpression of thioredoxin strongly inhibited the increase in ROS at both 10 and 25 mM glucose (Fig. 1E). These findings are consistent with previous reports showing the functional effects of adenoviral gene transfer of Txinp and thioredoxin (13, 14). Overexpression of Txinp results in increased protein levels of Txinp (>30-fold) and functional inhibition of thioredoxin activity by 61 ± 23%. In turn, adenoviral gene transfer of thioredoxin increases thioredoxin protein levels (>20-fold) and thioredoxin activity by 270 ± 40% (14). Txinp gene knockdown induced through post-transcriptional interference of specifically designed Txinp RNAi (Fig. 1F) inhibited the hyperglycemia-induced reduction of thioredoxin activity (96.6 ± 15.3% of base-line activity in cells transfected with Txinp RNAi versus 58.4 ± 19.3% in cells transfected with scrambled RNAi under 25 mM glucose; p < 0.05; Fig. 1G). Furthermore, gene knockdown of Txinp reduced the glucose-induced increase in oxidative stress by 27.4 ± 11.1% under 10 mM glucose and by 35.8 ± 39% under 25 mM glucose compared with controls (both p < 0.05 versus scrambled RNAi) without significant differences at base line (Fig. 1H). These findings support the specific inhibition of the thioredoxin antioxidative system in hyperglycemia through induction of Txinp, the endogenous inhibitor of thioredoxin function.

Induction of Txinp in Hyperglycemia Is Mediated by p38 MAPK—We next analyzed the underlying cellular signaling mechanisms that regulate glucose-induced expression of Txinp. Incubation of aortic SMCs with hyperglycemic concentrations of glucose (25 mM) resulted in a phosphorylation of p38 MAPK after 30 min that was prevented by preincubation with the p38 MAPK inhibitor PD169316 (Fig. 2A). In addition, inhibition of p38 MAPK abolished the glucose-induced expression of Txinp mRNA (Fig. 2B) and protein (Fig. 2C). Therefore, glucose-induced activation of p38 MAPK is required for the induction of Txinp expression.

Activation of PI 3-Kinase 3-Min kinase Suppresses Txinp—Further experiments revealed enhanced glucose-induced expression of Txinp in the presence of the PI 3-kinase inhibitor wortmannin (31.4 ± 19.9-fold versus 14.6 ± 5.6-fold in glucose-stimulated cells; p < 0.05). No effects were detectable after incubation with the ERK inhibitor U0126, G, inhibition by pertussis toxin, or protein kinase C (PKC) inhibition by GF109203X (data not shown).
PKC activation (phorbol myristate acetate, 100 nM), a central mediator of glucose-induced cellular signaling mechanisms (17), did not affect the expression of Txnip. As shown previously (18), incubation of SMCs with hyperglycemic levels of glucose reduced the PI 3-kinase-dependent phosphorylation status of Akt in response to stimulation with PDGF-BB (4 ng/ml) (Fig. 2D). Activation of PI 3-kinase through stimulation with PDGF-BB reduced glucose-induced expression of Txnip mRNA by 45.4 ± 16.4% (p < 0.01 versus non-stimulated cells) (Fig. 2E). Taken together, these findings indicate that the PI 3-kinase pathway inhibits Txnip expression, and its inhibition in hyperglycemia promotes the expression of Txnip.

Vascular Regulation of Txnip in Diabetes Mellitus in Vivo—We next investigated the functional regulation of the thioredoxin system in an animal model of diabetes mellitus in vivo. The diabetic state was induced in adult rats through intraperitoneal injection of STZ. First, we assessed increased formation of superoxide anion in vascular samples of animals with diabetes mellitus. Superoxide anion, an intermediate product of cellular redox scavenging pathways, can be detected on frozen tissue sections by the redox-sensitive compound DHE staining revealed increased levels of superoxide in vessels from diabetic animals compared with controls (Fig. 3A).

Vascular thioredoxin activity was reduced in animals with diabetes mellitus compared with controls (26.7 ± 22.6 versus 79.0 ± 44.6 ng of thioredoxin per μg of total protein; p < 0.01 versus base line) but normalized under insulin treatment (59.3 ± 1.3 ng of thioredoxin per μg of total protein; p = 0.78 versus base line; Fig. 3B). Vascular expression of Txnip increased dramatically in diabetic animals (379.7 ± 123.5% of base-line expression; p < 0.001) without changes in the expression of thioredoxin (83.8 ± 41.9% of base-line expression; p = 0.83; Fig. 3C). Normalization of serum glucose by insulin treatment reduced Txnip expression to base-line levels (81.3 ± 13.9% of base line; p = 0.79). In addition, vascular Txnip protein levels increased in diabetes mellitus and normalized with insulin treatment (Fig. 3D). Immunohistochemical analysis of vascular sections showed strong immunoreactivity for Txnip in the media of aortas from diabetic animals with no changes in thioredoxin levels (Fig. 3E).

**DISCUSSION**

The thioredoxin system regulates cellular redox balance through the reversible oxidation of its redox-active cysteine residues (—Cys-Gly-Pro-Cys—) to form a disulfide bond that in
turn is reduced by thioredoxin reductase and NADPH (11). Targeted deletion of thioredoxin causes early embryonic lethality (22). The thioredoxin system compensates for a lack of glutathione reductase in *Drosophila melanogaster*, emphasizing the role of this system as a central and highly conserved antioxidative cellular defense mechanism (23). In endothelial cells, S-nitrosylation at Cys-69 is required for the anti-apoptotic and redox regulatory function of thioredoxin (24). In addition, thioredoxin functions as a crucial mediator of redox-mediated transcriptional activation (25, 26). After nuclear translocation, thioredoxin can bind by reduction of its redox-active cysteine residues to the transcriptional activator Ref-1, which in turn increases the DNA binding activity of AP-1 and also activates ribonucleotide reductase, a key enzyme in DNA synthesis (25). As part of the cellular stress response, cells express and release a truncated thioredoxin isoform (10 kDa) through a non-typical secretion pathway (27). The truncated isoform has growth-inducing abilities and can act as a chemokine (27). However, it lacks the reducing capacity of full-length thioredoxin and does not participate in the regulation of ROS (28).

The endogenous thioredoxin inhibitor Txnip binds to the active site of thioredoxin and inhibits thioredoxin activity (15). Txnip competes with other thioredoxin-binding proteins such as apoptosis-signaling kinase-1 (ASK1) (16) and inhibits the thioredoxin-mediated degradation of ASK1 through ubiquitination (29), thereby sensitizing cells to apoptotic stimuli (13). In addition, Txnip prevents the nuclear translocation of thioredoxin (14) and regulates thioredoxin-dependent transcriptional mechanisms. Txnip has been described as an intracellular protein that interacts with thioredoxin at its redox-active cysteine residues (15). Since the thioredoxin/Txnip interaction occurs in the cytoplasm, we assume that it interacts only with thioredoxin-1. However, thioredoxin-2, the mitochondrial isoform of thioredoxin, also has the typical cysteine-containing motif, which potentially would allow the interaction with Txnip (30). Since no data are known regarding mitochondrial localization of Txnip, we can only speculate whether this interaction can occur in living cells.

Our results demonstrate the inhibition of thioredoxin activity in hyperglycemia, which can be explained by the specific induction of Txnip, the endogenous inhibitor of thioredoxin. Hyperglycemia enhances the thioredoxin/Txnip interaction leading to a functional inhibition of antioxidative thioredoxin function. This specific interaction results in a shift of the cellular redox balance that promotes increased intracellular oxidative stress. While previous reports described an increased formation of ROS (9, 10), our findings demonstrate the func-
tional inhibition of a central ROS-reducing cellular system in diabetic mellitus.

A pivotal mechanism leading to oxidative stress in hyperglycemia is the increased formation of superoxide anion by the mitochondria (9). Superoxide anion activates several major pathways of cellular damage in hyperglycemia: the polyol pathway, increased flux through the hexosamine pathway, activation of PKC, and the formation of advanced glycation end products (3, 9). Increased mitochondrial formation of superoxide anion has been attributed to increased glycolytic production of pyruvate and NADH that in turn leads to an increase of the mitochondrial proton gradient with excess production of superoxide anion (9). It can be prevented by overexpression of uncoupling protein-1 (UCP-1) leading to a breakdown of the proton gradient or overexpression of superoxide dismutase leading to increased degradation of superoxide anion (9). The activation of PKC plays a role in the activation of the membrane-bound NADPH oxidase resulting in further increase of superoxide production (10). Superoxide anion is also known to interact with nitric oxide (NO) producing the highly reactive metabolite peroxynitrite that in turn leads to uncoupling of endothelial NO synthase (31). Furthermore, uncoupling of NO synthase also occurs when this enzyme is deprived of its critical co-factor tetrahydrobiopterin or the substrate L-arginine. Uncoupling of endothelial NO synthase results in the production of molecular oxygen at the prosthetic heme site rather than formation of NO (31, 32).

Based on the experimental results of the present study, additional mechanisms might also contribute to increased levels of hydrogen peroxide in hyperglycemia. Gene silencing of Txnip prevented reduction of thioredoxin activity and reduces intracellular levels of ROS in hyperglycemia. Notably, adeno viral overexpression of thioredoxin reduces hyperglycemia-induced levels of ROS, while overexpression of Txnip enhances oxidative stress. Gene knockdown of Txnip reduced the hyperglycemia-induced increase in oxidative stress compared with controls without significant differences at baseline. However, hyperglycemia still increases levels of hydrogen peroxide in cells transfected with Txnip RNAi as measured by the cell-permeable and redox-sensitive compound DCFDA that interacts rather specifically with hydrogen peroxide. This increase might reflect levels of reactive oxygen species that overwhelm the scavenging capacity of the thioredoxin system even in the absence of the endogenous inhibitor Txnip. Notably, superoxide dismutase, glutathione peroxidase, and catalase, all central enzymes in the antioxidative defense mechanisms of the cell, are induced under hyperglycemic conditions, most probably reflecting a counter-regulatory mechanism of the cell (33). In addition, increased levels of hydrogen peroxide that result from impaired scavenging by thioredoxin are known to inhibit the Cu,Zn-superoxide dismutase activity (34) and activate NADPH oxidase (35). These mechanisms could in turn lead to an ambient increase of superoxide anion. Therefore, the induction of Txnip in hyperglycemia represents a unique molecular switch mechanism that results in a reduced scavenging capacity of the antioxidative thioredoxin system leading to increased oxidative stress.

The PI 3-kinase/Akt pathway negatively regulates the transcriptional activation of Txnip, while activation of p38 MAPK represents a necessary step in the increased expression of Txnip in hyperglycemia. Hyperglycemia inhibits activation of Akt; however, we can only speculate about the potential mechanisms of reduced activity of the PI 3-kinase/Akt pathway in hyperglycemia. One possible candidate for reduced Akt activity in hyperglycemia is the mammalian homolog of Drosophila tribbles, which functions as a negative modulator of Akt (36). By directly binding to Akt, TRB3 blocks the activation of this kinase.

The induction of Txnip in hyperglycemia is not restricted to vascular tissue. Through transcriptional screening of glucose-induced gene expression, Txnip has been identified as one of the genes with the highest induction in response to glucose incubation in fibroblasts (37), islet cells (38), and mesangial cells (39). Moreover, increased renal expression of Txnip was demonstrated recently in an animal model of STZ-induced diabetes mellitus (39). The induction of Txnip by glucose seems to be a sustained effect and can be detected up to 36 h after stimulation in mesangial cells (39). Our experimental findings suggest a direct and concentration-dependent regulation of Txnip by glucose in hyperglycemia. In addition to its ROS promoting effects leading to oxidative stress, Txnip may also play a crucial role in reducing cellular viability in hyperglycemia by sensitizing cell toward apoptosis (13). Therefore, this
recently discovered gene product may have diverse effects with broad pathophysiological implications in the development of end-organ damage in diabetes mellitus.

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