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Heredity of type 2 diabetes confers increased susceptibility to oxidative stress and inflammation

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ABSTRACT

Introduction and objective Heredity of type 2 diabetes mellitus (T2DM) is associated with greater risk for developing T2DM. Thus, individuals who have a first-degree relative with T2DM (FDRT) provide a natural model to study factors of susceptibility towards development of T2DM, which are poorly understood. Emerging key players in T2DM pathophysiology such as adverse oxidative stress and inflammatory responses could be among possible mechanisms that predispose FDRTs to develop T2DM. Here, we aimed to examine the role of oxidative stress and inflammatory responses as mediators of this excess risk by studying dynamic postprandial responses in FDRTs.

Research design and methods In this open-label case-control study, we recruited normoglycemic men with (n=9) or without (n=9) a family history of T2DM. We assessed plasma glucose, insulin, lipid profile, cytokines and F_2-isoprostanes, expression levels of oxidative and inflammatory genes/proteins in circulating mononuclear cells (MNC), myotubes and adipocytes at baseline (fasting state), and after consumption of a carbohydrate-rich liquid meal or insulin stimulation.

Results Postprandial glucose and insulin responses were not different between groups. Expression of oxidant transcription factor NRF2 protein ($p<0.05$ for myotubes) and gene ($p_{group}=0.002$, $p_{time\times group}=0.016$), along with its target genes TXNRD1 ($p_{group}=0.004$, $p_{time\times group}=0.007$), GPX3 ($p_{group}=0.011$, $p_{time\times group}=0.019$) and SOD-1 ($p_{group}=0.046$ and $p_{time\times group}=0.191$) was upregulated in FDRT-derived MNC after meal ingestion or insulin stimulation. Synergistically, expression of target genes of inflammatory transcription factor nuclear factor kappa B such as tumor necrosis factor alpha ($p_{group}=0.001$, $p_{time\times group}=0.007$) was greater in FDRT-derived MNC than in non-FDRT-derived MNC after meal ingestion or insulin stimulation.

Conclusions Our findings shed light on how heredity of T2DM confers increased susceptibility to oxidative stress and inflammation. This could provide early insights into the underlying mechanisms and future risk of FDRTs for developing T2DM and its associated complications.

Significance of this study

What is already known about this subject?

- Oxidative stress and inflammation can interfere with insulin signaling and contribute to the development of insulin resistance, type 2 diabetes mellitus (T2DM) and its associated complications.
- Heredity of T2DM is a major risk factor for developing T2DM but little is known of the factors mediating the increased risk.

What are the new findings?

- Individuals with a first-degree relative with T2DM (FDRT) show increased oxidative stress and inflammation in response to a high-carbohydrate challenge as compared with non-FDRT individuals. These observations are independent of age, body mass index and insulin sensitivity.
- The adverse oxidative stress and inflammatory responses following the meal challenge are observed in circulating mononuclear blood cells. Moreover, when an insulin stimulus was used to mimic the meal challenge in vitro, myotubes and adipocytes isolated from FDRT individuals also showed increased oxidative stress and inflammatory responses as compared with non-FDRT individuals.
- Individuals with heredity of T2DM are susceptible to adverse dynamic postprandial stress responses.

How might these results change the focus of research or clinical practice?

- Our observation that heredity of T2DM confers increased susceptibility to oxidative stress and inflammation following a high-carbohydrate meal could provide early insights into the underlying mechanisms and future risk of FDRTs for developing T2DM and its complications.

INTRODUCTION

Oxidative stress and inflammation induced by nutritional excess can contribute to impaired insulin receptor signaling in insulin-sensitive target tissues,[1–5] leading to insulin resistance, development of type 2 diabetes mellitus (T2DM) and diabetic vascular complications.[6–8] Defects in insulin signaling are inherited, and insulin action in offspring of patients with T2DM has been shown to be the

best predictor for development of the disease.[9–11] The cumulative incidence of T2DM among individuals with a first-degree relative with T2DM (FDRT) is 40%–76%.[12 13] Thus, normoglycemic individuals with heredity of T2DM constitute an ideal natural model for studying early pathophysiological events during the development and progression of T2DM. In the basal state, FDRTs demonstrate inherited defects in muscle fiber composition and function, for example, reversal of type I (oxidative) to type II (glycolytic) fiber ratio[14] and compromised muscle mitochondrial biogenesis and oxidative metabolism.[15 16]

Although a family history of T2DM is an established risk factor for development of T2DM, the factors mediating this excess risk remain poorly understood. Major T2DM risk factors such as anthropometric measures, lifestyle risk factors and genetic risk score all together have been shown to explain only 13% of the risk of T2DM associated with family history.[17] Accumulating evidence suggests an association of heredity of T2DM and enhanced levels of systemic inflammatory and oxidative stress in the fasted state,[18–20] which is aggravated after an acute high-fat meal challenge.[21 22] Cumulative perturbations in the regulation of oxidative stress and inflammatory responses to meal ingestion may contribute to the greater risk for T2DM and its cardiovascular complications among FDRT individuals.[23 24]

We have previously shown that a single meal rich in carbohydrate, but not fat or protein, elicits adverse inflammatory, metabolic, hormonal as well as oxidative stress responses in obese insulin-resistant individuals with normal glucose tolerance.[25–28] This work led us to enquire whether a high-carbohydrate meal challenge could also induce distinct oxidative and inflammatory responses in another high-risk metabolic phenotype, namely FDRTs. Here, we aimed to examine the role of oxidative stress and inflammatory responses as mediators of excess risk of T2DM in FDRTs by studying dynamic postprandial responses in them. This could provide early insights into underlying mechanisms and future risk of FDRTs for developing T2DM.

To this end, we characterized these responses in young, overweight and obese individuals with or without a family history of T2DM before and after a test meal. We investigated the effects of a single meal rich in carbohydrate on the expression profile of oxidative stress and inflammatory genes, proteins and metabolites in circulating mononuclear cells (MNC), myotubes, adipocytes and plasma.

RESEARCH DESIGN AND METHODS

Participants

This study was conducted at the National University of Singapore School of Medicine, Singapore. Study participants were recruited from October 2013 until February 2016. Data collection was carried out from March 2015 to November 2018. We studied normoglycemic (fasting blood glucose <5.6 mmol/L) Chinese men with (n=9) or without (n=9) a first-degree relative with type 2 diabetes (age: 21–40 years, body mass index (BMI): 23.0–27.5 kg/m^2). The study participants were selected from a larger group of subjects and matched one to one for BMI and insulin sensitivity (homeostatic model assessment of insulin resistance). Subjects in both groups had no history of smoking, alcohol consumption, overt disease, medication intake, atypical dietary habits, hospitalization or surgery in the last 6 months and weight change >5% in the last 3 months.

Experimental design

This was an open-label case-control study. Fasting blood samples were collected to measure serum insulin, glucose, non-esterified fatty acid (NEFA) concentrations, and lipid profile. Eligible participants, after having fasted overnight and abstained from vigorous physical activity for 24 hours, ingested a carbohydrate-rich liquid mixed meal (~600 kcal; 57% carbohydrate, 29% fat and 14% protein; ~400 mL; Ensure Plus, Abbott Nutrition), within 5 min. Fasting and postprandial (0, 30, 60, 90, 120, 180, 240, 300, and 360 min) venous blood samples were collected for the measurement of glucose, insulin, triglyceride (TG) and NEFA concentrations. Fasting and postprandial (360 min) midstream urine samples were also collected for the measurement of urinary F_2-isoprostanes, a biomarker of oxidative stress-induced lipid peroxidation.

To isolate MNCs by density gradient centrifugation, 9 mL of blood was collected into tubes containing EDTA at 0, 120, and 360 min.

Skeletal muscle and adipose tissue samples were taken from lateral aspect of the upper thigh of consenting subjects (n=5 per group) in the fasted state, as previously described.[5] Approximately 500 mg of subcutaneous adipose tissue and 200 mg of muscle tissue were excised and immediately placed in phosphate-buffered saline (PBS) and processed as detailed below.

Human preadipocyte isolation and primary culture

Subcutaneous white adipose tissue samples were minced and digested with collagenase (1 mg/mL) (Sigma-Aldrich, Israel) and supplemented with 2% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 1 hour. Digested tissue was then filtered through a 100 µm cell strainer (SPL Life Sciences, Gyeonggi-do, Korea), followed by several washes in PBS, and centrifugation (400 *g* for 5 min).

The stromal vascular fraction (SVF) containing preadipocytes was then resuspended in growth medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone-GE Healthcare, Logan, UT, USA), 15% fetal bovine serum (FBS) (Hyclone-GE Healthcare), 1% non-essential amino acids (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin (PS) (Hyclone-GE Healthcare) and 5 ng/mL fibroblast growth factor (Gibco, Frederick, MD, USA). Cells were grown at 37°C and 5% CO_2.

When preadipocytes displayed fibroblastic morphology, they were differentiated using Lonza's PGM-2 Adipocyte Differentiation Kit (catalog number PT8002, Lonza, Basel, Switzerland) for 14 days following the manufacturer's protocol. Mature adipocytes were then serum starved for 3 hours after which cells were stimulated with 100 and 1000 nM insulin (Gibco, Grand Island, NY, USA) for 10–30 min. Cells were subsequently harvested for glutathione (GSH) assay, RNA and protein.

Human myoblast isolation and primary culture

Skeletal muscle tissue biopsies collected in PBS were digested with collagenase (1 mg/mL) (Sigma-Aldrich, Israel) for 30–45 min at 37°C to isolate myoblast cells. Tissue digest was passed through a 100 µm cell strainer (SPL Life Sciences), washed several times in PBS, and pelleted down at 2200 g for 10 min. The cell pellet was then resuspended in myoblast growth media consisting of DMEM (Hyclone-GE Healthcare) supplemented with 20% FBS (Hyclone-GE Healthcare), 10% horse serum (Gibco, New Zealand), and 1% PS (Hyclone-GE Healthcare) and plated in an uncoated cell culture dish for 2 hours at 37°C and 5% CO_2.

Thereafter, the cell suspension containing myoblasts was transferred to a Matrigel-coated (Corning, Bedford, MA, USA) cell culture dish and grown to 80% confluence. Differentiation of myoblasts into myotubes was initiated using differentiation medium (DMEM supplemented with 2% horse serum and 1% PS) for 7 days in Matrigel-coated plates. Following differentiation, myotubes were serum starved for 3 hours, and subsequently underwent stimulation with 100 and 1000 nM insulin (Gibco, Grand Island, NY, USA) for 10–30 min, after which they were harvested for GSH assay, RNA and protein.

Biochemical analyses

Plasma glucose and TG concentrations were measured by using enzymatic and colorimetric methods, respectively (AU5800, Beckman Coulter, California, USA). Serum insulin was measured by using a chemiluminescence immunoassay (ADVIA Centaur, Siemens Healthcare Diagnostics, Hamburg, Germany). These analyses were carried out by a laboratory accredited by the College of American Pathologists. Plasma NEFA was measured at Mayo Medical Laboratories (Rochester, MN, USA) using an enzymatic colorimetric method (Cobas 6000, Roche Diagnostics, Indianapolis, USA).

Plasma interleukin-6 (IL-6) (catalog number HS600B) concentration was measured using Quantikine high-sensitivity ELISA kit (R&D Systems, Minneapolis, MN, USA). Plasma tumor necrosis factor alpha (TNFα) concentration was measured using an ultrasensitive ELISA kit (catalog number 45-TNFHUU-E01, Alpco Diagnostics, Salem, NH, USA). Intra-assay and interassay coefficients of variations for IL-6 and TNFα were <10%.

Plasma total (free and esterified) and urinary free F_2-isoprostanes were measured using a method described previously.[29 30] The isoprostane levels in plasma and urine were normalized against arachidonic acid and creatinine levels, respectively.

Gene expression assay

Real-time reverse transcription-PCR was performed using ViiA 7 Real-Time PCR System (Applied Biosystems). The expression of housekeeping gene GAPDH was stable and did not show significant variation across the different time points and subject groups. The panel of inflammatory genes assessed included IL-6, TNFα, IL-1β, IL-18, IL-8, IL-10, transforming growth factor-β, Toll-like receptor 4, monocyte chemoattractant protein-1, and nuclear factor kappa B (NF-κB) gene complex, that is, Rel-A (p65 subunit of NF-κB), p105 (precursor of p50 subunit of NF-κB), IκB-α and IκB-β (inhibitors of NF-κB). The panel of oxidative stress genes examined included NRF2, GPX3, TXN, TXNRD1, SOD1, SOD2, CYBA, CYBB, NCF-1, NCF-2 and NCF-4.

Reduced/oxidized glutathione ratio detection assay

Following 7 days of differentiation and 10–30 min of insulin stimulation, cells (adipocytes and myotubes) were washed, lifted, lysed and deproteinized (catalog number 204708, Abcam, Cambridge, UK). Reduced/oxidized glutathione (GSH/GSSG) ratio detection assay was performed subsequently using cell lysates (catalog number 205811, Abcam). GSH and GSSG were quantified on a fluorescent microplate reader at excitation/emission wavelength set to 490/520 nm. Absolute amounts of GSH and GSSG were determined using GSH and GSSG standard curves.

Western blots

Western blot was carried out from differentiated adipocytes and myotubes derived from a subset of individuals undergoing the meal challenge, subject to availability of tissue biopsies. NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, USA) was used for protein isolation according to the manufacturer's protocol. Concentration of protein lysates was determined using Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Cytosolic and nuclear lysates (30 µg) were resolved by 4%–20% Mini-PROTEAN polyacrylamide gradient gel (Bio-Rad Laboratories) electrophoresis. Separated proteins were then transferred to PolyVinylidene DiFluoride (PVDF) membranes (Bio-Rad Laboratories) and blocked with StartingBlock Blocking Buffer (Thermo Scientific) for 1 hour. Membranes were incubated overnight at 4°C with the following antibodies: Anti-NRF2 mAb (catalog number 12721; Cell Signaling Technology, Danvers, MA, USA), Anti-NF-κB p65 mAb (catalog number 32536; Abcam), Anti-GAPDH mAb (catalog number 5174; Cell Signaling Technology), and Anti-Histone H3 mAb (catalog number 4499, Cell Signaling Technology). Thereafter, membranes were incubated with appropriate secondary horseradish peroxidase-conjugated antibodies

(Bio-Rad Laboratories) for 2 hours in blocking solution, subsequent to washing in PBST.

Proteins of interest were visualized by enhanced chemiluminescence (Millipore, Billerica, MA, USA) and exposed to imaging film. Molecular sizes of targets were estimated using prestained protein ladder (Thermo, Rockford, IL, USA). Signal intensities were quantified by density analysis using ImageJ software.

Genetic loci analysis

We retrieved a list of T2DM susceptibility genetic loci (178 BMI-unadjusted loci and 10 BMI-adjusted loci) in East-Asian meta-analyses.[31] We determined if any of these 188 T2DM susceptibility genetic loci were cis-expression quantitative trait loci (eQTL) or in linkage disequilibrium (LD) with cis-eQTLs for the panel of inflammatory and oxidative stress genes included in this study, from Genome-Tissue Expression (GTEx) repository (https://www.gtexportal.org/home/). Cis-eQTLs in GTEx were determined as those with $p<5\times10^{-8}$ in any of the 44 tissues.[32] LD between T2DM genome-wide association study (GWAS) susceptibility single-nucleotide polymorphisms (SNP) and cis-eQTLs were assessed using East-Asian reference panel from the 1000 Genomes database (http://grch37.ensembl.org/index.html) and an $r^2>0.8$ was used to call loci that were in LD.

Statistical analysis

The primary outcome in this study was the change (from fasting to postprandial) in transcription of oxidative stress genes regulated by NRF2 in MNC, as an indicator of NRF2 transcriptional activity and binding. Post hoc power calculation was based on the postprandial NRF2 expression in terms of mean fold changes in FDRT and non-FDRT groups, in which a sample size of nine subjects per group showed the study power of detecting the difference between the two groups to be 85% at the 5% significance level.

The incremental area under the curve was computed using the trapezoidal method. A linear mixed model was used to analyze the differences in expression of genes in MNC following the meal challenge by group and time. Repeated measures analysis of variance was used to analyze the differences in all other variables with time series following meal challenge or insulin stimulation (levels of F_2-isoprostanes, GSH, and expression of genes in myotubes/adipocytes), by group and time. Independent samples t-test was used to analyze the differences in variables without time series. Statistical significance was set at $p<0.05$.

RESULTS

Baseline characteristics

Demographic and metabolic parameters at baseline were not significantly different between groups (table 1; online supplementary database 1).

Table 1 Baseline subject characteristics

	FDRT (n=9)	Non-FDRT (n=9)	P value
Age (years)	29.3 (2.50)	32.7 (2.90)	0.396
BMI (kg/m^2)	25.7 (0.50)	24.8 (0.50)	0.273
Fasting blood glucose (mmol/L)	4.96 (0.14)	4.70 (0.14)	0.223
Fasting serum insulin (mU/L)	8.68 (1.20)	7.87 (2.03)	0.735
Fasting total cholesterol (mmol/L)	4.76 (0.26)	4.55 (0.25)	0.562
Fasting triglyceride (mmol/L)	0.87 (0.10)	1.00 (0.17)	0.508
Fasting LDL-cholesterol (mmol/L)	3.02 (0.24)	2.94 (0.26)	0.834
Fasting HDL-cholesterol (mmol/L)	1.35 (0.11)	1.15 (0.05)	0.119
Fasting NEFA (mmol/L)	0.78 (0.27)	0.52 (0.08)	0.371
HOMA-IR	1.94 (0.31)	1.69 (0.47)	0.662

Data are mean±SEM. Statistical significance was set at $p<0.05$ using t-test.
BMI, body mass index; FDRT, first-degree relative with type 2 diabetes mellitus; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; NEFA, non-esterified fatty acid.

Glucose, insulin, TG and NEFA trajectories following meal challenge

Postprandial incremental glucose, insulin, TG and NEFA responses were not different between the FDRT and non-FDRT groups following a carbohydrate-rich liquid mixed meal challenge (online supplementary table 1; online supplementary database 1).

Oxidative stress response in circulating MNCs and plasma following a carbohydrate-rich liquid mixed meal challenge in FDRT and non-FDRT subjects

The expression of oxidative stress genes in MNCs during the fasting state was not significantly different between groups, with the exception of GPX3, which was lower in FDRT-derived MNC compared with non-FDRT-derived MNC ($p<0.05$) (online supplementary table 2). There were trends for greater expression of most pro-oxidant genes such as NCF-1, NCF-2, NCF-4, and CYBB ($p\leq0.1$) in FDRT-derived MNC compared with non-FDRT-derived MNC following the meal challenge (table 2; figure 1A). By contrast, pro-oxidant gene CYBA showed a lower postprandial expression in the FDRT group ($p_{group}=0.027$ and $p_{time\times group}=0.005$). This occurred alongside marked postprandial upregulation in expression of antioxidant transcription factor NRF2 ($p_{group}=0.002$ and $p_{time\times group}=0.016$) and its target genes GPX3 ($p_{group}=0.011$ and $p_{time\times group}=0.019$) and TXNRD1 ($p_{group}=0.004$ and $p_{time\times group}=0.007$) in MNC from FDRT subjects compared with non-FDRT subjects (table 2; figure 1B). In addition, antioxidant gene SOD-1 showed a significant increase in postprandial expression in the

Table 2 Fold changes in oxidative gene expression in MNC at 2 and 6 hours in FDRT (n=9) and non-FDRT (n=9) subjects after a meal challenge

			Δ 2 hours	Δ 6 hours	Group	Time	Interaction
Pro-oxidant genes	NCF-1	FDRT	1.09±0.06	1.29±0.14	0.080	0.075	0.090
		Non-FDRT	0.99±0.04	1.00±0.08			
	NCF-2	FDRT	1.08±0.04	1.22±0.13	0.115	0.076	0.281
		Non-FDRT	0.95±0.04	1.03±0.08			
	NCF-4	FDRT	1.12±0.05	1.37±0.14	0.131	**0.024**	0.093
		Non-FDRT	1.07±0.05	1.06±0.12			
	CYBB	FDRT	1.22±0.08	1.49±0.13	0.127	**0.002**	0.115
		Non-FDRT	1.19±0.12	1.16±0.10			
	CYBA	FDRT	0.97±0.04	0.96±0.04	**0.027**	**0.001**	**0.005**
		Non-FDRT	1.12±0.04	1.01±0.08			
Antioxidant genes	NRF2	FDRT	1.13±0.06	1.51±0.16	**0.002**	**0.005**	**0.016**
		Non-FDRT	0.91±0.05	1.01±0.08			
	GPX3	FDRT	1.12±0.09	3.18±0.68	**0.011**	**0.001**	**0.019**
		Non-FDRT	0.75±0.11	1.42±0.26			
	TXNRD1	FDRT	1.32±0.10	1.97±0.21	**0.004**	**0.001**	**0.007**
		Non-FDRT	0.99±0.13	1.14±0.16			
	TXN	FDRT	1.09±0.06	1.26±0.08	0.340	0.115	0.189
		Non-FDRT	1.10±0.10	1.03±0.13			
	SOD1	FDRT	1.20±0.04	1.39±0.14	**0.046**	0.078	0.191
		Non-FDRT	1.02±0.12	1.04±0.14			
	SOD2	FDRT	1.14±0.09	1.18±0.10	0.156	0.262	0.275
		Non-FDRT	1.04±0.06	0.99±0.07			

Data are mean±SEM. P<0.05 using a linear mixed model. Numbers in bold represent significant values.
FDRT, first-degree relative with type 2 diabetes mellitus; MNC, mononuclear cell.

FDRT group (p_{group}=0.046 and $p_{time\times group}$=0.191) (table 2; figure 1B).

There was no significant difference in plasma and urinary F_2-isoprostane levels between groups in the fasted and postprandial states (online supplementary table 2; online supplementary figure 1). The relevant source data is available in online supplementary database 2.

Comparison of oxidative stress responses to insulin stimulus in myotubes and adipocytes isolated from FDRT and non-FDRT subjects

To assess the effects of a high-carbohydrate meal challenge in cells derived from other metabolic tissues, adipocytes and myotubes from the FDRT and non-FDRT subjects were exposed to a 10 and 30 min insulin stimulus (100 nM) in vitro. Overall there were trends for a greater expression of antioxidant genes following insulin stimulation in FDRT-derived myotubes compared with myotubes derived from non-FDRT subjects (p≤0.1 for TXN and NRF2) (online supplementary table 3). Synergistically, expression of both pro-oxidant and antioxidant genes increased in FDRT-derived adipocytes (p≤0.05 for CYBB and NCF-1; $p_{time\times group}$=0.029 for TXN) (online supplementary table 3).

Baseline oxidative stress protein levels were not different between groups (online supplementary table 2), but expression of antioxidant transcription factor NRF2 protein was greater following insulin stimulation in FDRT-derived than non-FDRT-derived myotubes (p<0.01) (figure 2). Adipocyte responses between the two groups did not differ significantly (online supplementary figure 2).

GSH levels and GSH/GSSG ratio were not different between groups (online supplementary figure 3). The relevant source data is available online supplementary database 2.

Inflammatory response in circulating MNCs and plasma following a carbohydrate-rich liquid mixed meal challenge in FDRT and non-FDRT subjects

The baseline gene expression of IL-10 and IL-6 was lower in FDRT-derived MNC compared with non-FDRT-derived MNC (p<0.05), but expression of other genes did not differ significantly between groups (online supplementary table 2). The meal challenge induced greater expression of TNFα (p_{group}=0.001 and $p_{time\times group}$=0.007) in MNC derived from FDRT compared with MNC from non-FDRT subjects (table 3; figure 3). In addition, IL-6 (p_{group}=0.089) and IL-10 (p_{group}=0.057 and $p_{time\times group}$=0.077) showed a

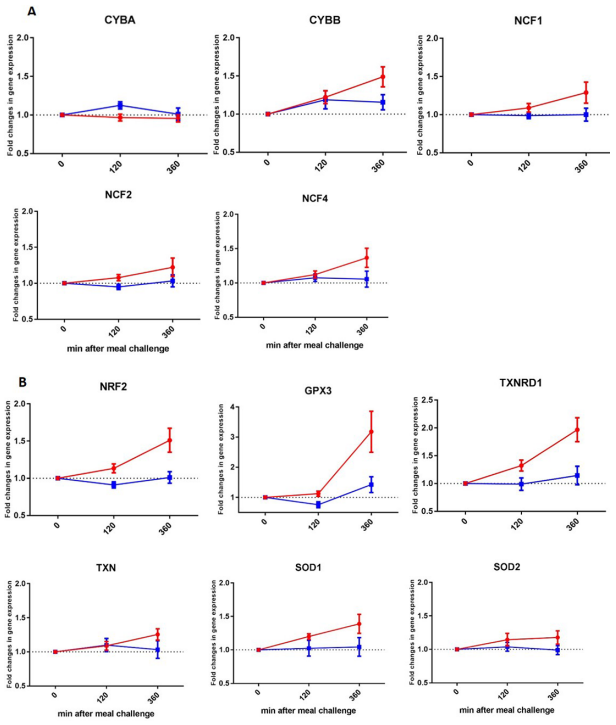

Figure 1 Fold changes from baseline in expression of genes involved in oxidative stress pathway in mononuclear cells (MNC) up to 6 hours following a high-carbohydrate meal challenge in first-degree relative with type 2 diabetes mellitus (FDRT; red circle) and non-FDRT (blue square) subjects. (A) Pro-oxidant genes. Significant or near-significant differential responses, as analyzed by linear mixed model, were found after meal ingestion in expression of: CYBA (p_{group}=0.027 and $p_{time\times group}$=0.005), NCF-1 (p_{group}=0.080 and $p_{time\times group}$=0.090), NCF-2 (p_{group}=0.115 and $p_{time\times group}$=0.281), NCF-4 (p_{group}=0.131 and $p_{time\times group}$=0.093), and CYBB (p_{group}=0.127 and $p_{time\times group}$=0.115). (B) Antioxidant genes. Significant differential responses, as analyzed by linear mixed model, were found after meal ingestion in expression of: NRF2 (p_{group}=0.002 and $p_{time\times group}$=0.016), GPX3 (p_{group}=0.011 and $p_{time\times group}$=0.019), TXNRD1 (p_{group}=0.004 and $p_{time\times group}$=0.007), and SOD1 (p_{group}=0.046 and $p_{time\times group}$=0.191). Data are mean±SEM. P<0.05.

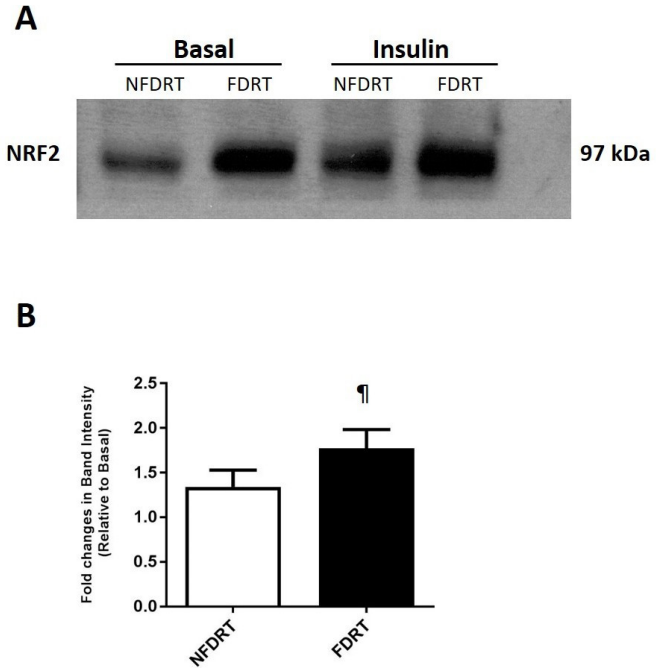

Figure 2 Changes in expression of oxidant transcription factor NRF2 in myotubes, assessed by western blot assay. (A) Representative western blot (n=5) of basal and insulin (1000 nM for 30 min) stimulated levels of nuclear NRF2, assessed in myotubes from FDRT and non-FDRT subjects. (B) Densitometry analysis of western blot. Bars represent relative expression levels of nuclear NRF2 band intensity (n=5), normalized to respective basal levels, in FDRT (black bar) and non-FDRT (white bar) subjects. Data are mean±SEM (¶p<0.01 using t-test). FDRT, first-degree relative with type 2 diabetes mellitus; NFDRT, non-FDRT.

near-significant increase in postprandial expression in the FDRT group.

Plasma levels of IL-6 did not differ between groups in the fasted and postprandial states (online supplementary table 2 and table 3), while plasma TNFα was generally undetectable (data not shown). The relevant source data is available in online supplementary database 3.

Comparison of inflammatory responses to insulin stimulus in myotubes and adipocytes isolated from FDRT and non-FDRT subjects

There were minimal changes in expression of inflammatory genes between FDRT and non-FDRT-derived myotubes following insulin stimulation (online supplementary table 4). By contrast, whereas expression of proinflammatory genes showed a near-significant increase in FDRT-derived adipocytes (p_{group} and $p_{time\times group}$≤0.1 for TNFα), expression of anti-inflammatory genes did not (online supplementary table 4).

Baseline protein levels were not significantly different between groups (online supplementary table 2), but expression of inflammatory transcription factor NF-κB protein was greater in the non-FDRT-derived myotubes compared with the FDRT-derived myotubes following insulin stimulation (p<0.05) (online supplementary figure 4). Adipocyte responses between the two groups did not differ significantly (online supplementary figure 5). The relevant source data is available in online supplementary database 3.

Genetic loci analysis

We examined whether T2DM susceptibility genetic loci were associated with the expression of inflammatory and oxidative stress genes that were found to be differentially expressed between the FDRT and non-FDRT subjects. Using data from GTEx, we found that none of the reported 188 T2DM susceptibility genetic loci in East Asians are cis-eQTLs for our panel of inflammatory and oxidative stress genes (online supplementary database 4 for GTEx data for T2DM susceptibility genetic loci in East Asians). Additionally, we identified 39 variants that are at LD with the T2DM susceptibility genetic loci but none of

Table 3 Fold changes in inflammatory gene expression in MNC and plasma IL-6 at 2 and 6 hours in FDRT (n=9) and non-FDRT (n=9) subjects after a meal challenge

			Δ 2 hours	Δ 6 hours	Group	Time	Group×time
Proinflammatory genes	TNFα	FDRT	1.36±0.10	1.80±0.16	**0.001**	**0.001**	**0.007**
		Non-FDRT	1.06±0.08	1.17±0.07			
	IL-6	FDRT	1.33±0.56	6.73±2.38	0.089	**0.002**	0.153
		Non-FDRT	0.56±0.16	2.68±0.89			
	MCP-1	FDRT	0.99±0.20	5.16±2.06	0.116	**0.005**	0.159
		Non-FDRT	0.61±0.09	2.00±0.37			
	TLR4	FDRT	1.03±0.08	1.34±0.12	0.397	**0.001**	0.417
		Non-FDRT	1.02±0.07	1.19±0.06			
	IL-1β	FDRT	0.91±0.08	1.30±0.20	0.916	**0.008**	0.817
		Non-FDRT	0.96±0.07	1.22±0.08			
	NF-κB/p105	FDRT	1.00±0.07	1.11±0.06	0.209	0.741	0.106
		Non-FDRT	0.99±0.05	0.93±0.07			
	Rel-A	FDRT	1.00±0.05	1.39±0.15	0.112	**0.001**	0.220
		Non-FDRT	0.90±0.06	1.13±0.07			
Anti-inflammatory genes	IκB-β	FDRT	1.00±0.09	1.64±0.29	0.137	**0.001**	0.355
		Non-FDRT	0.89±0.04	1.26±0.11			
	IκB-α	FDRT	0.89±0.03	1.03±0.05	0.781	**0.005**	0.254
		Non-FDRT	0.92±0.05	0.97±0.04			
	IL-10	FDRT	1.54±0.33	8.94±3.43	0.057	**0.004**	0.077
		Non-FDRT	0.72±0.19	2.57±0.67			
	TGFβ	FDRT	1.04±0.04	1.09±0.07	0.114	**0.004**	0.114
		Non-FDRT	1.24±0.12	1.31±0.13			
Cytokine	IL-6 (plasma)	FDRT	0.64±0.16	1.02±0.13	0.349	**0.001**	0.606
		Non-FDRT	0.43±0.10	0.98±0.18			

Data are mean±SEM. P<0.05 using a linear mixed model. Numbers in bold represent significant values.
FDRT, first-degree relative with type 2 diabetes mellitus; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MNC, mononuclear cell; NF-κB, nuclear factor kappa B; TGFβ, transforming growth factor-β; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha.

these variants are cis-eQTLs for our panel of inflammatory and oxidative stress genes.

DISCUSSION

We found that individuals with heredity of T2DM have increased inflammatory and oxidative stress responses to a high-carbohydrate meal challenge, independent of age, BMI and insulin sensitivity. Interestingly, there were no differences between FDRT and non-FDRT individuals in a variety of inflammatory and oxidative stress markers measured in the fasting state. These observations highlight the importance of using a dynamic challenge (eg, meal) to unravel defects in physiological regulation and function.

FDRT individuals are known to be more insulin resistant, metabolically inflexible, and have greater circulating NEFA concentrations[33–35] compared with their non-FDRT counterparts. Despite the strong association of heritability of T2DM with future risk of T2DM, the majority of this excess risk remain unexplained by major T2DM risk factors such as anthropometric measures, lifestyle factors and genetic risk scores.[17] Emerging key players in T2DM pathophysiology such as adverse oxidative stress and inflammatory responses could be among possible mechanisms that predispose FDRTs to develop T2DM. To our knowledge, this is the first report of a detailed characterization of inflammatory and oxidative stress responses following a high-carbohydrate meal challenge and insulin stimulation, in association with heredity of T2DM.

Oxidative stress, a persistent imbalance between reactive oxygen species (ROS) and/or reactive nitrogen species and antioxidant defenses, damages DNA and proteome with adverse effects on many cellular functions.[36] Our results in MNC demonstrate that heredity of T2DM is associated with increased postprandial inflammatory and oxidative stress responses. This has been suggested to be one of the earlier defects in atherogenic processes, and may thus increase risk of cardiovascular disease in these individuals.[37 38] It is known that circulating immune

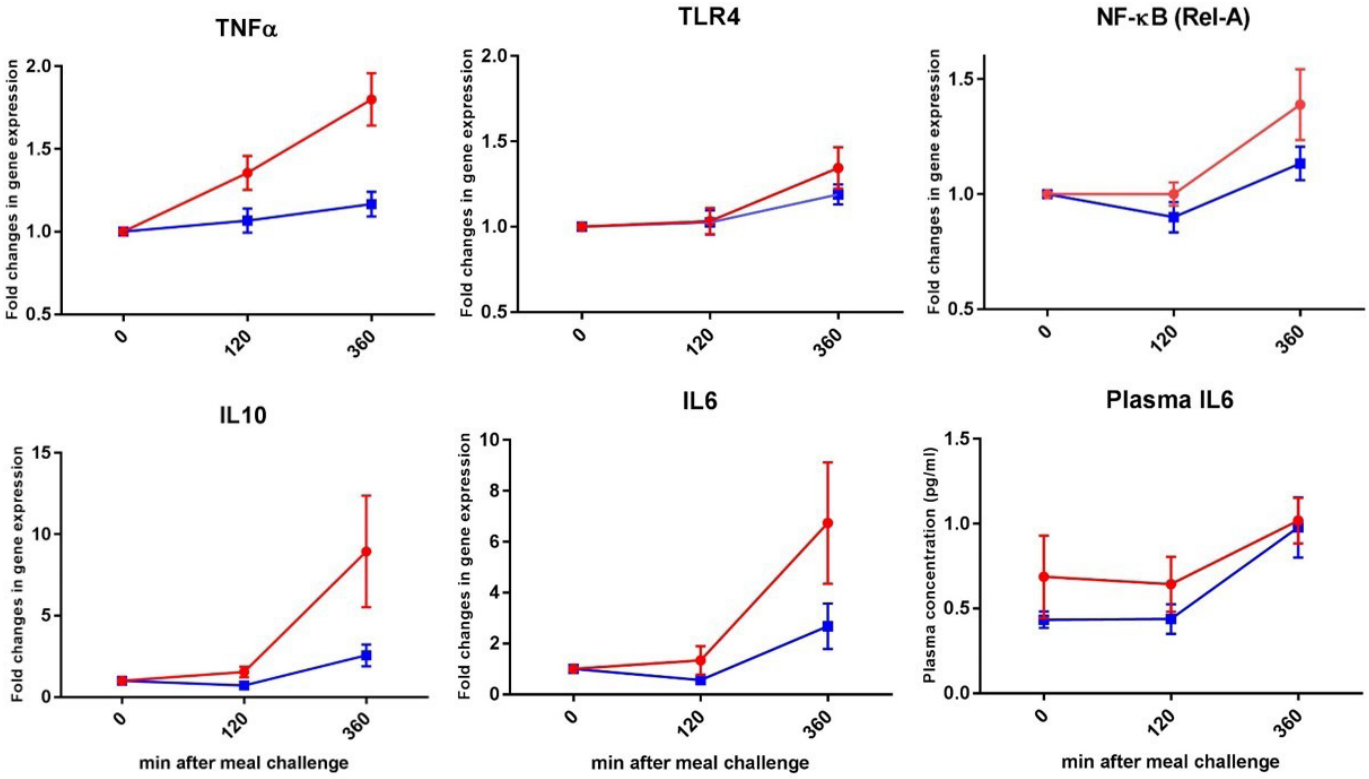

Figure 3 Fold changes from baseline in expression of genes involved in inflammatory pathway in mononuclear cells (MNC) up to 6 hours following a high-carbohydrate meal challenge in first-degree relative with type 2 diabetes mellitus (FDRT; red circle) and non-FDRT (blue square) subjects. Significant or near-significant differential responses, as analyzed by linear mixed model, were found after meal ingestion in expression of: TNFα (p_{group}=0.001 and $p_{time\times group}$=0.007), IL-6 (p_{group}=0.089 and $p_{time\times group}$=0.153), IL-10 (p_{group}=0.057 and $p_{time\times group}$=0.077), TGFβ (p_{group}=0.114 and $p_{time\times group}$=0.114), and MCP-1 (p_{group}=0.131 and $p_{time\times group}$=0.093). Data are mean±SEM. P<0.05. IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappa B; TGFβ, transforming growth factor-β; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha.

cells (neutrophils and monocytes) in overnight fasted, overweight insulin-resistant individuals with a heredity of T2DM are in a pro-oxidative, proinflammatory state.[20] This was evident by significant upregulation of plasma levels of TNFα, IL-6 as well as myeloperoxidase, an ROS-generating enzyme that is known to strongly correlate with increased risk for cardiovascular and inflammatory diseases.[39 40]

Assessment of the oxidative stress genes in MNC revealed trends towards a higher pro-oxidative post-prandial response in FDRT than non-FDRT subjects. This occurred alongside significant upregulation in the expression of antioxidant transcription factor NRF2 at the gene and protein levels as well as its target genes in FDRT subjects compared with non-FDRT subjects following the high-carbohydrate meal. Plasma and urinary F_2-isoprostanes showed no differences between groups in the fasted and postprandial states. These results may demonstrate the adaptive homeostatic responses to a nutritional challenge, as orchestrated by the antioxidant and anti-inflammatory stress defenses. However, such compensatory mechanisms could be overwhelmed with persistent and progressive hyperglycemia and hyperlipidemia in FDRT individuals, leading to a decompensated and irreversible disease state, that is, T2DM and its complications.

In the current study, a high-carbohydrate meal induced an overall greater postprandial proinflammatory response in MNC from FDRT subjects as evident by the marked upregulation of TNFα after meal ingestion. This was accompanied by a compensatory rise in expression of anti-inflammatory genes such as IL-10, presumably to mitigate inflammatory cellular damage. It is known that oxidative stress culminates in activation of many stress-sensitive signaling pathways including the NF-κB-mediated inflammatory pathway. This in turn may lead to impaired insulin signaling and glucose transport activity associated with metabolic dysregulation states, including the development and progression of T2DM.[6 41–44] Of note, despite marked upregulation of TNFα gene, a greater expression of its classical regulator—transcription factor NF-κB—at gene and protein levels was not observed in this study. It is known that transcriptional regulation of expression of TNFα, an early gene rapidly transcribed after cellular activation by inflammatory or stress signaling, could be independent of NF-κB.[45] For example, proteins of signal transducer and activator of transcription families are involved in activation of TNFα

gene transcription[46] which is also reported to be specific to cell type and stimulus.[47] Perhaps this could explain why the expressions of NF-κB and TNFα between myotube after insulin stimulation and MNC after meal intake were not aligned in our study.

We found that in vitro insulin stimulation exerts a modulating effect on the expression of inflammatory and oxidative stress genes on myotubes and adipocytes. This was evidenced by higher antistress defenses in response to higher stress in myotubes. However, GSH levels and reduced versus oxidized GSH ratio were not different between FDRT and non-FDRT groups. Aguer *et al* investigated the adaptive responses to oxidative stress in primary myotubes from obese individuals with heredity of T2DM.[48] On exposure to chronic high-glucose and insulin conditions, there was an increase in antioxidant defenses (SOD2 expression and uncoupled respiration) in myotubes derived from FDRT individuals, similar to our findings. Further, oxidative stress level did not differ in myotubes between groups. These observations may represent adaptive homeostatic responses to a physiological challenge in otherwise healthy normoglycemic individuals.

In this study, we demonstrated that currently known T2DM susceptibility genetic loci in East Asians had no association with the differential expression of inflammatory and oxidative stress genes. However, there may be other common and/or rare variants that are associated with these gene expression profiles, which remain to be characterized. Furthermore, post-translational and chromatin modifications are emerging as critical mediators in pathogenesis of oxidative stress and inflammation in the context of T2DM and may be contributing factors to the development of disease.[49]

To our knowledge, detailed changes in postprandial inflammatory and oxidative stress have not been assessed previously in association with heredity of T2DM. We studied the effects of a high-carbohydrate meal with a caloric content and macronutrient composition well representative of normal dietary intake, as opposed to high-fat meals used in previous studies.[21 22] We were able to observe significant differences in several but not all of biomarkers of stress-activated signaling pathways examined. It should be noted that, other than heredity of T2DM, all our subjects were otherwise healthy and normoglycemic and therefore capable of adaptive homeostatic responses. This may have led to the modest changes in a number of biomarkers observed in this study.

CONCLUSIONS

Our findings shed light on how heredity of T2DM is associated with increased susceptibility to oxidative stress and inflammation in the postprandial state, which may predispose FDRT individuals to T2DM and its associated complications. Findings from this study contribute to forming the basis for large, prospective cohort studies delineating novel factors specifically contributing to conversion to T2DM. Further, interventional studies and novel therapeutic approaches targeting these stress pathways could be envisioned.

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Contributors SB, MS and EPR contributed to the study execution, acquisition, analyses and interpretation of data, and manuscript preparation. SB, MS, MA, DSQO, NA, GN, VC, and YT performed various experiments/analyses (MNC isolation, adipocyte/myotube culture, gene/protein expression assay, glutathione assay, and F2-isoprostane assay). AVP and SAT contributed to the grant proposal. FM, MHL, DSQO, CC, RCSS and AVP contributed to critical revision of the manuscript. SAT contributed to the conception and design of the work and interpretation of data and critically revised the manuscript. SB, MS and SAT are the guarantors of this work, and as such, had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Patient consent for publication Not required.

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