Impaired cardiac anti-oxidant activity in diabetes: human and correlative experimental studies.

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ABSTRACT

Objectives. Increased reactive oxygen species (ROS) are traditionally viewed as arising from the metabolic flux of diabetes, although reduction in the activity of anti-oxidant systems has also been implicated. Among the latter is the major thiol reducing thioredoxin system, the activity of which may be diminished by high glucose-induced expression of its endogenous inhibitor, thioredoxin interacting protein (TxnIP).

Methods. We assessed TxnIP mRNA/protein expression along with thioredoxin activity in human right atrial biopsy specimens from subjects with and without diabetes undergoing coronary artery grafting. In correlative experimental studies, we examined TxnIP expression in both type 1 and type 2 rodent models of diabetic cardiomyopathy. Finally, we used *in vitro* gene silencing to determine the contribution of changes in TxnIP abundance to the high glucose-induced reduction in thioredoxin activity.

Results. In human right atrial biopsies, diabetes was associated with a >30 fold increase in TxnIP gene expression, and a 17% increase in TxnIP protein expression (both $p<0.05$). This was associated with a 21% reduction in thioredoxin activity when compared to human non-diabetic cardiac biopsy samples (all $p<0.05$). In correlative animal studies, both type 1 and type 2 diabetic rats demonstrated a significant increase in TxnIP mRNA and reduction in thioredoxin activity when compared to non-diabetic animals (all $p<0.05$). This was associated with a significant increase in reactive oxygen species ($p<0.05$ when compared with

control). In cultured cardiac myocytes, high glucose increased reactive oxygen species and TxnIP mRNA expression, in association with a reduction in thioredoxin activity ($p < 0.01$). These findings were abrogated by TxnIP siRNA. Scrambled siRNA had no effect upon ROS or TxnIP expression.

Conclusions. High glucose reduces thioredoxin activity and increases reactive oxygen species via TxnIP over-expression. These findings suggest that impaired thiol reductive capacity, through altered TxnIP expression, contributes to increased ROS in the diabetic heart.

Abbreviations:

ATCC - American Type Culture Collection

ANOVA – analysis of variance

DMEM - Dulbecco's Modified Eagle's Medium

EDP - end diastolic pressure

EDPVR - the slope of the end diastolic pressure volume relationship

EDV - end diastolic volume

ESP - end systolic pressure

FBS –fetal bovine serum

NADPH –nicotinamide adenine dinucleotide phosphate

OCT – optimal cutting temperature

PCR – polymerase chain reaction

PLSD – Fishers protected least significant difference

PRSW - the slope of the preload recruitable stroke work relationship

PV - left ventricular pressure-volume loop

RAS - renin-angiotensin system

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Ren-2 - TGR(mRen-2)²⁷ transgenic (Ren-2) rats

RVU –relative volume unit

ROS –reactive oxygen species

siRNA - small interfering RNA

STZ - streptozotocin

SW - stroke work

TRX – thioredoxin

TxnIP - thioredoxin interacting protein

Introduction

The presence of diabetes in the setting of coronary artery disease substantially worsens prognosis. For instance, when compared with their non-diabetic counterparts, patients with diabetes who have sustained a myocardial infarction have an approximate 50% increase in mortality, a doubling in their likelihood of developing heart failure and substantially worse quality of life [1, 2]. Moreover, these adverse outcomes are also seen in unstable angina [3] and persist after adjustment for clinical and angiographic variables [4]. These poor outcomes have dramatically impacted the appropriate management strategy of such individuals, with recent data demonstrating that coronary artery bypass grafting has superior outcomes to percutaneous coronary intervention in patients with diabetes and advanced coronary artery disease, albeit at a cost of increased stroke rates in the bypass graft cohort [5, 6].

A number of factors potentially contribute to the adverse outcome in diabetes such as the existence of a unique entity termed “diabetic cardiomyopathy” [7]. Furthermore, a large body of evidence now exists to support that notion that increased production of reactive oxygen species (ROS) which develops as a consequence of the hyperglycemia-driven glycolytic flux may be a primary pathogenic factor in the development of diabetes related complications [8].

In addition to enhancing ROS production, hyperglycemia may also lead to dysregulation of the anti-oxidant defence systems that not only remove ROS, but also repair oxidised molecules and target those that have been irretrievably damaged for degradation. Among these, the sulfur-containing amino acid residues, cysteine and methionine are **exquisitely** sensitive to oxidative stress [9] though they can also be readily reduced by the enzyme, thioredoxin [10] or by the enzyme glutaredoxin [11]. This highly and near-ubiquitously expressed dithiol protein efficiently reduces oxidised sulfhydryl groups. Its activity, however, is tightly regulated by an endogenous inhibitor, thioredoxin interacting protein (TxnIP), a small, 38 amino acid protein [12], that not only impedes the reductive capacity of thioredoxin but also promotes cardiomyocyte apoptosis [13].

Among the factors known to regulate TxnIP expression, the induction of its transcription in response to high glucose is particularly dramatic and widespread, having been documented in numerous cell types in culture [14-18]. More recently, these effects have also been reported in the *in vivo* setting with increased TxnIP expression in the kidneys of rodents with diabetes and in biopsies

from patients with diabetic nephropathy [19]. However, despite its clinical importance, changes in TxnIP expression in the hearts of patients with diabetes have not been examined. Accordingly, we sought to assess TxnIP expression in the heart. Having found a dramatic increase in TxnIP in the hearts of patients with diabetes undergoing coronary artery bypass surgery, we then used an experimental approach to explore the impact of such changes on thioredoxin activity.

METHODS

Human Biopsies

Right atrial appendage samples were obtained from patients undergoing routine coronary artery bypass surgery (N=7 per group). Excised samples were either snap frozen at -80°C, stored in formalin or embedded in OCT and also

stored at -80°C for subsequent analysis. Informed consent was obtained from all patients for being included in the study in accordance with the requirements of the St Michael's Hospital human research ethics committee. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Quantitative Real Time RT-PCR

Thioredoxin (TRX) and TxnIP gene expression were measured and quantified using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, as previously described [20]. Primer and probe sequences were: human Txnip, forward GGCACCTGTGTCTGCTAAAA, reverse CGGGAACATGTATTCTCAAA; human TRX, forward GTCAGACTCCAGCAGCCAAGA, reverse TTATCACCTGCAGCGTCCAA, probe TGAAGCAGATCGAGAGCA. RPL32 forward 5' CAA CAT TGG TTA TGG AAG CAA CA 3' reverse 5' TGA CGT TGT GGA CCA GGA ACT 3'. Data output was analyzed using the Applied Biosystems Comparative CT method (Applied Biosystems User Bulletin #2). Experiments were performed in triplicate for each sample and no template controls were added to ensure that amplification was not due to contamination of other components within the PCR mixture.

Western blotting

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Protein was isolated from tissue after washing in phosphate-buffered saline (PBS), with Cell Lysis Buffer (Cell Signaling) containing 1 mmol/l phenylmethylsulfonyl fluoride. Protein concentration was determined by the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, California, USA). Samples with equal concentrations of protein were subjected to SDS-PAGE and Western blot analysis, as previously described[21], loading gels with 50mg protein, before transferring to nitrocellulose membranes. Immunoblotting was performed with primary antibodies (TxnIP antibody, 1:1000: Invitrogen, CA, USA). Following incubation with anti-rabbit secondary antibody (1:2500: Dako, Glostrup, Denmark) for 1 hour at room temperature, proteins were detected by ECL detection system (Amersham, Buckinghamshire, UK). The membranes were reprobed with β -actin (1:5000:Abcam) which served as loading control.

Thioredoxin activity:

Thioredoxin activity was measured in lysates using the insulin disulfide reduction assay as previously described [22]. In brief, 40 μ l of reaction mixture (200 μ l HEPES buffer, 40 μ l EDTA, 40 μ l NADPH and 500 μ l of insulin) were added to cellular protein extracts and incubated at 37 °C for 20 min following the addition of 10 μ l of bovine thioredoxin reductase (American Diagnostica Inc., Greenwich, CT). The reaction was then terminated by adding 500 μ l of stop buffer (0.4 mg/ml DTNB/6M guanidine HCl in 0.2 M Tris-HCl) after which absorption at 412 nm was measured.

Animal studies: Experimental diabetic cardiomyopathy in both type 1 and type 2 diabetes

Following on from the human studies, experimental animal studies were undertaken to examine changes in TxnIP expression in the diabetic heart, using a hemodynamically-validated model of type 1 diabetic cardiomyopathy, the streptozotocin injected homozygous TGR(mRen-2)²⁷ transgenic (Ren-2) rats [23] and a model of type 2 diabetes, the Goto-Kakizaki rat [24, 25]. All animal studies were approved by the St Michael's Animal ethics care committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Type 1 model of DCM

At six weeks of age, homozygous TGR(mRen-2)²⁷ transgenic (Ren-2) rats (n=8 per group) were randomized to receive either 55 mg/kg of streptozotocin (STZ; Sigma, St Louis, MO, USA) diluted in 0.1 M citrate buffer pH 4.5 or citrate buffer alone (non-diabetic) by tail vein injection following an overnight fast. Once diabetes was confirmed, diabetic animals received 2-4 units of isophane insulin (Humulin NPH, Eli Lilly, NSW, Australia) 3 times per week to promote weight gain and to reduce mortality. Each week, rats were weighed and blood glucose was

determined using Accu-check Advantage (Roche, Mississauga, ON, Canada). Animals were housed at constant room temperature ($21 \pm 1^{\circ}\text{C}$) with a 12 hour light/dark cycle and were fed standard rat chow and water *ad libitum*. After 6 weeks of diabetes, animals were anesthetized (2% isoflurane). Effective anaesthesia was defined as lack of response to toe pinch and lack of spontaneous respiration. The abdomen, neck and chest were shaved, followed by *in-vivo* left ventricular pressure-volume (PV) loop acquisition. Animals were then euthanized (60mg/kg pentobarbitone I.V. followed by cervical dislocation), and their heart and lungs excised.

Cardiac catheterization

Cardiac catheterization was performed, as previously published [26] for the diabetic Ren-2 rats. In brief, animals were placed on a warming pad (37°C), intubated using a 14 gauge catheter, and ventilated using positive pressure with a tidal volume of 10% body weight at 70 breaths per minute using room air. Rats were secured in a recumbent position and the right jugular vein was cannulated. Pressure was calibrated after warming the catheter (Model SPR-838 Millar instruments, Houston, TX) in 0.9% NaCl at 37°C for 30 minutes. The right internal carotid was then identified and ligated cranially. A 2F miniaturized combined conductance catheter-micro-manometer was inserted into the carotid artery to obtain aortic blood pressure, then advanced into the left ventricle until stable PV loops were obtained. The abdomen was opened and the inferior vena cava

and portal vein identified. Elastic bands were placed around these vessels to allow rapid reduction in cardiac preload. All loops were obtained with the ventilator turned off for 5 -10 seconds and the animal apnoeic. Data were then acquired under steady state conditions and during preload reduction with parallel conductance values obtained by the injection of approximately 200 μL of 10% NaCl into the right atrium[27]. Calibration from Relative Volume Units (RVU) conductance signal to absolute volumes (in μL) was undertaken using a previously validated method of comparison to known volumes in Perspex wells [28, 29]. Using the pressure conductance data, a range of functional parameters were then calculated (Millar analysis software PVAN 3.4). These included: end diastolic pressure (EDP), end systolic pressure (ESP), the slope of the end diastolic pressure volume relationship (EDPVR).

Histopathology

Changes in cardiac structure were assessed in a masked protocol in six animals from each group, focussing on cardiac fibrosis, myocyte hypertrophy and oxidative stress. Paraffin embedded sections, each 4 μm thick were examined in at least 6 animals per group in the diabetic Ren-2 rats. The accumulation of matrix was assessed by immunostaining for collagen I (anti-type I collagen: Southern Biotechnology Associates, Inc. Birmingham, AL), by the streptavidin-biotin technique using alkaline phosphatase as the labelling enzyme, as previously reported[30]. The extent of labelling was quantified using computer-

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assisted image analysis, as also previously reported [30]. In brief, random non-overlapping fields from 6 rats per group were captured and digitized using a BX50 microscope attached to a Fujix HC2000 digital camera. Digital images were then loaded onto a Pentium III IBM computer. Areas of brown on immunostained sections were selected for their color ranges. Calculation of the proportional area stained was then determined using image analysis (AIS, Analytical imaging Station Version 6.0, Ontario, Canada).

The extent of cardiac myocyte hypertrophy was determined on haematoxylin and eosin stained sections, as adapted from the methods described by Kai and colleagues [31]. In brief, spindle-shaped cardiac myocytes with elliptical nuclei in transverse section were selected. The transverse diameter was measured 3 times per cell at the level of the nucleus, and the values were averaged. Approximately 20 cells per field at x200 magnification were found with 3 to 4 fields randomly selected per sample slide. The average diameter of at least 50 myocytes was then calculated for 6 animals in each group.

To assess the extent of oxidative stress, sections were also stained with anti-nitrotyrosine antibody (Upstate, NY, USA) and quantified, as described above for type I collagen immunohistochemistry.

Type 2 model of DCM

The Goto-Kakizaki (GK) rat is a non obese, non hypertensive model of type 2 diabetes bred on a Wistar background. GK rats and control Wistar rats (Taconic

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Ind. NY, USA) were followed for 20 weeks. Diabetes was confirmed at 8 weeks of age in the GK rats. Each week, rats were weighed and blood glucose was determined using Accu-check Advantage (Roche, Mississauga, ON, Canada). HbA1c was measured using A1cNow+ (Bayer, Sunnyvale, CA). Animals were housed at constant room temperature ($21 \pm 1^{\circ}\text{C}$) with a 12 hour light/dark cycle and were fed standard rat chow and water *ad libitum*. After 12 weeks of diabetes, animals were euthanized and their heart and lungs excised.

In the GK and Wistar rats, oxidative stress was assessed using (dihydroethidium) DHE fluorescence. Cryostat sections ($6\ \mu\text{m}$) of OCT embedded heart tissue from GK ($n=5$) and Wistar rats ($n=6$) both at age of 20 weeks, were stained with $5\ \mu\text{M}$ DHE at 37°C for 30 minutes in dark. After 3 washes with PBS, slides were mounted with a fluorescence mounting medium (Dako) and cell fluorescence was observed under fluorescence microscopy. The fluorescence positivity was quantified using Image J software.

TxnIP /TRX mRNA expression

In brief, Thioredoxin (TRX) and TxnIP gene expression were measured in type 1 and type 2 diabetic rats and quantified using ABI Prism 7000 Sequence

Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, as previously described [20].

Thioredoxin activity:

TRX activity was determined in both type 1 and type 2 diabetic rats as outlined above using the insulin disulfide reduction assay as previously described [22].

Txnip Western blot:

TxnIP protein concentration was determined using western blot as outlined above and previously described.

***In vitro* studies**

To assess the effects of high glucose on the thioredoxin system in cardiac cells, neonatal Sprague Dawley cardiomyocytes were obtained, as previously reported [23]. In brief, 2 day old Sprague Dawley rats underwent cervical dislocation. Primary cardiomyocyte cultures were then isolated using the Worthington neonatal cardiomyocyte isolation system (Worthington Biochemical Corp.) following the manufacturer's instructions. Specifically, ventricular tissues are first rinsed in sterile calcium- and magnesium-free Hank's balanced salt solution (CMF HBSS) solution lacking Ca^{2+} and Mg^{2+} and incubated overnight with 50 ng/ml trypsin, then with a 5 M excess of soybean trypsin inhibitor. After 3 successive washings in HBSS, tissues were then treated

with 300 U/ml of collagenase in HBSS for 45 min under constant stirring. The cell suspension was then filtered (70- μ m pores) and centrifuged for 5 min at 100 g. To achieve myocyte enrichment, dissociated cells were preplated twice in T75 flasks for 90 min, a time period during which nonmyocyte cells attached to the bottom of the cultured flask. Cardiomyocytes (125×10^3 cells/cm²) were then cultured in L-15 Leibovitz medium (Worthington, Lakewood, NJ) supplemented with 5% (vol/vol) fetal calf serum and 1% (vol/vol) penicillin and streptomycin solution (Life Technologies; Invitrogen, Cergy-Pontoise, France).

In addition to these primary cells, rat H9c2 transformed cardiomyoblasts were also obtained from American Type Culture Collection (ATCC). Cells were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), streptomycin (100g/ml), penicillin (100 U/ml), and 2 mM glutamine at 37°C in 95% air/5% CO₂. Cells from passage 5–10 were cultured in either DMEM (which contains 5.6 mM glucose) or DMEM supplemented with a further 19.4 mM glucose (final concentration of glucose 25 mM) for 48 h. Mannitol (19.4 mM) added to DMEM served as the osmotic control. Protein concentration was determined by the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA).

To assess the impact of high glucose on the thioredoxin system, TRX and TxnIP gene expression were measured and quantified using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to

the manufacturer's instructions, with specific primers for human and rat sequences, as described previously [19]. Thioredoxin activity was measured in cultured cells using the insulin disulfide reduction assay as described above. In brief, 40 μ l of reaction mixture (200 μ l HEPES buffer, 40 μ l EDTA, 40 μ l NADPH and 500 μ l of insulin) were added to cellular protein extracts and incubated at 37 °C for 20 min following the addition of 10 μ l of bovine thioredoxin reductase (American Diagnostica Inc., Greenwich, CT). The reaction was then terminated by adding 500 μ l of stop buffer (0.4 mg/ml DTNB/6M guanidine HCl in 0.2 M Tris-HCl) after which absorption at 412 nm was measured.

To examine the effects of high glucose induced TxnIP expression on thioredoxin activity, H9c2 cells were transfected (Lipofectamine 2000, Invitrogen) with 60 nM small interfering RNA (siRNA) for selectively silencing TxnIP (rACAGACUUCGGAGUACCUGdTT), as previously reported [18]. Knockdown of gene expression was confirmed by quantitative PCR as indicated above. Scramble RNAi was used as control.

To examine the impact of high glucose upon oxidative stress, we assessed both dihydroethidium fluorescence and 5-(and 6) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes), as described previously[19].

In brief, hydrogen peroxide and peroxidases oxidize the intracellular DCFH to the fluorescence compound 2',7'-dichlorofluorescein. H9c2 cells were incubated

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with TxnIP or scrambled siRNA, as described already. After 48 h of exposure to 25 mM glucose or normal culture medium, cells were washed, trypsinized, resuspended in PBS, loaded with 10 μ M CM-H2DCFDA, and incubated at 37°C for 30 min. The measurement of intracellular ROS was performed on 10,000 cells through a flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ). Flow cytometry data were analyzed using WinMDI 2.9 (Scripps Institute, La Jolla, CA).

To assess DHE fluorescence, rat H9C2 cells were seeded in a 96-well plate (3×10^4 cells/well) and cultured in DMEM medium containing 5.6 mM glucose (low glucose). After 24 hours, cells were transfected with either Txnip-shRNA plasmid or scramble plasmid using Lipofectamine Reagent (Life technologies). 24 hours post transfection, medium was changed with either low glucose or high glucose medium (25mM) and low glucose medium containing 20 mM mannitol served as osmotic control. Cells were maintained for another 24 hours and then incubated with 200 μ l of DHE solution (10 μ M in PBS) for 15 minutes at 37°C in dark. The fluorescence intensity was measured by a SpectraMax M5 Multi-Mode Microplate Reader with excitation set at 518nm and emission at 605 nm. Non transfected cells treated with medium containing 200 μ M hydrogen peroxide (H_2O_2) for 30 minutes prior to DHE staining was used as positive control.

Statistical Analysis

Unless otherwise stated, data were expressed as mean $\pm$ SEM. Differences between groups were determined by unpaired t-test where appropriate. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Thioredoxin interacting protein mRNA and protein is over-expressed in human hearts and associated with reduced thioredoxin activity

Human right atrial biopsy specimens were obtained from patients undergoing coronary artery bypass surgery (Table 1). Subjects with diabetes were matched in terms of age, sex, medication use and cardiac risk factors apart from diabetes. Quantification of TxnIP gene expression by real time PCR revealed a 38-fold increase in TxnIP mRNA in human right atrial biopsies from patients with diabetes compared with control tissue. Thioredoxin gene expression, on the other hand, was not different in the control compared with the diabetic group. Furthermore, TxnIP protein was increased by 17% when compared to control subjects. The increase in TxnIP expression/protein was associated with a 21% decrease in thioredoxin activity (figure 1, all $p < 0.05$).

TxnIP overexpression in experimental diabetes

In order to assess the role of oxidative stress in experimental diabetic cardiomyopathy, we first assessed whether experimental diabetes altered TRX and TxnIP gene expression in animal models of both type 1 and type 2 diabetes.

Type 1 diabetes

Plasma glucose confirmed the presence of diabetes in STZ-treated rats (mRen)2-27 transgenic rats. Diabetes was associated with reduced body mass and heart weight to body weight ratio when compared with control animals. Diabetic animals demonstrated diastolic dysfunction as evidenced by increased slope of

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the end-diastolic pressure volume relationship, as well as impaired early active relaxation as evidenced by prolonged Tau (Table 2).

The STZ treated (mRen)2-27 transgenic rats demonstrated increased extracellular matrix deposition in the subendocardial region of the left ventricle, along with cardiomyocyte hypertrophy. Diabetes was associated with a significant increase in cardiac oxidative stress as measured by 3-nitrotyrosine abundance (figure 2).

Quantitation of TxnIP gene expression by real time PCR revealed a ~2-fold increase in mRNA in LV specimens in experimental diabetic cardiomyopathy when compared with control animals. Thioredoxin gene expression, on the other hand, was substantially lower in the experimental diabetic cardiomyopathy relative to its the control group (Figure 3). TxnIP protein expression was mildly increased (11%, $p<0.05$) when compared to Ren-2 control animals. However, there was a 23% reduction in thioredoxin activity seen in diabetic Ren-2 rats ($p<0.05$, figure 3).

Type 2 diabetes

Goto-Kakizaki rats developed diabetes at around 8 weeks of age. By 20 weeks of age, they develop left ventricular hypertrophy and diastolic dysfunction (all $p<0.05$) when compared to Wistar control rats (see table 3).

Diabetic GK rats demonstrated evidence for cardiac oxidative stress with a significant increase in DHE expression compared with Wistar controls. Furthermore, GK rats at 20 weeks of age demonstrated an ~10 fold increase in TxnIP expression when compared with Wistar control. Thioredoxin mRNA expression was not different between GK and Wistar control animals. Like the human diabetic samples

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and the rodent model of type 1 diabetes, the increased TxnIP expression was associated with a significant reduction in thioredoxin expression (~49% reduction, $p < 0.05$, see figure 4).

TxnIP overexpression mediates high glucose induced reduction in thioredoxin activity in cardiac myocytes

Given the *in vivo* findings of TxnIP over-expression in patients and rodents with diabetes, we next examined its expression in the *in vitro* setting in a range of cardiac cell types. Cultured rat neonatal Sprague Dawley cardiomyocytes and H9c2 rat cardiomyoblasts in high glucose for 48 hours led to a 2-3 fold increase in TxnIP expression (Figure 5) accompanied by a reduction in the enzymatic activity of thioredoxin, as assessed by the insulin disulphide reaction ($p < 0.01$). Furthermore, high glucose increased oxidative stress as assessed by CFDA fluorescence and DHE staining (all $p < 0.05$ when compared with 5.6 mmol/L glucose or mannitol). To confirm that these latter effects were mediated by high glucose-induced TxnIP overexpression, we used a specific siRNA to prevent it. These studies showed that despite a persistently high glucose, TxnIP siRNA restored thioredoxin activity to control levels, and reduced oxidative stress. Scrambled siRNA had no effect upon TRX activity or oxidative stress. (Figure 6).

DISCUSSION

The excessive production of ROS is a consistent feature of cells that have been exposed to high glucose concentrations, providing a unifying mechanism in the pathogenesis of diabetic complications [8]. This paradigm is based on the knowledge that increased intracellular glucose leads to the overproduction of superoxide by the mitochondrial electron-transport chain resulting in increased ROS formation [32]. Activity of anti-oxidant systems, such as thioredoxin, is accordingly an important defence against diabetes-associated, ROS-mediated cell injury. In the present report we show that diabetes leads to a dramatic and paradoxical rise in the abundance of TxnIP, an inhibitor of the thioredoxin system. These findings were not only evident in the hearts of patients with diabetes and coronary artery disease but in correlative experimental studies, similar changes were also seen in animals with experimental diabetic cardiomyopathy and shown to impact on the reductive capacity of cardiac myocytes.

Thioredoxin (TRX) is a selenium containing disulphide reductase that reversibly catalyses the reduction of disulphide bonds on cysteine residues by binding them to its active Cys-Gly-Pro-Cys site [33]. The activity of TRX1 is functionally regulated by an endogenous inhibitor, TxnIP [34] which impedes its activity by interacting directly with its catalytic site [35]. In the present study, we not only found that TRX activity was reduced by high glucose, but that attenuating TxnIP translation by siRNA enhanced thioredoxin activity. This finding suggests that TxnIP mediated

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inhibition of TRX represents a logical therapeutic target in order to combat diabetic complications. Indeed, Chen et al demonstrated that despite severe diabetes, diabetic animals treated with calcium channel blockers reduced TxnIP expression which was associated with a significant reduction in cardiomyocyte apoptosis [36]. Furthermore, the increase in TxnIP occurred in both experimental models of type 1 and type 2 diabetes, as well as in human biopsy specimens from patients with type 2 diabetes, which represent the vast majority of patients at risk of significant diabetic complications.

In the non-diabetes setting, increased ROS, formed as a consequence of ischemia-reperfusion injury in the heart [37], or in response to photic injury in the retina [38], leads to enhanced activity of the thioredoxin system, providing an important defence against oxidative injury. However, in contrast to these adaptive responses, the present study shows that despite the increase in oxidative stress induced by high glucose, cardiac myocytes showed a paradoxical reduction in thioredoxin system activity, as a consequence of TxnIP overexpression. Not only was this maladaptive response evident in cells and rodents with experimental diabetes, but the over-expression of TxnIP was even more apparent in the human setting where diabetes was accompanied by a 38-fold increase in TxnIP mRNA, along with a 21% reduction in TRX activity.

The diabetes-induced over-expression of TxnIP and consequent depression of thioredoxin activity that we found in the heart is not unique to this organ. In

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addition to a range of in vitro and experimental animal studies [15-18, 39], reports of similar findings in the human kidney biopsies [19] complement our findings in the hearts of patients with diabetes undergoing coronary artery bypass surgery. Indeed, the presence of a carbohydrate response element (ChoRE) in the TxnIP promoter [39] suggest that increased TxnIP expression and consequent diminished thioredoxin activity may account for the increased ROS in tissues where glucose entry in to cells follows its ambient concentration within plasma.

In addition to its role in ROS, the TxnIP also has important roles in the regulation of intermediary metabolism. For instance, TxnIP gene deletion shifts heart and skeletal muscle fuel dependence from fat and ketone bodies to glucose [40] suggesting that the increase in cardiac TxnIP, as demonstrated in the present study, may underlie the reverse metabolic shift, characteristic of diabetic heart disease [41]. Indeed, Yoshioka et al, using TxnIP deletion mice, demonstrated that TxnIP deletion suppresses mitochondrial function, but in the setting of ischemia reperfusion coordinates a shift to enhanced anaerobic metabolism, thus providing an alternative energy source outside the mitochondria [42]. Furthermore, using proteomic screening with glutathione S-transferase interaction pulldown assays in lysates from cells overexpressing TxnIP, the authors demonstrated several proteins involved in metabolism for which there was a putative interaction – suggesting a direct role for TxnIP overexpression in modulating metabolism. However, at present, the exact role is unclear and future studies are required to define the role increased TxnIP expression plays in modulating cellular metabolism in the diabetes setting.

Our study does have limitations. Firstly, we assessed human right atrial tissue, whereas the experimental studies assessed TxnIP expression in the left ventricles of rats. Unfortunately, we were unable to obtain human LV biopsy specimens from patients with diabetes. However, hyperglycemia has been shown to increase TxnIP expression in multiple tissue cell types such as renal proximal tubular cells, mesangial cells, hepatic cells, endothelial cells, atrial and ventricular cardiomyocytes [19, 43], suggesting that the results are generalizable to human LV specimens. Indeed, the ubiquitous expression of the ChoRE in glucose responsive tissues (such as cardiomyocytes) suggest this response is a fundamental mechanism in energy metabolism and modifying the cellular response to oxidative stress [44].

Secondly, we observed some variability in the response of tissues studied to upregulate TxnIP. Both human atrial tissue and ventricular tissue from GK rats demonstrated a substantially greater increase in TxnIP mRNA than the H9c2 cells and diabetic Ren-2 rats. Whilst hyperglycemia is the *sine qua non* of diabetes, type 2 diabetes represents a spectrum of diseases associated with insulin resistance, obesity, hypertension etc which develops over a significant time period, as opposed to the streptozotocin model of diabetes which manifests as a profound insulin deficiency over a short time frame, akin to type 1 diabetes. The multifactorial nature and duration of type 2 diabetes likely accounts for such a difference. Furthermore, our results are limited by the small sample size, and the potential influence of oral hypoglycemic medications and agents which

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antagonize the renin angiotensin system, itself known to modify oxidative stress which may also influence TxnIP expression.

CONCLUSION

In summary, using human biopsy findings in conjunction with experimental animal and *in vitro* studies, the present report shows that in addition to increased ROS production, dysregulation of the thioredoxin system as a consequence of diabetes-induced changes to TxnIP and also TRX may contribute to oxidative stress and thereby the pathogenesis of heart disease in diabetes. TxnIP overexpression may serve as a new target for therapeutic development in the treatment of diabetic complications.

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Conflict of Interest: Kim A Connelly, Andrew Advani, Suzanne L Advani, Yuan Zhang, Young M Kim, Vanessa Shen, Kerri Thai, Darren J Kelly and Richard E Gilbert declare that they have no conflict of interest.

References:

1. Alter DA, Khaykin Y, Austin PC, et al. (2003) Processes and Outcomes of Care for Diabetic Acute Myocardial Infarction Patients in Ontario: Do physicians undertreat? *Diabetes Care* 26:1427–1434. doi: 10.2337/diacare.26.5.1427

2. Uchmanowicz I, Lobo-Grudzien K, Jankowska-Polanska B, Sokalski L (2011) Influence of diabetes on health-related quality of life results in patients with acute coronary syndrome treated with coronary angioplasty. *Acta Diabetol* 50:217–225. doi: 10.1007/s00592-011-0280-2
3. Donahoe SM, Stewart GC, McCabe CH, et al. (2007) Diabetes and mortality following acute coronary syndromes. *JAMA* 298:765–775. doi: 10.1001/jama.298.7.765
4. Woodfield SL, Lundergan CF, Reiner JS, et al. (1996) Angiographic Findings and Outcome in Diabetic Patients Treated With Thrombolytic Therapy for Acute Myocardial Infarction: The GUSTO-I Experience. *JAC* 28:1661–1669. doi: 10.1016/S0735-1097(96)00397-X
5. Gao F, Zhou YJ, Shen H, et al. (2012) Meta-analysis of percutaneous coronary intervention versus coronary artery bypass graft surgery in patients with diabetes and left main and/or multivessel coronary artery disease. *Acta Diabetol* 50:765–773. doi: 10.1007/s00592-012-0411-4
6. Farkouh ME, Domanski M, Sleeper LA, et al. (2012) Strategies for Multivessel Revascularization in Patients with Diabetes. *N Engl J Med* 367:2375–2384. doi: 10.1056/NEJMoa1211585
7. Tarquini R, Lazzeri C, Pala L, et al. (2010) The diabetic cardiomyopathy. *Acta Diabetol* 48:173–181. doi: 10.1007/s00592-010-0180-x
8. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820. doi: 10.1038/414813a
9. Berlett BS (1997) Protein Oxidation in Aging, Disease, and Oxidative Stress. *Journal of Biological Chemistry* 272:20313–20316. doi: 10.1074/jbc.272.33.20313
10. Yamawaki H, Berk BC (2005) Thioredoxin: a multifunctional antioxidant enzyme in kidney, heart and vessels. *Current Opinion in Nephrology and Hypertension* 14:149.
11. Emiroglu MY, Esen ÖB, Bulut M, et al. (2010) GGT levels in type II diabetic patients with acute coronary syndrome (does diabetes have any effect on GGT levels in acute coronary syndrome?). *Acta Diabetol* 50:21–25. doi: 10.1007/s00592-010-0208-2
12. Chen K-S, DeLuca HF (1994) Isolation and characterization of a novel cDNA from HL-60 cells treated with 1,25-dihydroxyvitamin D-3. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1219:26–32. doi: 10.1016/0167-4781(94)90242-9
13. Xiang G, Seki T, Schuster MD, et al. (2005) Catalytic degradation of vitamin D up-regulated protein 1 mRNA enhances cardiomyocyte survival and prevents

- left ventricular remodeling after myocardial ischemia. *J Biol Chem* 280:39394–39402. doi: 10.1074/jbc.M502966200
14. Cheng DW, Jiang Y, Shalev A, et al. (2006) An analysis of high glucose and glucosamine-induced gene expression and oxidative stress in renal mesangial cells. *Archives of Physiology and Biochemistry* 112:189–218. doi: 10.1080/13813450601093518
 15. Hirota T, Okano T, Kokame K, et al. (2002) Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J Biol Chem* 277:44244–44251. doi: 10.1074/jbc.M206233200
 16. Kobayashi T, Uehara S, Ikeda T, et al. (2003) Vitamin D3 up-regulated protein-1 regulates collagen expression in mesangial cells. *Kidney Int* 64:1632–1642. doi: 10.1046/j.1523-1755.2003.00263.x
 17. Qi W, CHEN X, Gilbert RE, et al. (2007) High Glucose-Induced Thioredoxin-Interacting Protein in Renal Proximal Tubule Cells Is Independent of Transforming Growth Factor- β 1. *The American Journal of Pathology* 171:744–754. doi: 10.2353/ajpath.2007.060813
 18. Schulze PC, Yoshioka J, Takahashi T, et al. (2004) Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* 279:30369–30374. doi: 10.1074/jbc.M400549200
 19. Advani A, Gilbert RE, Thai K, et al. (2009) Expression, localization, and function of the thioredoxin system in diabetic nephropathy. *J Am Soc Nephrol* 20:730–741. doi: 10.1681/ASN.2008020142
 20. Advani A, Kelly DJ, Cox AJ, et al. (2009) The (Pro)renin receptor: site-specific and functional linkage to the vacuolar H⁺-ATPase in the kidney. *Hypertension* 54:261–269. doi: 10.1161/HYPERTENSIONAHA.109.128645
 21. Connelly KA, Advani A, Kim S, et al. (2011) The cardiac (pro)renin receptor is primarily expressed in myocyte transverse tubules and is increased in experimental diabetic cardiomyopathy. *J Hypertens* 29:1175–1184. doi: 10.1097/HJH.0b013e3283462674
 22. Holmgren A, Björnstedt M (1995) Thioredoxin and thioredoxin reductase. *Meth Enzymol* 252:199–208.
 23. Connelly KA, Kelly DJ, Zhang Y, et al. (2007) Functional, structural and molecular aspects of diastolic heart failure in the diabetic (mRen-2)27 rat. *Cardiovasc Res* 76:280–291. doi: 10.1016/j.cardiores.2007.06.022
 24. Kuliszewski M, Kabir G, Fujii H, et al. (2011) 724 Characterisation of a novel rodent model of diabetes induced heart failure with preserved ejection fraction - The Goto Kakizaki rat. *Canadian Journal of Cardiology* 27:S328–S328. doi:

10.1016/j.cjca.2011.07.598

25. Vahtola E, Louhelainen M, Merasto S, et al. (2008) Forkhead class O transcription factor 3a activation and Sirtuin1 overexpression in the hypertrophied myocardium of the diabetic Goto-Kakizaki rat. *J Hypertens* 26:334–344. doi: 10.1097/HJH.0b013e3282f293c8
26. Connelly KA, Prior DL, Kelly DJ, et al. (2006) Load-sensitive measures may overestimate global systolic function in the presence of left ventricular hypertrophy: a comparison with load-insensitive measures. *Am J Physiol Heart Circ Physiol* 290:H1699–705. doi: 10.1152/ajpheart.00577.2005
27. Georgakopoulos D, Mitzner WA, Chen CH, et al. (1998) In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. *Am J Physiol* 274:H1416–22.
28. Pacher P, Nagayama T, Mukhopadhyay P, et al. (2008) Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc* 3:1422–1434. doi: 10.1038/nprot.2008.138
29. Yang B, Larson DF, Beischel J, et al. (2001) Validation of conductance catheter system for quantification of murine pressure-volume loops. *J Invest Surg* 14:341–355. doi: 10.1080/089419301753435710
30. Kelly DJ, Zhang Y, Hepper C, et al. (2003) Protein kinase C beta inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension. *Diabetes* 52:512–518.
31. Kai H, Muraishi A, Sugiu Y, et al. (1998) Expression of proto-oncogenes and gene mutation of sarcomeric proteins in patients with hypertrophic cardiomyopathy. *Circulation Research* 83:594–601.
32. Brownlee M (2005) The pathobiology of diabetic complications a unifying mechanism. *Diabetes* 54:1615–1625.
33. Berndt C, Lillig CH, Holmgren A (2006) Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. *AJP: Heart and Circulatory Physiology* 292:H1227–H1236. doi: 10.1152/ajpheart.01162.2006
34. Nishiyama A, Matsui M, Iwata S, et al. (1999) Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem* 274:21645–21650.
35. Junn E, Han SH, Im JY, et al. (2000) Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol* 164:6287–6295.

36. Chen J, Cha-Molstad H, Szabo A, Shalev A (2009) Diabetes induces and calcium channel blockers prevent cardiac expression of proapoptotic thioredoxin-interacting protein. *Am J Physiol Endocrinol Metab* 296:E1133–9. doi: 10.1152/ajpendo.90944.2008
37. Turoczi T, Chang VW-H, Engelman RM, et al. (2003) Thioredoxin redox signaling in the ischemic heart: an insight with transgenic mice overexpressing Trx1. *Journal of Molecular and Cellular Cardiology* 35:695–704.
38. Tanito M, Masutani H, Nakamura H, et al. (2002) Cytoprotective effect of thioredoxin against retinal photic injury in mice. *Invest Ophthalmol Vis Sci* 43:1162–1167.
39. Minn AH, Hafele C, Shalev A (2005) Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. *Endocrinology* 146:2397–2405. doi: 10.1210/en.2004-1378
40. Hui STY, Andres AM, Miller AK, et al. (2008) Txnip balances metabolic and growth signaling via PTEN disulfide reduction. *Proc Natl Acad Sci USA* 105:3921–3926. doi: 10.1073/pnas.0800293105
41. Boudina S, Abel ED (2007) Diabetic Cardiomyopathy Revisited. *Circulation* 115:3213–3223. doi: 10.1161/CIRCULATIONAHA.106.679597
42. Yoshioka J, Chutkow WA, Lee S, et al. (2012) Deletion of thioredoxin-interacting protein in mice impairs mitochondrial function but protects the myocardium from ischemia-reperfusion injury. *J Clin Invest* 122:267–279. doi: 10.1172/JCI44927DS1
43. Li X, Rong Y, Zhang M, et al. (2009) Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells. *Biochem Biophys Res Commun* 381:660–665. doi: 10.1016/j.bbrc.2009.02.132
44. Ma L, Robinson LN, Towle HC (2006) ChREBP*MLx Is the Principal Mediator of Glucose-induced Gene Expression in the Liver. *Journal of Biological Chemistry* 281:28721–28730. doi: 10.1074/jbc.M601576200

Figure legends.

Figure 1. TxnIP and TRX in human right atrial biopsy specimens (n=7 per group). Diabetes was associated with no change in TRX expression (A), a ~38 fold rise in TxnIP mRNA expression (B, * $p<0.0001$), and a reduction in thioredoxin activity (C, * $p<0.05$). Diabetes patients demonstrated a 17% increase in TxnIP protein expression (D and E, * $p<0.05$).

Figure 2: 3-Nitrotyrosine expression as a marker of oxidative stress in control (A) and diabetic (B) rats. Diabetes was associated with increased 3-NT expression ($p<0.05$).

Figure 3. Experimental diabetes in the diabetic Ren-2 rat was associated with reduced TRX mRNA (a), increased TxnIP mRNA (B) and thioredoxin activity was

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reduced by 23% in diabetic Ren-2 rats (C), c/w control animals (N=10 per group, * $p<0.01$). TxnIP protein was increased in diabetic Ren-2 rats (D and E, * $p<0.05$).

Figure 4: Experimental type 2 diabetes in the GK rat was associated with increased oxidative stress, as assessed by dihydroethidium (DHE) staining in the cytoplasm of the left ventricle. GK rats demonstrated no change in thioredoxin mRNA, increased TxnIP mRNA and reduced thioredoxin activity when compared to Wistar controls (n=6 per group, * = $p<0.05$ c/w Wistar control).

Figure 5: TxnIP mRNA in neonatal cardiomyocytes and H9c2 cardiomyoblasts exposed to normal (5.6 mmol/l) and high (25 mmol/l) glucose for 48 hours. Both neonatal cardiomyocytes and H9c2 cells demonstrated increased TxnIP expression when exposed to high glucose (* $p<0.01$).

Figure 6: High glucose (25 mmol/l) was associated with reduced thioredoxin activity and increased oxidative stress as measured by CFDA and DHE fluorescence in cardiac H9c2 cells. The increase in TxnIP and ROS under high glucose conditions was inhibited by TxnIP siRNA ($p<0.01$) but not scramble RNA, consistent with TxnIP overexpression as a key mediator of glucose-induced reduction in thioredoxin activity. Mannitol as an osmotic control had no effect upon TRX activity or ROS production. * $p<0.01$ c/w 5.6 mmol/l glucose (NG). † $p<0.05$ c/w 25mmol/l glucose (HG) ‡ $p<0.05$ c/w siRNA treated cells.

Figure 1:

Figure 2:

Figure 3:

Figure 4:

Figure 5:

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Figure 6:

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Table 1. Clinical Characteristics of patients in whom right atrial biopsies were obtained. Data is presented as mean $\pm$ S.D. Number in parentheses represent

	No diabetes (N=7)	Diabetes (N=7)
Age, years	66 $\pm$ 16	68 $\pm$ 13
Sex, males:females	5:2	5:2
HbA1c (%)	-	7.2 $\pm$ 0.1
Surgical procedure, n (%)		
CABG	6 (86)	7 (100)
AVR	1 (14)	0
Medical history, n (%)		
Smoking	0	0
Hypertension	5 (71)	6 (86)
Diabetes mellitus	0	7 (100)
Dyslipidemia	6 (86)	7 (100)
Previous MI	4 (57)	4 (57)
Medications, n (%)		
Aspirin	7 (100)	6 (86)
β-blockers	6 (86)	6 (86)
Statins	6 (86)	6 (86)
Nitrates	4 (57)	1 (14)
Calcium antagonists	1 (14)	1 (14)
ACE inhibitors/ARBs	4 (57)	5 (71)
Insulin	0	3 (43)
Oral anti-hyperglycemic drugs	0	5 (71)

%.

Table 2: Animal characteristics of control and diabetic TGR(mRen)2-27 rats. Data is presented as mean +/-S.E.M. 3-NT is 3 –nitrotyrosine. EDPVR is the slope of the end-

	Control	Diabetic
N	10	10
Body weight (g)	302 ±15	236±7*
Plasma glucose (mmol/L)	6±0.5	26±0.5 *

diastolic pressure volume relationship. A steeper value represents worse diastolic function. * is $p < 0.05$ c/w control.

3-NT (% proportional area)	1.14 ± 0.47	3.8 ± 1.3 *
Cardiomyocyte diameter (μm)	17.3 ± 0.45	22.35 ± 0.32*
Collagen I (AU)	0.98 ± 0.18	3.5 ± 0.38*
EDPVR (mmHg/μl)	0.026 ± 0.006	0.055 ± 0.006*
	Wistar	GK rats

Table 3: Animal characteristics of Wistar control and Goto Kakizaki rats. Data is presented as mean +/-S.E.M. Tau is the time constant of relaxation. DHE is dihydroethidium staining. * is p<0.05 c/w control.

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N	6	6
Body weight (g)	614 ±25	401 ±10*
HbA1c (%)	4.5 ± 0.1	8.8 ± 1.2 *
Height weight: body weight (mg/g)	2.5 ± 0.07	3.0 ± 0.2 *
Lung weight: Body weight (mg/g)	2.7 ± 0.07	3.8 ± 0.3*
DHE staining (AU)	19.35 ± 2.4	38 ± 1.3 *
Tau (msec)	6.3 ± 0.2	8.1 ± 0.4 *