Fructose induces gluconeogenesis and lipogenesis through a SIRT1-dependent mechanism

Paul W Caton, Nanda K Nayuni, Noorafza Q Khan, Elizabeth G Wood and Roger Corder

Department of Translational Medicine and Therapeutics, Bart's and the London School of Medicine and Dentistry, William Harvey Research Institute, Queen Mary University of London, London EC1M 6BQ, UK

(Correspondence should be addressed to P W Caton; Email: p.w.caton@qmul.ac.uk)

Abstract

Consumption of a fructose-rich diet leads to insulin resistance and dyslipidemia in part due to elevated gluconeogenesis and lipogenesis. SIRT1, an NAD⁺-dependent protein deacetylase, can induce gluconeogenesis and lipogenesis. The aim of this study was to determine whether fructose increased hepatic SIRT1, leading to induction of gluconeogenesis and lipogenesis. Rat hepatocytes were incubated with fructose (1–5 mM). SIRT1 protein, SIRT1 activity, and NAD⁺/NADH ratio were measured. The effects of SIRT1 inhibitors (EX-527 and nicotinamide) and activators (SIRT1 activator 3 and SIRT1720) and the mitochondrial complex I inhibitor rotenone were examined on fructose-induced increases in gluconeogenesis and lipogenesis. Fructose increased SIRT1 protein, SIRT1 activity, and NAD⁺/NADH ratio. Fructose also induced gluconeogenesis, with increases in peroxisome proliferator-activated receptor coactivator 1- α (PGC1 α) and phosphoenolpyruvate carboxykinase (PEPCK; gene code *Pck1*)

gene expression, PEPCK activity, and hepatocyte glucose production. In addition, levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Hmgcr*) and acetyl-coA carboxylase (*Acc*) mRNA, and intracellular cholesterol were increased. Increases in gluconeogenesis, *Hmgcr*, *Acc*, and cholesterol were abolished by SIRT1 inhibitors and rotenone, while SIRT1 activators increased gluconeogenesis, *Hmgcr*, *Acc*, *Pgc1 β* , and sterol regulatory element-binding protein 1c (*Srebp1c*) gene expression. In conclusion, fructose induces gluconeogenesis and lipogenesis through a SIRT1-dependent mechanism, suggesting that induction of hepatic SIRT1 could play a pivotal role in the metabolic changes observed in humans and animals consuming a fructose-rich diet. These results highlight the need for a greater understanding of the role of SIRT1 in metabolic regulation and indicate the potential for adverse effects of SIRT1 activators if used therapeutically.

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Introduction

Dietary sugar consumption has risen by more than 30% over the past 40 years with the widespread use of high fructose corn syrup as a sweetener in many foods and beverages being a major contributor to this increase (Bray *et al.* 2004, Gaby 2005, Johnson *et al.* 2007). The prevalence of obesity, metabolic syndrome, and type 2 diabetes mellitus (T2DM) has increased in parallel with sugar consumption, particularly fructose (Bray *et al.* 2004, Johnson *et al.* 2007). Consistent with this, even a modest daily intake of sugar-sweetened soft drinks or fruit juice is associated with weight gain or an increased risk of metabolic syndrome and T2DM (Bazzano *et al.* 2008, Malik *et al.* 2010).

In rodents, fructose feeding leads to onset of symptoms of the metabolic syndrome, including hyperglycemia, hyperinsulinemia as well as obesity, hypertension, and increased levels of triglycerides, fatty acids and cholesterol (Fiebig *et al.* 1998, Kelley *et al.* 2004, Jurgens *et al.* 2005, Bilz *et al.* 2006, Rajasekar & Anuradha 2007, Nagai *et al.* 2009). In humans,

consumption of a fructose-rich diet causes insulin resistance, increases uric acid, and stimulates *de novo* lipogenesis, resulting in increased levels of triglycerides, cholesterol, and small pro-atherogenic low-density lipoprotein particles (Hollenbeck 1993, Faeh *et al.* 2005, Nakagawa *et al.* 2006, Aeberli *et al.* 2007, Abdel-Sayed *et al.* 2008, Parks *et al.* 2008, Stanhope *et al.* 2009).

Induction of gluconeogenesis by overexpression of phosphoenolpyruvate carboxykinase (PEPCK; gene code *Pck1*) is sufficient to cause a T2DM-like state with insulin resistance and hyperglycemia (Valera *et al.* 1994, Sun *et al.* 2002), while knockdown of hepatic *Pck1* corrected hyperglycemia and dyslipidemia in rodent models of T2DM (Gomez-Valades *et al.* 2006, 2008). Hence, observations that *Pck1* gene expression and activity are increased in response to a high-fructose diet with elevated hepatic glucose production and hyperglycemia (Blakely *et al.* 1981, Rajasekar & Anuradha 2007) could represent a key element of the metabolic response to excessive dietary fructose. Despite clear evidence for a relationship between excess fructose

consumption and impaired glucose and lipid homeostasis, the mechanisms responsible have yet to be elucidated.

Recent studies have demonstrated the essential role of SIRT1, an NAD^+ -dependent protein deacetylase, in the regulation of hepatic gluconeogenesis. SIRT1 deacetylates peroxisome proliferator-activated receptor coactivator 1- α (PGC1 α), a key coactivator of *Pck1* gene expression, leading to increased hepatic glucose production (Rodgers *et al.* 2005, Rodgers & Puigserver 2007). Hepatic SIRT1 also regulates the expression of genes controlling cholesterol and lipid metabolism and is able to induce lipogenesis (Rodgers & Puigserver 2007, Chen *et al.* 2008). However, the specific nutrient signals that regulate hepatic SIRT1 have yet to be identified.

Activation of SIRT1 has been reported in the hearts of fructose-fed mice through increases in NAD^+/NADH ratio (Pillai *et al.* 2008). Similarly, increased NAD^+/NADH ratio is associated with increased SIRT1 protein levels in the livers of fasted rats (Rodgers *et al.* 2005). Fructose-induced gluconeogenesis can be suppressed by inhibition of the electron transport chain. This may be due to a decrease in NAD^+/NADH ratio that occurs in parallel (Pryor *et al.* 1987), but the role of SIRT1 in the hepatic response to fructose has yet to be examined.

In this study, we demonstrate that fructose induces hepatic gluconeogenesis and lipogenic gene expression through a SIRT1-dependent mechanism. This occurs through a complex I-mediated increase in NAD^+/NADH ratio. This study indicates that SIRT1 activators may cause adverse metabolic consequences if used as a therapeutic strategy for T2DM.

Materials and Methods

Materials

Primary hepatocytes isolated from male Sprague–Dawley rats were purchased from 3H Biomedical (Uppsala, Sweden). SIRT1 activator 3 (Catalogue #; 10009797) and SRT1720 (Catalogue #; CAY10559) were obtained from Cayman Chemical, Ann Arbor, MI, USA. EX-527 (6-chloro-2,3,4,9-tetra-hydro-1-H-carbazole-1-carboxamide) was obtained from Alexis Biochemical, Nottingham, UK. Rotenone was from CalBiochem, Nottingham, UK. All other materials were from Sigma, unless otherwise stated.

Cell culture

Rat H4IIEC3 hepatoma cells were cultured in DMEM containing 5 mM glucose, non-essential amino acids, and 10% (v/v) FCS. Primary hepatocytes were suspended in William's E Glutamax media (Invitrogen) and cultured for 4 h prior to treatment, to allow adherence to collagen-coated cell culture plates (Invitrogen). Cells were incubated with fructose (5 mM), lactate (20 mM), pyruvate (2 mM), or with SIRT1

activators for 6 h in glucose-free Earle's Balanced Salt Solution (EBSS; 0.264 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.4 g/l KCl; 0.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2.2 g/l NaHCO_3 ; 0.158 g/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4), or co-incubated with EX-527 (5–10 μM) and fructose (5 mM) for 6 h in glucose-free EBSS. Pretreatment with nicotinamide and rotenone was for 24 h in DMEM containing 5 mM glucose prior to fructose incubations. For cholesterol measurements, cells were incubated with fructose (5 mM) or co-incubated with fructose plus nicotinamide (2 mM) or rotenone (50 nM) for 24 h in DMEM containing 5 mM glucose. For rotenone and SIRT1 activator 3 experiments, H4IIEC3 cells were pretreated with rotenone (1 h; 50 nM), prior to 6 h incubation with fructose (5 mM), or co-incubated with SIRT1 activator 3 (60 μM).

Glucose measurements

Glucose was measured using a colorimetric glucose oxidase assay based on the method described by Hugget and Nixon (Bergmeyer 1963). Glucose levels are expressed as nmol/ 2.5×10^5 cells (H4IIEC3) or mM (primary hepatocytes).

PEPCK activity

PEPCK activity was assayed as described previously (Caton *et al.* 2009) using a three-sequential reaction process based on the stoichiometric transformation of oxaloacetate (OAA), via phosphoenolpyruvate (PEP) into ATP. PEPCK activity is expressed as nM PEP produced per mg protein.

Quantitative RT-PCR

Expression of all genes was measured using qRT-PCR, according to the procedure described previously (Douthwaite *et al.* 2003). Relative mRNA levels were determined by standard curve methodology normalized against 18S ribosomal control RNA (Applied Biosystems, Warrington, UK). Changes in gene expression are represented as fold change relative to 1, where control equals 1.

Western blotting

Solubilized protein samples (10 μg ; measured and equalized in each fraction using the Bio-Rad RC-DC protein assay; Bio-Rad) were separated by SDS-PAGE and transferred onto nitrocellulose Hybond membrane or PVDF membrane (GE Healthcare, Amersham, UK). Membranes were blocked with 3% (w/v) milk protein in Tris-buffered saline containing 0.1% Tween-20, and then incubated overnight with primary antibody. Antibodies used in this study were ovalbumin-conjugated mouse monoclonal anti-SIRT1 IgG (1:1000 dilution; clone 2G1/F7; Upstate Biotech, Billerica, NY, USA), anti-phospho(Thr¹⁸³/Tyr¹⁸⁵)SAPK/JNK, anti-total JNK (Cell Signaling Technologies, Danvers, MA, USA). Detection of bands was achieved by using the chemiluminescent substrate SuperSignal West Pico (Pierce, Rockford, IL, USA).

Blots are representative of three separate blots. Densitometry was determined using Image J software (National Institutes of Health, Bethesda, Maryland, USA). Reference protein measurements were made with mouse monoclonal anti- β -actin (clone AC-15) primary antibody in a 3% (w/v) milk/TNT solution (data not shown).

SIRT1 activity

SIRT1 activity was assayed in nuclear fractions isolated from primary hepatocytes or H4IIEC3 cells using a two-step fluorometric technique based on deacetylation of lysine in the substrate Boc-Lys(Ac)-AMC (Bachem, St Helens, UK), followed by trypsin treatment to release fluorescent 7-amino-4-methyl coumarin (AMC; Ishdorj *et al.* 2008). Nuclear isolation from cell samples was carried out by following the published methods (Kain *et al.* 2000). Protein content was measured and equalized in each fraction using the Bio-Rad RC-DC protein assay. Trichostatin A (TSA) as a class I and II histone deacetylase (HDAC) inhibitor and nicotinamide or EX-527 as SIRT1 inhibitors were used to confer specificity for SIRT1 activity as opposed to general HDAC or nuclear sirtuin activity. SIRT1 activity is expressed as AMC produced (μ moles) per mg protein.

NAD⁺/NADH ratio and ATP levels

ATP levels and NAD⁺/NADH ratio were measured according to the published methods (San *et al.* 2002, Moynihan *et al.* 2005). ATP levels are expressed as relative luminescence units. ATP levels and NAD⁺/NADH ratio are represented as fold change relative to 1, where control equals 1.

Intracellular cholesterol assays

Cells (1.2×10^6) were washed twice in 1 ml ice-cold PBS and scraped into 1 ml ice-cold extraction buffer (320 mM sucrose, 1 mM EDTA, 1 mg/ml BSA, and 10 mM Tris-HCl, pH 7.4; 1% Triton-X). Harvested cells were centrifuged at 1000 *g* for 10 min at 4 °C to produce a cell pellet, which was resuspended in 1 ml extraction buffer. Cell lysate samples or supernatants (150 μ l) were added to reaction buffer (50 μ l) containing 0.1 M potassium phosphate (pH 7.4), 50 mM NaCl, 5 mM cholic acid, 1 U/ml cholesterol oxidase, 1 U/ml cholesterol esterase, 1 U/ml HRP, and 150 μ M Amplex Red reagent, mixed vigorously and incubated at 37 °C for 30 min. The assay is based on a two-sequential enzymatic reaction process. Initially, cholesterol esters are hydrolyzed by cholesterol esterase into cholesterol. Cholesterol is then oxidized by

Figure 1 H4IIEC3 cells were incubated for 6 h with fructose (1–5 mM) in glucose-free EBSS (A, D–G; *n*=6); (A) glucose production, (D) PEPCK activity, (E) *Pck1* and (F) *Pgc1α* mRNA levels, (G) cellular NAD⁺/NADH ratio, (H) SIRT1 protein levels (representative blot; *n*=3), (I) SIRT1 activity (co-incubated with TSA (3 μ g/ml)). Co-incubation of EX-527 (5–10 μ M) and fructose (5 mM; *n*=6) for 6 h inhibits fructose-induced increases in (J) SIRT1 activity, (K) glucose production, (L) PEPCK activity, (M) *Pck1* and (N) *Pgc1α* mRNA levels. Alternatively, H4IIEC3 cells were incubated for 6 or 24 h with fructose (5 mM; F), pyruvate (2 mM; P), and lactate (20 mM; L) in glucose-free EBSS (B and C; *n*=6). Data are expressed as mean $\pm$ S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001 versus fructose alone; ++*P*<0.01 versus P and L alone.

cholesterol oxidase to produce H_2O_2 . In the presence of HRP, Amplex Red reacts with H_2O_2 with a 1:1 stoichiometry to produce resorufin, a fluorescent compound (Ex: 560 nm; Em 590 nm) measured using a Cytofluor II multiwell plate reader (PerSeptive Biosystems, Framingham, MA, USA).

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Statistical differences were determined using StatView (SAS Institute, Inc., Cary, NC, USA) with either a two-tailed unpaired *t*-test or a one-way ANOVA followed by a Fisher's *post-hoc* test, where appropriate. A *P* value of 0.05 was considered significant.

Results

Fructose induces gluconeogenesis through a SIRT1-dependent mechanism

H4IIEC3 cells were incubated with fructose at concentrations of 1–5 mM. These concentrations were chosen because levels of fructose present in the hepatic portal vein in humans and rats following consumption of a fructose-rich meal are estimated to be ~ 1.1 – 2.2 mM (Mayes 1993, Pagliassotti *et al.* 2003). Incubation of H4IIEC3 cells with fructose induced increases in glucose production (Fig. 1A). Fructose (5 mM) alone induced glucose production to a greater extent than pyruvate (2 mM) and lactate (20 mM) over 6 h, while co-incubation with fructose, pyruvate, and lactate did not further increase glucose production compared with fructose alone (Fig. 1B). Interestingly, over 24 h, co-incubation with fructose, lactate, and pyruvate induced a twofold increase in glucose production compared with fructose alone (Fig. 1C). Fructose (alone)-induced increases in glucose production occurred in parallel with increased PEPCK activity (Fig. 1D), and mRNA levels of *Pck1* (Fig. 1E) and *Pgc1 α* (Fig. 1F). SIRT1, a nuclear NAD^+ -dependent protein deacetylase enzyme (Landry *et al.* 2000), has been shown to induce gluconeogenic gene expression (Rodgers *et al.* 2005, Rodgers & Puigserver 2007). Consistent with fructose-induced gluconeogenesis being mediated through SIRT1, fructose increased NAD^+ / $NADH$ ratio (Fig. 1G), SIRT1 protein level (Fig. 1H), and nuclear HDAC activity (Fig. 1I). HDAC activity was not inhibited by TSA, an inhibitor of class I and II HDAC that does not affect class III HDACs such as SIRT1 (Marmorstein 2004, Li *et al.* 2007). This indicated that the observed increase in HDAC occurs through increased activity of the nuclear sirtuins: SIRT1, 6, or 7 (Michan & Sinclair 2007). The effect of fructose on nuclear sirtuin activity was also inhibited by the SIRT1 inhibitors EX-527, a specific SIRT1 inhibitor (Fig. 1J), and nicotinamide (Supplementary Figure 1A, see section on supplementary data given at the end of this article) at similar concentrations to those used in previous studies of SIRT1 activation (Napper *et al.* 2005, Rodgers *et al.* 2005). This indicated that the fructose-induced

increase in nuclear sirtuin activity occurred as a result of increased SIRT1 activity.

To establish whether fructose-induced gluconeogenesis is dependent on SIRT1, hepatocytes were co-incubated with EX-527 and fructose. EX-527 inhibited fructose-induced increases in hepatic glucose production (Fig. 1K), PEPCK activity (Fig. 1L) as well as *Pck1* (Fig. 1M) and *Pgc1 α* (Fig. 1N) mRNA levels. Fructose-induced gluconeogenesis was also inhibited by nicotinamide (Supplementary Figure 1B–E). However, pretreatment with TSA had no effect on fructose-induced gluconeogenesis (Supplementary Figure 1F and G).

Previous studies have reported that fructose can induce an acute stress response in hepatocytes and liver through

Figure 2 Primary hepatocytes isolated from male Sprague–Dawley rats were incubated for 6 h with fructose (5 mM) in glucose-free EBSS (A and B); (A) SIRT1 protein levels (representative blot; *n*=3), (B) SIRT1 activity (*n*=6). Co-incubation of EX-527 (5–10 μ M) with fructose (5 mM; *n*=6) for 6 h inhibits fructose-induced increases in (C) SIRT1 activity, (D) glucose production, (E) PEPCK activity, (F) *Pck1* and (G) *Pgc1 α* mRNA levels. Data are expressed as mean $\pm$ S.E.M. **P*<0.05, ***P*<0.01, ****P*<0.001 versus untreated control (U); #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus fructose alone.

activation of c-JUN N-terminal kinase (JNK; Kelley *et al.* 2004, Wei & Pagliassotti 2004, Wei *et al.* 2005). In agreement, we report protein expression of phospho(Thr¹⁸³/Tyr¹⁸⁵)-SAPK/JNK, indicating JNK activation, after 30-min fructose incubation. Similarly, SIRT1 levels were high following 30-min fructose incubation. However, phospho(Thr¹⁸³/Tyr¹⁸⁵) SAPK/JNK significantly decreased between 30 min and 1 h, becoming undetectable at 3–6 h, indicating that JNK activation occurs as an acute response to fructose and is not sustained over longer periods (Supplementary Figure 2A, see section on supplementary data given at the end of this article). In contrast, SIRT1 protein levels decreased significantly between 30 min and 1 h, increased slightly between 1 and 3 h, before increasing markedly between 3 and 6 h (Supplementary Figure 2A). This is suggestive of an initial acute fructose-induced SIRT1 response, perhaps mediated by JNK activation, followed by a longer-term, JNK-independent, effect over 6 h. Interestingly, only at 6 h were significant increases in PEPCK activity observed, which corresponded with changes in hepatic glucose production being markedly increased at 6 h, compared with 1 h (Supplementary Figure 2B).

To further evaluate physiological relevance, results were confirmed in primary rat hepatocytes. Consistent with the findings in H4IIEC3 cells, incubation of primary hepatocytes with fructose (5 mM) increased the levels of SIRT1 protein (Fig. 2A) and activity (Fig. 2B), while fructose-induced increases in SIRT1 activity were suppressed by EX-527 (Fig. 2C). In primary hepatocytes, fructose also increased glucose production (Fig. 2D), PEPCK activity (Fig. 2E), and mRNA levels of *Pck1* and *Pgc1α* (Fig. 2F and G). These changes were inhibited by EX-527, further indicating that fructose induces gluconeogenesis through a SIRT1-mediated mechanism. Observed changes in glucose production, PEPCK and SIRT1 activities were markedly higher in primary hepatocytes than in H4IIEC3 cells, likely reflecting the increased metabolic activity of these hepatocytes.

SIRT1 activators induce gluconeogenesis

To establish the role of SIRT1 in the induction of gluconeogenesis, we analyzed the ability of a potent SIRT1 activator, SIRT1 activator 3 (Nayagam *et al.* 2006), to stimulate gluconeogenesis in H4IIEC3 cells and primary hepatocytes. SIRT1 activator 3 increased SIRT1 activity in the presence of TSA (Fig. 3A and B). It also raised PEPCK activity (Fig. 3C and D), mRNA levels of *Pck1* (Fig. 3E and F) and *Pgc1α* (Fig. 3G and H) as well as glucose production (Fig. 3I and J).

SIRT1720 has been described as a small molecule activator of SIRT1 (Milne *et al.* 2007), although a recent study has suggested that this compound is not a direct activator of SIRT1 and may act on multiple targets (Pacholec *et al.* 2010). However, since SIRT1720 is currently under investigation as a therapy for T2DM, we assessed whether this compound could also induce gluconeogenesis. Incubation of H4IIEC3

cells and primary hepatocytes with SIRT1720 increased SIRT1 activity in the presence of TSA (Fig. 4A and B), as well as increasing PEPCK activity (Fig. 4C and D), mRNA levels of *Pck1* (Fig. 4E and F) and *Pgc1α* (Fig. 4G and H), and elevating glucose production (Fig. 4I and J).

Taken together, these data show that induction of hepatic SIRT1 can induce gluconeogenesis, and that fructose induces gluconeogenesis through a SIRT1-mediated mechanism.

Figure 3 H4IIEC3 cells and primary hepatocytes were incubated for 6 h with SIRT1 activator 3 (20–60 μM) in glucose-free EBSS ($n=6$); (A and B) SIRT1 activity, (C and D) PEPCK activity, (E and F) *Pck1* and (G and H) *Pgc1α* mRNA levels. For glucose measurements, (I and J) H4IIEC3 and primary hepatocytes were incubated for 6 h with 10 mM lactate and 2 mM pyruvate (LP) with or without SIRT1 activator 3 (20 μM). Data are expressed as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus untreated control (0).

Figure 4 H4IIEC3 cells and primary hepatocytes were incubated for 6 h with SIRT1720 (50–100 and 10 nM respectively) in glucose-free EBSS ($n=6$); (A and B) SIRT1 activity, (C and D) PEPCK activity, (E and F) *Pck1* and (G and H) *Pgc1 α* mRNA levels. For glucose measurements, (I and J) H4IIEC3 and primary hepatocytes were incubated for 6 h with 10 mM lactate and 2 mM pyruvate (LP) with or without SIRT1720 (100 or 10 nM respectively). Data are expressed as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus untreated control (0).

Mitochondrial complex I regulates SIRT1-dependent response to fructose

Activation of complex I of the mitochondrial electron transport chain increases NAD^+/NADH ratio through increased NAD^+ production as well as increasing ATP production from ADP (Voet & Voet 1994). Fructose induced gluconeogenesis through a SIRT1-mediated mechanism in parallel with increasing NAD^+/NADH ratio (Figs 1 and 2

and ATP levels at 6 h (Supplementary Figure 3A, see section on supplementary data given at the end of this article). Therefore, we tested whether changes in SIRT1 resulted from increased complex I activity by using rotenone, a potent complex I inhibitor. Rotenone inhibited fructose-induced increases in NAD^+/NADH ratio (Fig. 5A), ATP levels (Fig. 5B), SIRT1 protein (Fig. 5C), and SIRT1 activity (Fig. 5D). Rotenone also inhibited fructose-induced increases in hepatocyte glucose production (Fig. 5E), PEPCK activity (Fig. 5F), and mRNA levels of *Pck1* (Fig. 5G) and *Pgc1 α* (Fig. 5H). These results suggest that the changes in NAD^+/NADH ratio and SIRT1 in response to fructose occur through increased activity of complex I of the mitochondrial electron transport chain. Consistent with observations of increased SIRT1 expression and activity at 6 h, time course studies indicated that NAD^+/NADH ratios were only increased with 6 h fructose incubation and were unchanged at 1 and 3 h (Supplementary Figure 3B).

To further elucidate the role of complex I in fructose-induced SIRT1-mediated gluconeogenesis, we investigated whether the inhibitory effects of rotenone could be overcome by SIRT1 activator 3. Pretreatment with rotenone inhibited fructose-induced hepatic glucose production to basal level. Activation of SIRT1 by incubation of fructose with SIRT1 activator 3 after pretreatment with rotenone partially countered the effect of rotenone, as hepatic glucose production increased to 60% of the levels achieved by incubation with fructose alone (Fig. 5I). This suggests that while the electron transport chain exerts some SIRT1-independent flux control over gluconeogenesis in response to fructose, $\sim 60\%$ of the fructose-mediated induction of hepatic glucose production observed in these experiments occurs through SIRT1.

Taken together, these data show that fructose-induced increases in SIRT1 occur through activation of complex I of the electron transport chain causing increased NAD^+/NADH and ATP levels, leading to increased SIRT1 activation and induction of gluconeogenesis.

Fructose induces lipogenic gene expression through a SIRT1-mediated mechanism

To determine whether fructose also induces *de novo* lipogenesis and cholesterol synthesis through a SIRT1-dependent mechanism, we measured alterations in gene expression of acetyl-coA carboxylase (*Aca*) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Hmgcr*), the rate-limiting enzymes for lipogenesis and cholesterol synthesis (Hobbs *et al.* 1990, Rodgers & Puigserver 2007). The response of primary hepatocytes to SIRT1 activators and fructose alone or co-incubated with EX-527 was evaluated. In addition, we measured the impact of SIRT1 activators on gene expression of two key regulators of lipogenic gene expression; *Pgc1 β* and sterol regulatory element-binding protein 1c (*Srebp1c*).

Figure 5 Pretreatment of H4IIEC3 cells for 24 h with rotenone (50 nM) inhibited fructose-induced (6 h; 5 mM; $n=6$) increases in (A) cellular NAD⁺/NADH ratio, (B) cellular ATP levels, (C) SIRT1 protein levels (representative blot; $n=3$), (D) SIRT1 activity, (E) glucose production, (F) PEPCK activity, (G) *Pck1* and (H) *Pgc1α* mRNA levels. (I) H4IIEC3 cells were pretreated with rotenone (50 nM; R) for 1 h, prior to 5 h incubation with fructose (5 mM; F) or co-incubation with fructose (5 mM) and SIRT1 activator 3 (60 μM; S). Data are expressed as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus untreated control (U); # $P<0.05$, ## $P<0.01$, ### $P<0.001$ versus fructose alone; +++ $P<0.001$ vs F+R alone.

Fructose induced gene expression levels of *Hmgcr* and *Acc*. These responses were inhibited by EX-527 (Fig. 6A and E). *Hmgcr* and *Acc* gene expression was also increased by both SIRT1 activator 3 (Fig. 6B and F) and SRT1720 (Fig. 6C and G). In addition, fructose-induced increases in *Hmgcr* and *Acc* were inhibited by rotenone (Fig. 6D and H), further

highlighting the importance of complex I activity for induction of SIRT1 by fructose. Fructose also induced intracellular cholesterol production over 24 h, an effect that was inhibited by nicotinamide and rotenone (Fig. 6I–K). Moreover, SIRT1 activator 3 and SRT1720 induced gene expression of hepatic *Pgc1β* (Fig. 6L and M) and *Srebp1c* (Fig. 6N and O) in primary hepatocytes.

Taken together, these data indicate that fructose induces *Hmgcr* and *Acc* gene expression through a SIRT1-dependent mechanism, and that these responses may occur through SIRT1-mediated induction of PGC1β and SREBP1c.

Discussion

Previous studies have described the function of hepatic SIRT1 in the induction of gluconeogenesis, focusing on its role in hepatic glucose production in response to fasting, and in glucose-starved cultured hepatocytes (Rodgers *et al.* 2005, Rodgers & Puigserver 2007). In this study, we show that fructose as a nutrient substrate, at concentrations similar to estimates for the hepatic portal vein, stimulates gluconeogenesis via SIRT1. This indicates that activation of hepatic SIRT1 could contribute to the metabolic abnormalities observed following excess dietary fructose consumption. This is significant since consumption of a fructose-rich diet impairs glucose and lipid homeostasis leading to a metabolic syndrome-like state and has been associated with the development of obesity and T2DM (Bray *et al.* 2004, Gaby 2005, Johnson *et al.* 2007). While the effect of fructose feeding on hepatic SIRT1 has yet to be directly examined *in vivo*, SIRT1 is reportedly induced in the hearts of fructose-fed rats (Pillai *et al.* 2008).

Other animal studies support the idea that dysregulation of hepatic SIRT1 could lead to hyperglycemia through induction of abnormally elevated hepatic gluconeogenesis. RNA interference and antisense oligonucleotide-mediated knockdown of hepatic SIRT1 resulted in the correction of hyperglycemia in two different rodent models of T2DM (Rodgers & Puigserver 2007, Erion *et al.* 2009). SIRT1 is reported to induce gluconeogenesis through removal of STAT3-dependent inhibition of gluconeogenesis (Nie *et al.* 2009) and by deacetylation-mediated facilitation of FOXO1 nuclear retention (Frescas *et al.* 2005), indicating that inappropriate activation of SIRT1 can contribute to hyperglycemia. Interestingly, *SIRT1* null mice that survive to adulthood display a phenotype that includes low blood glucose (Chen & Guarente 2007), indicating inability to induce gluconeogenesis.

Others have shown that SIRT1 activators increase *Pck1* gene expression (Feige *et al.* 2008). Chronic treatment of ob/ob mice fed a high-fat diet with the putative SIRT1 activator, SRT1720 (30 mg/kg), led to increased food intake and body weight (Pacholec *et al.* 2010), and SRT1720 did not lower plasma glucose or improve mitochondrial capacity in this study.

Figure 6 Primary hepatocytes were co-incubated for 6 h with either EX-527 (5–10 μM) in combination with fructose (5 mM), or for 6 h with SIRT1 activator 3 (20 μM) or SRT1720 (10 nM), without fructose, in glucose-free EBSS ($n=6$), and mRNA levels for *Acc* (A–C), *Hmgcr* (E–G), *Pgc1β* (L and M), and *Srebp1c* (N and O) were measured. H4IIEC3 cells were pretreated with rotenone (24 h; 50 nM) followed by fructose incubation (6 h; 5 mM), and levels of *Acc* and *Hmgcr* mRNA (D, H) were measured ($n=4$). For cholesterol assays, (I–K) H4IIEC3 cells were incubated with fructose (1–5 mM) or co-incubated with fructose (5 mM) and nicotinamide (0.1–2 mM) or fructose (5 mM) and rotenone (50 nM) for 24 h. Data are expressed as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus untreated control (U); # $P<0.05$, ### $P<0.001$ versus fructose alone.

We report in this study that SIRT1 activators can induce hepatic lipogenesis. Moreover, liver-specific SIRT1 knockout mice are reported to gain less weight and display lower fat accumulation in liver and WAT compared with wild-type controls, as a result of decreased SIRT1-induced hepatic lipogenesis (Chen *et al.* 2008). Hence, increased weight gain in high-fat-fed ob/ob mice administered SRT1720 may occur through induction of hepatic lipogenesis.

We also report that fructose incubation results in increased electron transport chain activity, demonstrated by increased NAD^+/NADH ratio and ATP levels and by the observation that rotenone, an inhibitor of complex I of the electron transport chain, inhibits fructose-induced gluconeogenesis. This is in agreement with previous studies reporting inhibition of fructose-mediated induction of electron transport chain activity and gluconeogenesis (Pryor *et al.* 1987). However, the mechanisms responsible for fructose-mediated activation of the electron transport chain are unclear. It is widely accepted that hepatic ATP levels fall on exposure to high fructose levels

(Clark *et al.* 1979, Latta *et al.* 2000). However, this is based on observations that assessed ATP levels after acute fructose exposure. Consistent with such observations the results of this study show a $\sim 20\%$ fall after 10-min fructose incubation, but ATP increases progressively after 1 h to reach a level 80% above baseline at 6 h. The initial fall is probably caused by the first step in hepatocyte fructose metabolism, which involves ATP-dependent fructokinase-catalyzed conversion of fructose to fructose 1-phosphate, as proposed by other authors (Johnson *et al.* 2007). The rise in ATP coincides with an increase in NAD^+/NADH ratios. It is possible that initial fructose-mediated depletion in ATP levels drives electron transport chain activity, resulting in the later increases in NAD^+/NADH ratios and consequent induction of SIRT1 and higher ATP levels observed in this study. Other studies have reported an increase in mitochondrial volume following gluconeogenic stimulation leading to stimulation of electron transport chain flux (Pryor *et al.* 1987). Such a mechanism could be operative in hepatocytes in response to fructose incubation.

Consumption of a fructose-rich diet has previously been shown to induce *de novo* lipogenesis leading to elevated triglyceride and cholesterol levels and dyslipidemia (Fiebig *et al.* 1998, Kelley *et al.* 2004, Jurgens *et al.* 2005, Bilz *et al.* 2006, Rajasekar & Anuradha 2007, Nagai *et al.* 2009). We report in this study that fructose induces gene expression of *Hmgcr* and *Aac* as well as intracellular cholesterol production through a SIRT1-dependent mechanism. This likely occurs through SIRT1-mediated activation of PGC1 β and SREBP1c. Overexpression of hepatic PGC1 β has been shown to induce expression of genes for lipid and cholesterol synthesis (Lin *et al.* 2005), while PGC1 β has been reported to mediate the lipogenic effects of both fructose and saturated fat through coactivation fatty acid genes (Lin *et al.* 2005, Nagai *et al.* 2009). Furthermore, previous studies have reported that SIRT1 can deacetylate PGC1 β in skeletal muscle, inducing the expression of PGC1 β gene targets. We provide evidence that a similar mechanism could operate in the liver.

SREBP1c is a key transcription factor for lipogenic genes and is also induced by fructose, potentially through activation of PGC1 β and liver X receptor (LXR; Rutledge & Adeli 2007, Nagai *et al.* 2009). Moreover, SIRT1 has been demonstrated to activate LXR (Li *et al.* 2007). In agreement with such observations, hepatic SIRT1 knockout mice have lower levels of lipogenesis and are protected from the negative metabolic effects of a high-fat diet (Chen *et al.* 2008). PGC1 β is reported to induce hepatic lipogenesis through induction of the lipogenic transcription factor SREBP1c and LXR (Lin *et al.* 2005, Nagai *et al.* 2009), while SIRT1 has separately been shown to deacetylate and activate LXR (Li *et al.* 2007, Chen *et al.* 2008). Whether the deacetylation of LXR by SIRT1 is separate to its action on PGC1 β or whether they are part of the same regulatory system requires further investigation.

Conversely, activators of SIRT1, particularly SRT1720, are reported to have anti-diabetic actions and are under investigation as treatments for T2DM, having been shown to reduce blood glucose levels and increase insulin sensitivity in rodent models of diabetes (Milne *et al.* 2007), potentially by facilitating degradation of CREB-regulated transcription coactivator 2 (CRTC2), a coactivator of gluconeogenic gene expression (Liu *et al.* 2008). However, this study also reported that following SIRT1-mediated degradation of CRTC2 after short-term fasting, SIRT1 induced gluconeogenic gene expression over a longer fast through a FOXO1-mediated mechanism. Additionally, we have previously reported increased SIRT1 following metformin administration in db/db mice, associated with reduced gluconeogenesis. However, this observation occurred in parallel with increased levels of GCN5, an acetyl transferase that can inhibit gluconeogenic gene expression. Increases in GCN5 likely masked the gluconeogenic stimulatory effects of SIRT1, while also contributing to the observed reductions in gluconeogenesis (Caton *et al.* 2010). In addition, reports have highlighted the potential benefits of increased hepatic

SIRT1 for protection against fatty liver through promotion of fatty acid oxidation (Feige *et al.* 2008, Pfluger *et al.* 2008, Purushotham *et al.* 2009). These effects are consistent with induction of PGC1 α , which along with SIRT1 is required for activation of peroxisome proliferator-activated receptor- α (PPAR α ; Purushotham *et al.* 2009), and may account for some of the insulin-sensitizing effects of SIRT1 activators. Despite this, knockdown of hepatic *Pgc1 α* lowers blood glucose in db/db and normal mice, while overexpression of *Pgc1 α* leads to mild hyperglycemia (Rodgers & Puigserver 2007). Moreover, *Pgc1 α* knockout mice display increased insulin sensitivity (Burgess *et al.* 2006).

Nevertheless, the metabolic consequences of SIRT1 activators in the liver have yet to be fully described. In this study, we demonstrated that SIRT1-activating compounds induced gluconeogenesis and lipogenesis in cultured primary hepatocytes, although in the case of SRT1720 it is currently unclear whether this compound activates SIRT1 directly. In this respect, *in vivo* studies of the effects of direct activators such as SIRT1 activator 3 would be beneficial. Given the data reported in this study and elsewhere, caution should be exercised with respect to the use of SIRT1 activators as therapies for T2DM and metabolic syndrome.

In summary, we have identified fructose as an activator of hepatic SIRT1, raising the possibility that induction of hepatic SIRT1 represents a key process in the onset of metabolic syndrome occurring in response to the consumption of a fructose-rich diet.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-10-0190>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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