Mechanism of Oxidative DNA damage in diabetes: tuberin inactivation and downregulation of DNA repair enzyme OGG1

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Objective: To investigate potential mechanisms of oxidative DNA damage in a rat model of type I diabetes and in murine proximal tubular epithelial cells (MCT) and primary culture of rat proximal tubular epithelial cells (RPTE).

Research Design and Methods: Phosphorylation of Akt and tuberin, 8-oxodG levels and OGG1 expression were measured in kidney cortical tissue of control and type I diabetic animals as well as in proximal tubular cells incubated with normal or high glucose.

Results: In the renal cortex of diabetic rats, the increase in Akt phosphorylation is associated with enhanced phosphorylation of tuberin, decreased OGG1 protein expression and 8-oxodG accumulation. Exposure of proximal tubular epithelial cells to high glucose (HG) causes a rapid increase in ROS generation that correlates with the increase in Akt and tuberin phosphorylation. HG also resulted in downregulation of OGG1 protein expression, paralleling its effect on Akt and tuberin. Inhibition of PI-3K/Akt significantly reduced HG-induced tuberin phosphorylation and restored OGG1 expression. Hydrogen peroxide stimulates Akt and tuberin phosphorylation and decreases OGG1 protein expression. The antioxidant, N-acetylcysteine, significantly inhibited ROS generation, Akt/PKB and tuberin phosphorylation and resulted in deceased 8-oxodG accumulation and upregulation of OGG1 protein expression.

Conclusions: Hyperglycemia in type I diabetes and treatment of proximal tubular epithelial cells with HG leads to phosphorylation/inactivation of tuberin and downregulation of OGG1 via a redox-dependent activation of Akt in renal tubular epithelial cells. This signaling cascade provides a mechanism of oxidative stress-mediated DNA damage in diabetes.

Key words: High glucose; ROS; Tuberin and OGG1
Systemic complications are the major causes of morbidity and mortality in patients with diabetes (1). Oxidative stress leads to protein, lipid and DNA modifications that cause cellular dysfunction and contributes to the pathogenesis of macro and microvascular complications of diabetes including diabetic nephropathy (2-6). Mitochondrion and nucleus, two major targets of oxidative stress, contain a variety of DNA repair enzymes to repair oxidant-induced DNA modifications (7,8). Damage most likely occurs when the endogenous antioxidant network and DNA repair systems are overwhelmed (9-11). However, it is essential for the cell to repair DNA damage induced by oxidants.

8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is a sensitive marker of ROS-induced DNA damage (12,13). There is an increase in 8-oxodG levels in tissue of diabetic rats and in the urine of patients with type I and type II diabetes (13-15) with the levels being significantly higher in patients with albuminuria or with other diabetic complications (16). The steady state level of 8-oxodG in DNA reflects its rate of generation as well as repair. 8-OxodG in DNA is repaired primarily via the DNA base excision repair pathway (17). The DNA repair enzyme that recognizes and excises 8-oxo-dG is 8-oxoG-DNA glycosylase (OGG1) (18). Deficiency in DNA repair enzyme OGG1 has important functional consequences, compromising the ability of cells to repair DNA. Indeed, the steady-state levels of 8-oxo-dG are significantly higher in tissues of OGG1 knock out mice compared to wild-type animals (19). OGG1 expression in the kidney of rats heterozygotes for tuberin is lower than that in wild type rats (20). Moreover, treatment of tuberin-deficient rats with an oxidative DNA damaging agent greatly decreases renal OGG1 expression with concomitant increase in 8-oxodG levels compared to wild type rats (20). We have recently shown that OGG1 is regulated by tuberin, the product of the tumor suppressor gene, TSC-2 (21). Tuberin normally exists in an active state physically bound to hamartin, the product of TSC-1 gene, to form a stable complex (22). These two proteins function within the same pathway(s) regulating cell cycle, cell growth, adhesion, and vesicular trafficking (23, 24). Activation of phosphatidylinositol 3-kinase (PI3-K) and phosphorylation of serine/threonine kinase Akt/protein kinase B by certain agonists, lead to inactivation of tuberin (25-28). The PI3-K/Akt pathway is activated in diabetes (29) and there is evidence that this activation is redox-dependent in different cell types (30-32), including renal cells.

Little is known about DNA repair disturbances potentially contributing to DNA damage in diabetes. In the present study, we determined a potential mechanism by which ROS result in 8-oxodG accumulation and explored the role of tuberin phosphorylation and OGG1 in the kidney cortex of rats with type I diabetes. We also investigated the effect of high glucose (HG) on tuberin phosphorylation, OGG1 expression and 8-oxodG accumulations in proximal tubular epithelial cells.

RESEARCH DESIGN AND METHODS

Animals. Two-month-old male Long Evans rats, weighing between 200 and 225 g were purchased from Charles River laboratories (Wilmington, MA). The animals were allowed food and water ad libitum prior to and during the experiments. The rats were divided into two groups of 6 rats/group. Group 2 was
injected intravenously via the tail vein with 55 mg/kg body weight streptozotocin (STZ) (Sigma, St. Louis, MO) in sodium citrate buffer (0.01 M, pH 4.5) under isofluorane inhalation anesthesia (Abbott, Abbott Park, IL) to induce type 1 diabetes. Group 1 (controls) was injected with an equivalent amount of sodium citrate buffer alone. Average serum glucose levels and body weight of both groups were measured at 4 weeks of diabetes. Animals were euthanized at 4 weeks and the kidneys were removed rapidly. Cortical tissue was used for isolation of primary proximal tubular epithelial (RPTE) cells and samples of cortical tissue used for biochemical analysis.

**Isolation and culture of RPTE cells.** Primary RPTE cells were isolated and cultured following the method of Glynne with minor modifications (33). Renal cortical tissue was collected in cooled Hanks’ balanced salt solution (HBSS) containing penicillin (50 U/ml), streptomycin (50 µg/ml), and amphotericin B (0.125 µg/ml). After the capsule was removed, the cortex was cut into small pieces and the tissue fragments were suspended in 1 mg/ml (in HBSS) of type II collagenase (Worthington Biochemical) and incubated for 1 h at 37°C. The cells were centrifuged (200 g, 5 min, 4°C) and seeded into 75-cm² tissue-culture flasks that had been coated with collagen S. The cells were grown in serum-free medium (DMEM/F-12; glucose concentration, 17 mM) containing 15 mM HEPES buffer, L-glutamine, and pyridoxine hydrochloride. The medium was supplemented with epidermal growth factor (10 ng/ml), insulin (10 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronin (4 pg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), and amphotericin B (0.125 µg/ml). The cells were incubated at 37°C in humidified 5% CO₂ in air.

**SV-40 immortalized murine proximal tubular epithelial (MCT) cells.** The MCT cells (provided by Dr. Eric Neilson, Vanderbilt University, Nashville, TN) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 7% fetal bovine serum, 5 mmol/l glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l glutamine. Confluent cells were growth-arrested for 24 h in serum-free DMEM before experiments. MCT cells express in vivo properties of proximal tubular epithelial cells (34).

**Reagents.** N-acetylcysteine (NAC), hydrogen peroxide and wortmannin, were purchased from Sigma (St. Louis, MO). LY294002 and Akt inhibitor IV were obtained from Calbiochem (La Jolla, CA).

**In vivo experiments.** Homogenates of kidney cortex were prepared in radioimmune precipitation assay buffer [1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS containing phenylmethyisulfonyl fluoride, aprotinin, sodium orthovanadate and protease inhibitor tablet ([complete™ Mini, Boehringer-Mannheim) containing antipain dihydrochloride (50 mg/ml), bestatin (40 mg/ml), chymostatin (60 mg/ml), E-64 (10 mg/ml), leupeptin (0.5 mg/ml), pepstatin (0.7 mg/ml), phosphoramidon (300 mg/ml), pefabloc SC (1 mg/ml), EDTA disodium salt (0.5 mg/ml), and aprotinin (2 mg/ml)].

The tissue was centrifuged at 14,000 xg for 30 min at 4°C and protein concentrations were determined with the Bradford assay (35) using bovine serum albumin as a standard. For immunoblotting, protein (100 µg) was subjected to 8% SDS–polyacrylamide gel electrophoresis. Proteins were transferred
to polyvinylidene difluoride (PVDF) membranes at a constant voltage of 200 V for 16 h. The PVDF membranes were blocked for 1 h in 5% nonfat dried milk in TBS-0.1% tween buffer [25 mM Tris–HCl, 0.2 mM NaCl; 0.1% Tween 20 (v/v) pH 7.6] (TBST). The membrane was washed 2X with TBST and then incubated overnight at 4°C with the respective primary antibodies. Phospho-tuberin, Phospho-Akt and Akt antibodies were from Cell Signaling (Beverly, MA); OGG1 antibody was from Novus Biologicals; tuberin and GADPH antibodies were obtained from Santa Cruz Biotechnology. After extensive washing of membrane with TBST buffer, anti-rabbit immunoglobulin conjugated with horseradish peroxidase was added at a 1:5000 dilution and incubated for 1 h at room temperature. An enhanced chemiluminescence kit (Amersham, NJ) was used to identify protein expression. Expression of each protein was quantified by densitometry using National Institutes of Health image 1.62 software and normalized to a loading control.

**In vitro experiments.** MCT and primary RPTE cells were seeded at a density of 0.5 x 10^6 cells on 60 mm petri dishes in 5 mM glucose +25 mM Mannitol (NG). When cells reached 90% confluency, serum was withdrawn for 24 h and cells were treated with high glucose (25 mM HG), hydrogen peroxide and/or PI3-K and Akt inhibitors under serum-free conditions for various time points as indicated. The cells were lysed in radioimmune precipitation assay buffer.

**Measurement of intracellular ROS production.** The peroxide-sensitive fluorescent probe 2’,7’-dichlorodihydrofluorescein diacetate (Molecular Probes, CA) was used to assess the generation of intracellular ROS as described previously (30). This compound is converted by intracellular esterases to 2’,7’-dichlorodihydrofluorescein, which is then oxidized by hydrogen peroxide to the highly fluorescent 2’,7’-dichlorodihydrofluorescein (DCF). MCT cells were grown in chamber slides. Serum-deprived cells were washed with Hank’s balanced salt solution (HBSS) loaded with 10µM 2’,7’-dichlorodihydrofluorescein diacetate and incubated for 30 min at 37°C; 25 mM glucose was added for the indicated time periods. Differential interference contrast images were obtained simultaneously using an Olympus inverted microscope with x40 Aplanfluo objective and an Olympus fluoview confocal laser-scanning attachment. The DCF fluorescence was measured with an excitation wavelength of 488 nm of light, and its emission was detected using a 510-550-nm band pass filter. Alternatively, cells were grown in 6 well plates and serum-deprived for 24 h. Immediately before the experiments, cells were washed with Hanks’ balanced salt solution without phenol red and then incubated for 30 min in the dark at 37°C with the same solution containing the peroxide-sensitive fluorophore 2,7-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, CA) at 5 µmol/liter. They were then incubated with 25 mM glucose for various time points. DCF-DA fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, as measured with a multiwell fluorescence plate reader (Wallac 1420 Victor², Perkin-Elmer Life Sciences), as described (31).

**Immunostaining of OGG1.** OGG1 expression was also assessed by immunofluorescence histochemistry as previously described (36). Acetone-fixed frozen kidney sections (4 µm) were incubated with nonimmune donkey IgG to
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block nonspecific binding, then incubated with rabbit anti-OGG1 primary antibodies followed by fluorescein isothiocyanates (FITC) FITC-labeled donkey anti-rabbit IgG (Chemicon International, Inc., Temecula, CA, USA) as secondary antibodies for signal detection. All incubations of primary and secondary antibodies were for 30 minutes with three washes with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), 5 minutes each between steps. Controls consisted of PBS/BSA in place of primary antibody followed by detection procedures as outlined above. Kidney sections were viewed and photographed using an Olympus Research microscope equipped for epifluorescence with excitation and band pass filters. FITC red signals for OGG1 were detected using a filter with excitation at 535 nm.

Immunostaining of 8-OxoD. A double fluorescent labeling method was used as previously described (20) with minor modifications. MCT cells in chamber slides were serum-starved for 24 h and incubated with 25 mM glucose for indicated time periods. The cells were washed with PBS, fixed and stained with mouse antibody against 8-oxo-dG (Biodesign International) followed by treatment with anti-mouse IgG (1:200) conjugated with FITC. The slides were reacted with Vectashield Mounting Medium with propidium iodide (PI) (Vector Laboratories, Burlingame, CA). In this assay, DNA was labeled with PI and 8-oxo-dG was identified by the primary monoclonal antibody and FITC-conjugated secondary antibody. FITC green signals for 8-oxo-dG were detected using a filter with excitation range 450–490 nm and PI red signals for nuclear DNA using a filter with excitation at 535 nm. FITC and PI were detected using Olympus FV-500 Laser Scanning Confocal microscopy. To demonstrate staining specificity, control cells were stained without primary antibody.

8-OxodG assay. Mitochondria and nuclear DNA fractions were isolated from rat kidney cortex using mitochondria and nuclear fractionation kit according to the manufacturer's instructions (Pierce, IL). Detection of dG and 8-oxodG was performed on DNA hydrolyzed with nuclease P1 and alkaline phosphatase as previously described and validated (20). Aliquots (90 µl) of DNA hydrolysates were injected onto a Partisil 5 (m ODS-3 reverse-phase analytical column for HPLC analysis with the eluate monitored with a UV photodiode array (Shimadzo, SPD M10A) and electrochemical (EC) detectors (ESA Coul Array). Authentic standards of 8-oxodG and dG were analyzed along with every batch of samples. Salmon sperm DNA (5-50 µg) was used as a positive control for DNA digestion reactions. Standard curves for dG and 8-oxodG were prepared and quantitation performed by linear regression analyses. Data were expressed as pmol 8-oxodG/dG x 10^-5 in 90 µl of DNA hydrolysate.

Statistics. Data are presented as mean ± standard error. Statistical differences were determined using ANOVA followed by Student Dunnett’s (Exp. vs. Control) test using 1 trial analysis. P-values less than 0.05 and 0.01 were considered statistically significant.

RESULTS

Diabetes is associated with an increase in Akt and tuberin phosphorylation and OGG1 downregulation in the renal cortex. Akt is known to phosphorylate tuberin on threonine (Thr) 1462, resulting in its
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inactivation (25). Recent studies suggest that HG activates Akt in various cell types, including renal cells (37). In order to test the hypothesis that hyperglycemia stimulates Akt and tuberin phosphorylation and downregulation of OGG1 in vivo, we used a rat model of type I diabetes. Type I diabetes was induced in rats by intravenous injection of STZ (55 mg/kg body wt). Blood glucose levels were 108 ±5.9 and 426.8 ± 49.3 and body weight 391.7 ± 22.7 and 284.0 ± 9.6 in control and diabetic animals at 4 weeks after STZ injection, respectively. Rats were sacrificed at 4 weeks after STZ or vehicle injection. Phosphorylation of Akt on the Ser 473 and the Akt-dependent phosphorylation site on tuberin, Thr 1462, were assessed in kidney cortex homogenates by western blotting analysis (Fig.1A-B). Akt phosphorylation on Ser 473 results in Akt activation, whereas phosphorylation of tuberin on Thr 1462 results in its inactivation. Data in Figure 1 show an increase in phosphorylation of Akt and tuberin in diabetic animals compared to controls. Partial deficiency in tuberin causes a decrease in OGG1 expression, suggesting that OGG1 is downstream of tuberin (20). To investigate the effect of hyperglycemia on OGG1 expression, we evaluated OGG1 protein expression in the kidney cortex of diabetic and control animals. The increase in tuberin phosphorylation is associated with a decrease in the expression of OGG1 protein in the diabetic animals compared to control animals (Fig.1C).

Diabetes is associated with a decrease in OGG1 expression and with increase in 8-oxodG levels. OGG1 is the major DNA base excision repair enzyme that recognizes and excises 8-oxodG. Therefore we determined whether the change in OGG1 abundance influenced the accumulation of 8-oxodG in vivo. Immunohistochemical analysis of OGG1 shows that diabetes caused a decrease in OGG1 staining in kidney cortex of diabetic rats compared to control rats (Fig. 2A). In addition, mitochondria and nuclear DNA fractions were isolated from renal cortex and 8-oxodG levels were analyzed by HPLC-EC. Mitochondrial 8-oxodG levels were significantly higher in kidney cortex of diabetic animals compared to kidney cortex in the control group. There was very little increase in nuclear 8-oxodG levels in diabetic rat kidney cortex compared to kidney cortex from the control group (Fig. 2B). These data suggest that the low levels of OGG1, observed in the kidney cortex of diabetic animals, may not be sufficient to repair the generated 8-oxodG.

High glucose induces Akt and tuberin phosphorylation, increases 8-oxodG and downregulates OGG1 in MCT cells. To investigate the role of HG in Akt and tuberin phosphorylation, MCT cells were incubated for the indicated time periods in serum-free medium containing either NG or HG. HG caused a rapid increase in Akt and tuberin phosphorylation in a time-dependent manner, with effects that peaked at 2.5-5 min and subsided by 60 min (Fig. 3A-B). To assess the effect of HG on OGG1 expression, we evaluated OGG1 protein expression and 8-oxodG accumulation in MCT cells treated with HG for different time periods. A decrease in OGG1 protein expression was observed in MCT cells incubated with HG (Fig. 3C) with a maximum effect at 60 min. Consistent with OGG1 downregulation, immunohistochemical fluorescence staining of 8-oxodG was increased predominantly in mitochondria and to much lower extent in nuclei after 60 min and more so after 2 and 4h of exposure.
of the cells to HG (Fig. 3D). Mitotraker was used as a mitochondrial marker to confirm mitochondrial localization (data not shown). Collectively these data demonstrate that high glucose induces Akt and tuberin phosphorylation results in downregulation of OGG1 and an increase 8-oxodG levels.

Phosphatidylinositol 3-kinase/Akt pathways mediates high glucose-Induced Akt phosphorylation and OGG1 protein downregulation in MCT cells. Activation of PI3-K has been shown to be necessary and sufficient for growth factor-induced increase in Akt phosphorylation (38). PI3-K-dependent activation of Akt in response to glucose has been demonstrated in MCT cells (37). The correlation between HG-induced Akt and tuberin phosphorylation and OGG1 downregulation led us to test the hypothesis that the PI3-K/Akt pathway activated by glucose results in tuberin phosphorylation and OGG1 downregulation. Serum starved MCT cells were pretreated with two structurally unrelated PI3-K inhibitors, LY294002 (50 µM) and wortmannin (100 nM) before exposure of the cells to HG for 60 min. Figure 4A shows that glucose-induced Akt phosphorylation is prevented by both inhibitors. Moreover, a decrease in Akt phosphorylation in the presence of LY294002 (Fig. 4A) and wortmannin (Fig. 4C) significantly attenuated the downregulation of OGG1 by HG (Fig. 4B & D). These data indicate that PI3-K is an upstream mediator of Akt activation and OGG1 downregulation in response to HG.

We next studied whether glucose-stimulated Akt is involved in OGG1 downregulation. MCT cells were preincubated with Akt inhibitor IV (25 µM) for 1 h before exposure to HG. Inhibition of Akt prevented tuberin phosphorylation and restored the levels of OGG1 to those of cells incubated in normal glucose (Fig 5A & B). These findings demonstrate that Akt mediates HG–induced downregulation of OGG1 in MCT cells.

Phosphorylation of tuberin and downregulation of OGG1 are redox-sensitive in MCT cells. We next investigated a potential role for ROS in Akt and tuberin phosphorylation and downregulation of OGG1. Hydrogen peroxide has recently been shown to activate Akt in various cell types, including renal proximal tubular epithelial cells or mesangial cells (30-32, 39 & 40). However, the effect of ROS generation on tuberin phosphorylation and OGG1 expression has not been investigated. MCT cells were treated with hydrogen peroxide (100 µM) for the indicated time periods in serum-free medium. Hydrogen peroxide induced Akt and tuberin phosphorylation with an effect seen as early as 2.5 min and a peak effect occurring at 5-15 min (Fig. 6A and 6B) while the maximum decrease in OGG1 was observed at 60 min (Fig. 6C). The time course kinetics of phosphorylation of Akt and tuberin paralleled those of downregulation of OGG1 by HG. These data demonstrate that phosphorylation of Akt and tuberin and downregulation of OGG1 are redox-sensitive in MCT cells.

Reactive Oxygen Species are required for high glucose-induced Akt and tuberin phosphorylation and OGG1 downregulation in MCT cells. HG increases ROS production in vascular cells as well as in renal cells including tubular epithelial cells or mesangial cells (2, 4 & 41). The fact that oxidant-induced effects on Akt and tuberin phosphorylation correlates well with that of HG, suggests that ROS may mediate the action of HG on Akt, tuberin and OGG1. First, we evaluated the role of HG on the generation of intracellular ROS.
MCT cells were incubated for the indicated time periods in serum-free medium containing either NG or HG. HG significantly increased the fluorescence of DCF, a peroxide-sensitive probe, with a maximal effect apparent 60 min after treatment, as measured by confocal microscopy (Fig. 7A). Additionally, measurements were performed with a multiwell fluorescence plate reader, in order to provide a better quantification of HG-induced ROS generation. Incubation of MCT cells with HG resulted in a rapid and time-dependent increase in DCF fluorescence seen as early as 5 min after treatment (Fig. 7B). These findings demonstrate that high glucose increases intracellular ROS generation in MCT cells. Next, we assessed whether ROS generation mediates the effect of HG-induced Akt and tuberin phosphorylation and OGG1 downregulation in MCT cells. Serum-starved cells were pretreated with the antioxidant NAC, a ROS scavenger. Pretreatment with NAC (20 mM) significantly inhibited ROS generation measured by DCF fluorescence in cells exposed to with HG for 30 min (Fig. 7C). Moreover, pretreatment of the cells with NAC inhibited Akt and tuberin phosphorylation and upregulation of OGG1 protein expression in cells exposed to HG (Fig. 7D). Furthermore, pretreatment of the cells with NAC significantly reduced 8-oxodG staining compared to cells treated with HG alone (Fig. 7E). These data indicate that ROS are critical for HG-induced Akt and tuberin phosphorylation as well as downregulation of OGG1. 

High glucose increases Akt and tuberin phosphorylation and downregulates OGG1 in primary proximal tubular epithelial cells. To confirm the validity of these data obtained in the MCT immortalized cells, we studied the effects of HG on the phosphorylation of Akt and tuberin and on OGG1 protein expression in primary rat proximal tubular epithelial cells (RPTE). Serum-deprived RPTE cells were treated with 25 mM glucose for different periods of time. HG concentration caused an increase in phosphorylation of Akt and tuberin with a peak at 30 min that subsided by 60 min, compared to cells incubated with 5 mM glucose (Fig. 8A). Moreover, the increase in phosphorylated Akt and phosphorylated tuberin is associated with a decrease in OGG1 expression in the cells incubated with 25 mM glucose for 60 min (Fig. 8A). These data confirm our observation in the MCT immortalized cells.

To confirm that HG increases also the intracellular ROS generation in RPTE cells, the production of intracellular ROS was measured by quantification of the DCF fluorescence with a multiwell fluorescence plate reader. Stimulation of RPTE cells with 25 mM glucose resulted in a rapid and time-dependent increase in DCF fluorescence, with the maximal effect (a threefold increase over control) apparent at 60 min after treatment (Fig. 8B). Collectively, these results confirm that HG elicits an increase in ROS also in primary proximal tubular epithelial cells. Furthermore, it appears that the time course of intracellular hydrogen peroxide generation in response to HG is consistent with a potential role of ROS in downstream signaling events, particularly the regulation of redox-sensitive protein kinases and DNA repair.

DISCUSSION

In this study, we provide evidence that diabetes is associated with enhanced phosphorylation and inactivation of tuberin via the redox-dependent
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activation of Akt. We also demonstrate that diabetes-induced Akt and tuberin phosphorylation are associated with a decrease in OGG1 expression and accumulation of 8-oxodG in kidney cortex of diabetic rats. Our data also indicate that this pathway is activated in cultured renal tubular epithelial cells, the major cell type in the kidney cortex, after exposure of the cells to high glucose concentration. These findings indicate that the PI3-K/Akt/tuberin/OGG1 pathway is highly relevant in the diabetic kidney and may contribute to oxidative stress-induced renal injury observed in diabetes. In diabetes, the renal tubule is subject to both direct and indirect insults. Tubular and interstitial lesions are prominent in diabetic patients (42). In a number of cells, HG promotes an increase in Akt phosphorylation (43 & 44). We show that HG phosphorylates tuberin on Thr 1462, a site known to be targeted by Akt. HG activates the PI3-K/Akt/mTOR signalling cascade to stimulate protein synthesis (37). Our data confirm these observations and show an early and sustained increase in Akt phosphorylation following exposure of the cells to HG. Thus, activation of Akt represents a very proximal step in the intracellular signalling pathway triggered by HG. The observation that PI3-K and Akt inhibitors block the effect of HG on tuberin phosphorylation, indicate that the PI3-K/Akt pathway mediates the action of HG on tuberin. Of interest is that insulin or insulin growth factor (IGF) 1-induced tuberin phosphorylation is also inhibited by the PI3K inhibitor LY294002 (45). The concept that PI3-K/Akt pathway regulates tuberin is supported by other observations that the expression of a constitutively active PI3-K or active Akt, including Akt1 or Akt2, induce tuberin phosphorylation (27). Phosphorylation of hamartin and/or tuberin may play an important role in the regulation of the tuberin-hamartin complex (46). Deficiency and/or enhanced phosphorylation of tuberin on threonine 1462 results in its inactivation (26-28). Indeed, Akt is known to phosphorylate tuberin at this site and results in its inactivation (26-28). Phosphorylation of tuberin by Akt affects its function through at least two mechanisms: first, phosphorylation decreases the activity of tuberin; second, phosphorylation destabilizes tuberin by disrupting the complex formation between hamartin and tuberin resulting in ubiquitination of free tuberin and its degradation by the proteosome (27). Our data show that HG causes a rapid Akt-dependent increase in tuberin phosphorylation in MCT cells. The fact that the acute exposure of cells to HG is sufficient to elicit proliferation and then apoptosis of human proximal tubule epithelial cells suggests that episodes of increases in glucose may contribute to cell injury and to epithelial cell dysfunction (47).

We also found that HG-induced tuberin phosphorylation by Akt is associated with biological consequences, namely a decrease in OGG1 expression and increased 8-oxodG levels. The decrease in tuberin protein expression is associated with a decrease in OGG1 expression (20). Therefore, tuberin deficiency, through its phosphorylation is upstream of OGG1 in the pathway linking HG to DNA damage. Collectively, the data indicate that glucose acts through the PI-3K/Akt/ tuberin pathway to downregulate OGG1 resulting in the accumulation of oxidized DNA. 8-OxodG is a product of oxidative DNA damage following specific enzymatic cleavage after the ROS-induced 8-hydroxylation of guanine bases in the mitochondrial and nuclear DNA (11 & 12). 8-OxodG is
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known to be a sensitive marker of oxidative DNA damage and of the total systemic oxidative stress in vivo (13). Importantly, 8-oxoG appears to play a role in tissue cell injury via the induction of apoptotic cell death (48). Increased number of 8-oxodG positive islet cells was found in the pancreas from humans with type II diabetic subjects (49). In addition, our data confirm a previous report of the accumulation of 8-oxodG primarily in the mitochondrial DNA and to a lesser extent nuclear DNA in kidney cortex of diabetic rats. 8-OxodG levels were rapidly normalized by insulin treatment, suggesting the involvement of hyperglycemia in oxidative DNA damage (50).

It is known that Akt is activated by oxidative stress in a variety of cell types including renal cells. Importantly, we show that ROS, and specifically hydrogen peroxide, directly induce Akt activation and tuberin phosphorylation resulting in downregulation of OGG1 in MCT cells, indicating that ROS are potential upstream mediators of the effects of HG on oxidative DNA damage. We also show that exposure of MCT to HG elicits a rapid increase in intracellular ROS production. This rapid effect supports the contention that ROS mediate early signalling events, such as Akt activation in response to HG. Moreover, the effect of HG on ROS generation, Akt/tuberin phosphorylation, OGG1 downregulation and 8-oxodG accumulation is markedly reduced by the antioxidant NAC. This is in agreement with a recent study showing that pretreatment of human proximal tubule epithelial cells with NAC reversed glucose-mediated ROS production (51). Collectively, our data indicate that ROS are signalling molecules responsible for Akt phosphorylation initiated by HG leading to tuberin phosphorylation and OGG1 protein downregulation. To confirm and validate our observations in the immortalized MCT cells, we isolated primary RPTE cells from rat kidney cortex. Similar to MCT cells, glucose enhances ROS generation in primary RPTE cells, and is associated with an increase in Akt and tuberin phosphorylation, as well as downregulation of OGG1. These data show that MCT cells are a relevant model and confirm that phosphorylation and inactivation of tuberin via the redox-dependent activation of Akt play a major role in OGG1 downregulation and 8-oxodG accumulation.

In summary, our data provide the first evidence that hyperglycemia and HG lead to phosphorylation/inactivation of tuberin and downregulation of DNA repair enzyme OGG1 via the redox-dependent activation of Akt. This signaling cascade may play a role in oxidative stress-mediated DNA damage induced by hyperglycemia during diabetic nephropathy. Recurrent acute exposure of renal cells to HG during diabetes has been recently proposed to be involved in renal injury (51). Our data shed light on the molecular mechanisms implicated in these events. The present study provides additional rationale for maintaining tight control of plasma glucose to prevent oxidative DNA damage in diabetes.

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FIGURE 1. Diabetes increases Akt and tuberin phosphorylation and decreases OGG1 protein expression. A-C. Representative immunoblot shows an increase in phospho-Akt (p-Akt) (A) and phospho-tuberin (p-tuberin) (B), and a decrease in OGG1 expression (C) in homogenized kidney cortex of diabetic compared to control rats. GAPDH was used as a loading control.
FIGURE 2. Diabetes causes a decrease in OGG1 protein expression and increases 8-oxodG levels. A. Kidney cortex sections were stained with anti-OGG1 antibody. There is a decrease in OGG1 staining in kidney cortex of diabetic animals compared to the controls. B. 8-OxodG levels are higher in mitochondrial DNA fraction of kidney cortex of diabetic rats compared to control rats. Data are expressed as pmol 8-oxodG/dG x 10^{-5} in 90 μl of DNA hydrolysate. Significant difference from wild type cells is indicated by ** $P < 0.01$. 
FIGURE 3. Effects of high glucose on Akt/PKB and tuberin phosphorylation and OGG1 expression in MCT cells. A-C. Representative immunoblot shows an increase in of phospho-Akt (p-Akt) (A), phospho-tuberin (p-tuberin) (B) in MCT cells treated with HG (25 mM glucose D-glucose) for the time periods indicated. GAPDH was used as loading control. Histograms in the bottom panel represent means ± S.E of 3 independent experiments. Significant difference from non-treated cells is indicated by * $P < 0.05$ and ** $P < 0.01$. D. Immunoflourescence shows an increase mitochondrial and nuclear 8-oxodG staining in MCT treated with HG for 1h and more intense at 2h and 4h compared to NG. FITC for 8-oxo-dG (green color) and PI for nuclear staining (red color) were detected with excitation wavelengths at 450–490 nm and 535 nm, respectively.
FIGURE 4. Role of PI3-K activation by high glucose on OGG1 expression in MCT cells. A & C. Representative immunoblot shows a decrease in phospho-Akt (p-Akt) expression in cells preincubated with LY294002 (50 µM) and wortmannin (100 nM) before exposure to HG for 60 min, respectively. B & D. Representative immunoblot shows an increase in OGG1 expression in cells preincubated with LY294002 (50 µM) and wortmannin (100 nM) before exposure to HG for 60 min, respectively. Histograms in the bottom panel represent means ± S.E of 3 independent experiments. Significant difference from non-treated cells is indicated by * $P < 0.05$ and ** $P < 0.01$. 
FIGURE 5. Role of Akt/PKB phosphorylation by high glucose on OGG1 expression in MCT cells. (A) Representative immunoblot shows a decrease in phospho-Akt (p-Akt) and phospho-tuberin (A) and an increase in OGG1 expression (B) in cells preincubated with Akt inhibitor IV (25 µM) before exposure to HG for 60 min. Histograms in the bottom panel represent means ± S.E of 3 independents experiments. Significant difference from non-treated cells is indicated by * $P < 0.05$ and ** $P < 0.01$. 

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\begin{align*}
\text{Akt inhibitor IV} & \\
\text{NG} & \text{HG} & \text{NG} & \text{HG} \\
\text{P-Akt} & \text{Akt} & \text{Thr}^{1462} & \text{Ser}^{473} \\
\text{P-tuberin} & \text{Tuberin} & & \\
\text{GAPDH} & & & \\
\end{align*}
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\[
\begin{align*}
\text{OGG1} & \text{GAPDH} \\
\text{NG} & \text{HG} & \text{NG} & \text{HG} & \text{NG} & \text{HG} & \text{NG} & \text{HG} \\
\text{P-Akt/GAPDH} & \text{P-tuberin/GAPDH} & & & & & & \\
\end{align*}
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FIGURE 6. Effects of hydrogen peroxide on tuberin and Akt/PKb phosphorylation and OGG1 expression in MCT cells. A-C. Representative immunoblot shows an increase in phospho-Akt (p-Akt) (A), phospho-tuberin (p-tuberin) (B) and a decrease in OGG1 expression (C) in MCT cells treated with 100 μM H₂O₂. GAPDH was used as loading control. Histograms in the bottom panel represent means ± S.E of 3 independents experiments. Significant difference from non-treated cells is indicated by * P < 0.05 and ** P < 0.01.
FIGURE 7. Effect of high glucose concentration on the production of reactive oxygen species (ROS) in MCT cells. A. 2',7'-Dichlorodihydrofluorescein (DCF) fluorescence reflecting the relative levels of ROS was imaged with a confocal laser scanning fluorescence microscope in serum-deprived MCT cells treated with HG for the indicated time periods. B. DCF fluorescence was measured using the peroxide-sensitive fluorescent probe DCF-DA by a multiwell fluorescence plate reader in intact MCT cells treated with HG. Histogram represents means ± S.E of 3 independents experiments. Significant difference from non-treated cells is indicated by * $P < 0.05$ and ** $P < 0.01$. C. NAC blocks ROS generation measured using the peroxide-sensitive fluorescent probe DCF-DA in MCT cells treated with HG. Histogram represents means ± S.E of 3 independents experiments. Significant difference from normal glucose is indicated by ** $P < 0.01$ and from cells treated with NAC vs HG alone is indicated by †† $P < 0.01$. D. Effect of N-acetylcysteine (NAC) on HG-induced Akt/PKB and tuberin phosphorylation and OGG1 expression in MCT cells. Representative immunoblot shows a decrease in phospho-Akt (p-Akt) and phospho-tuberin (p-tuberin) and increase in OGG1 expression in cells preincubated with NAC before exposure to HG. E. NAC blocks 8-oxodG generation in cells treated with HG. Immunofluorescence shows a decrease in mitochondrial and nuclear 8-oxodG staining in MCT pretreated with NAC compared to cells treated with HG alone for 2h.
Figure 7C-E
FIGURE 8. Effect of high glucose concentration on Akt/PKB and tuberin phosphorylation and OGG1 expression in primary cultures of RPTE cells. A. Representative immunoblot shows an increase in phospho-Akt (p-Akt) and phospho-tuberin (p-tuberin) and a decrease in OGG1 expression in cells treated with HG for the time periods indicated. GAPDH was used as loading control. B. DCF fluorescence was measured using the peroxide-sensitive fluorescent probe DCF-DA by a multiwell fluorescence plate reader in intact RPTE cells treated with HG. Histogram represents means ± S.E of 3 independents experiments. Significant difference from non-treated cells is indicated by * \( P < 0.05 \) and ** \( P < 0.01 \).