In Vitro Culture of Human Brown Adipocytes: Effects of Fructose

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***Abstract*-Brown adipocytes can be distinguished from their white counterparts by markers such as UCP-1 or P2RX5. This study cultured brown human adipocytes in growth media containing 0.1 g/L and 1 g/L fructose in addition to glucose and monitored the cell growth. Unexpectedly, the cultured cells co-expressed markers for white adipocytes (ASC-1) and markers for brown adipocytes (P2RX5). Cells developed multilocular lipid vesicles and exhibited similar kinetics of mRNAs involved in lipogenesis with and without fructose. Excessive amounts of fructose induce negative effects on insulin sensitivity, blood pressure, and liver metabolism. These adverse outcomes were attributed to disturbances in key metabolic pathways in the liver. However, possible consequences of high fructose levels when in direct contact with human white adipocytes in vivo have been considered. No such data existed on the effects of fructose on human brown adipocytes. Results indicate that in addition to toxic effects on liver cells, human white and brown adipocytes consume extra fructose and generate new lipid vesicles, further deregulating energy homeostasis.**

Keywords- Human Brown Adipocytes; Fructose; mRNA Expression; Obesity; Flow Cytometry

I. INTRODUCTION

Obesity has become a global problem due to changes in food consumption and increasingly sedentary lifestyle. This obesity epidemic has been accompanied by many factors including a steep rise in the consumption of sucrose and high-fructose corn syrup through sugar-sweetened beverages and other products. In fact, even adults attempting to lose weight consume an average of more than 700 mL of sugary drinks in a day [1]. Accordingly, the imbalance between caloric intake and energy expenditure results in the accumulation of lipids within white adipose tissue and leads to obesity. The development of obesity is also related to the balance between white adipose tissue, which is the primary site of energy storage, and brown adipose tissue, which is specialized for energy expenditure [2]. It is hoped that activation of brown adipose tissue that can burn excessive calories via uncoupling respiration can counter the fat accumulation.

A number of studies have implicated the role of high fructose consumption in obesity as well as in hypertension, hyperuricemia, hyperinsulinemia, dyslipidemia, hepatic insulin resistance, non-alcoholic fatty liver disease (NAFLD), leptin resistance, and type-2-diabetes, as summarized by [3]. Most studies that have investigated the adverse effects of fructose have analysed the hepatic effects of fructose. A recent study investigated whether white adipocytes in culture tolerate high amounts of fructose and if fructose may promote obesity by acting directly on adipocytes, as has been suggested in previous human studies [4]. The current study aims to investigate the claims that brown adipocytes can mitigate the effects of high caloric intake via uncoupled respiration [5]. For this purpose, primary human brown preadipocytes were cultured in growth medium containing high amounts of glucose (3g/L, representing the concentration in the brown fat maintenance medium (BAMM) sold by the manufacturer) with added fructose (0.1 g/L and 1 g/L). After differentiation, the cells were observed for the presence of multilocular fat vesicles [6] and screened for expression levels of genes involved in hexose uptake, nutrient sensing, and de novo lipogenesis.

II. MATERIALS AND METHODS

A. Cell Culture

Primary human brown preadipocytes were obtained from Creative Bioarray (Shirley, NY, USA). The brown fat cell progenitors were isolated from the brown fat depot of the scapular region from a human fetus; standard collagenase digestion and isolation of the stromal vascular fraction with a shortened collagenase digestion protocol was employed by the manufacturer. Preadipocytes were grown in preadipocyte growth medium (BPGM, Creative Bioarray) containing fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM/F12) and HEPES, and stored at 37 ℃ in humidified air containing 5% CO_2. All cell media contained glucose. Fructose when added at concentrations of 0.1 g/L or 1 g/L. Cells were visually inspected once per day, and fresh growth medium was added every second or third day. An aliquot of the depleted medium was stored at -80 ℃ and later used to measure fructose and glucose concentrations.

B. Differentiation

Twenty four hours after cells reached 100% confluence, cells were differentiated by 72 hours of treatment in preadipocyte differentiation medium (BADM, Creative Bioarray) containing serum, DMEM/F12, HEPES, biotin, pantothenate, insulin,

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dexamethasone, isobutylmethylxanthine, and C-59 with or without fructose. Subsequently, the media was replaced with brown fat maintenance medium (BAMM, Creative Bioarray) containing FBS, DMEM/F12, HEPES, biotin, pantothenate, insulin, and dexamethasone, with or without added fructose, and cell cultures were visually inspected once per day. The multilocular fat droplets were stained with Nile Red [7]. Aliquot flasks in sufficient amounts were cultured to allow harvesting for up to five weeks. The medium contained 3 g/L glucose, measured using standard clinical laboratory methods (GLU-Flex, Dimension Vista, SIEMENS Healthcare Diagnostics, Newark, USA; our laboratory is ISO 9001:2008 certified). Fructose concentrations were measured using a commercial kit (EFRU-100, Bioassay Systems, Hayward, USA).

C. RT-PCR

Cells were lysed in Trizol Reagent (Life Technologies, Darmstadt, Germany) and RNA was purified according to manufacturer instructions. To this end, aliquot flasks of differentiating/differentiated adipocytes were rinsed with Trizol™ for immediate mRNA preservation and extraction after the culture medium was collected (and frozen at -70 ℃ for later processing). The mRNA was reverse transcribed directly, and the cDNA was stored at -70 ℃. This protocol ensured the highest possible quality of mRNA, preserving the mRNA within seconds of nuclease digestion.

Random primers, desoxynucleotide triphosphates, protector RNAase inhibitor and reverse transcriptase were obtained from Roche (Basel, Switzerland). PCR was performed using Taqman primers and probes for the following molecules: fatty acid synthase (FASN; Hs01005622), glycerol-3-phosphate acyltransferase (GPAM; Hs0157368), Solute Carrier Family 2, member 4 (GLUT4, also known as SLC2A4; Hs00168966), Solute Carrier Family 2, member 5 (GLUT5, also known as SLC2A5; Hs0016172), Adiponectin (Hs00605917), Leptin (Hs00174877), Actin B (ACTB; Hs01060665_g1), Ribosomal Protein L3 (RPL3 = ASC-1; Hs01581771_g1), Uncoupling Protein 1 (UCP1; Hs00222453_m1), Solute carrier family 36, member 2 (SLC36A2; Hs04186501_m1), and Purinic receptor (P2RX5; Hs01112471_m1) obtained from Life Technologies (Thermo Fisher Scientific, Vienna, Austria). PCR was performed on an ABI Prism 7000 detection system (Life Technologies) with 5x HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), (PCR efficiency = 2). Duplicate experiments utilized two µL of the reverse transcribed sample, 4 µL mastermix, 1 µL primermix (ad 20 µL with H_2O); the Ct values of the two PCRs were less than 0.3 cycles apart). During the course of five weeks of monitoring the cells by PCR, actin Cts showed a mean of 19 ± 1 cycles. In previous experiments [4], actin was determined to be the appropriate reference gene. FAM fluorescence was used as a readout. The amplification blots were visually checked, and the baseline was set manually.

D. Flow Cytometry

Human brown preadipocytes at confluence were harvested by trypsinization, and aliquots were immediately frozen in 10% DMSO at -80 ℃. For immunostaining, one vial (containing approximately 10^7 cells/mL) was thawed at 37 ℃, cells were washed with PBS (containing 0,2% Na-Azide and 1% FBS). Cells were obtained by centrifugation at 350 ×G for five minutes and diluted in PBS at 10^5 cells per 100 µL. One-hundred µL aliquots of cell suspension were utilized for immunofluorescence staining. Five µL of fluorescently-labelled monoclonal antibodies P2RX5-FITC (LS-C216572/65103) and/or TRIP4/ASC-1-PE (LS-C272287/65072) (LSBio, LifeSpan Biosciences, Inc., Vienna, Austria) were added, and cells were incubated for 15 minutes at 4 ℃. Staining with nonspecific FITC- and PE-labelled control antibodies (Beckman-Coulter, Brea, CA, USA) was performed in each experiment. Flow cytometry analysis of stained brown preadipocytes was performed on a Beckman-Coulter Cytomics FC500 (Beckman-Coulter, Brea, CA, USA). Ten thousand events of each antibody combination of each sample were acquired. Instrument setup was performed for every experiment using Flowcheck controls (Beckman-Coulter). The intraassay coefficient of variation was <5%. Data analysis was performed with CXP Analysis software (Beckman-Coulter). Data are expressed as median channel fluorescence (MCF) of either FITC (Fl-1; P2RX5) or PE (Fl-2; ASC-1) fluorescence.

E. Analysis

All experiments were performed twice. Cells with different amounts of fructose were each grown in two 10 cm^2 flasks. After harvesting, the mRNAs of the two flasks were pooled and PCR was performed twice for each time point. The figures show the time courses and depict the raw data (mean of two PCRs) of one representative experiment. Glucose and fructose intake by the cells was calculated by subtracting the concentrations found in spent media from the baseline value. The values of consumed hexoses were normalized to the consummation of one day in culture to account for the change of media every second or third day.

III. RESULTS

A. Brown Preadipocytes

The primary aim of this study was to determine whether or not brown human adipocytes can tolerate the presence of fructose in culture medium. Cells were cultured in brown preadipocyte growth medium with or without added fructose (0.1 g/L and 1 g/L) for up to two weeks. Cells (with or without added fructose) reached confluence simultaneously, could be replated after trypsin incubation, and were morphologically indistinguishable among all three culture media.

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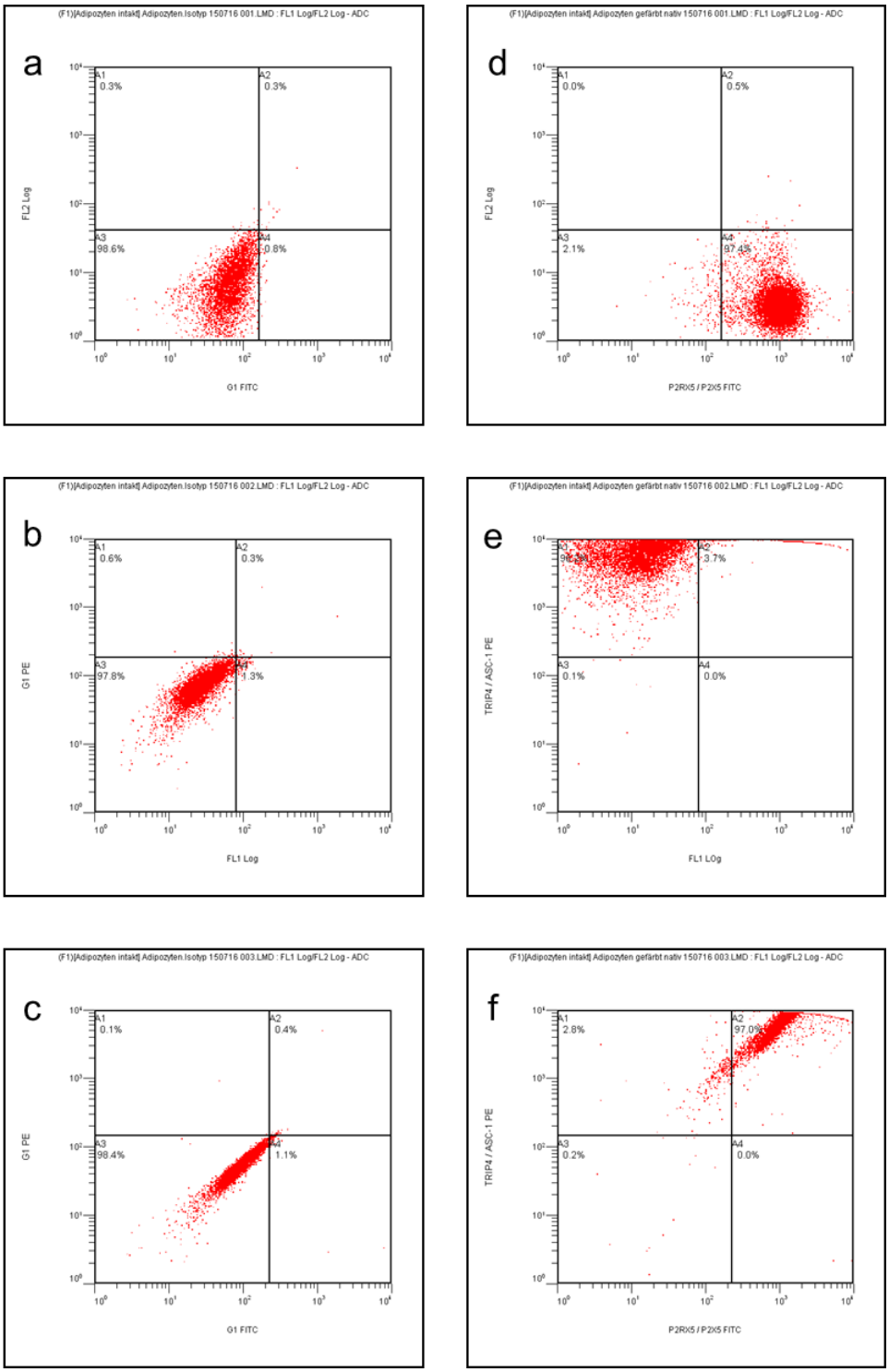

Fig. 1 Human brown pre-adipocytes analyzed by flow cytometry for ASC-1 and P2RX5 antigens

One-hundred μL aliquots of cell suspension were used for immunofluorescence staining. Five μL of fluorescently-labelled monoclonal antibodies P2RX5-FITC and/or TRIP4/ASC-1-PE were added, and cells were incubated for 15 minutes at 4 ℃. Data are expressed as median channel fluorescence (MCF) of either FITC (Fl-1; P2RX5) or PE (Fl-2; ASC-1) fluorescence. (a-c): isotype controls; (d) cells stained with ASC-1; (e) cells stained with P2RX5; (f) cells labeled with both antibodies (all cells are double-positive).

When it was obvious that the cultured cells demonstrated tolerance to the fructose in the medium, different biochemical mechanisms reflecting the addition of the two hexoses were sought by analyzing the expression of various genes responsible for the uptake, utilization, and effects of fructose. To this end, the mRNA levels of target genes were compared to the amount of actin mRNA. Prolonged exposure of the cells incubated with 0.1 g/L versus 1 g/L fructose showed different patterns of mRNA expression for the various genes: whereas the expression level of P2RX5 and UCP-1 in 1 g/L fructose did not increase as much as that in 0.1 g/L (ratio of 1:4), GPAM expression levels showed a seven-fold increase in 1 g/L fructose as compared to 0.1 g/L fructose. The amount of mRNA representing the other studied markers (FASN, GLUT4, GLUT5, leptin) showed

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similar increases after incubation of the cells with 0.1 g/L or 1 g/L fructose for one week (data not shown). Interestingly, GLUT4 (glucose transporter) mRNA levels were 100-fold less than GLUT5 (fructose transporter) mRNA levels (the same as in brown adipocytes, see below) in media containing fructose.

Unexpectedly, levels of mRNA expression of the white adipocyte marker ASC-1 were 200-fold higher than the levels of P2RX5 mRNA. To verify that the utilized cells were brown preadipocytes, immunostaining was performed with fluorescence-labeled antibodies specific to white and brown adipocytes. Single- and double-staining experiments on a flow cytometer confirmed the presence of both antigens on the surface of the cells (double-positive cells, shown in Fig. 1) with a mean FITC fluorescence (P2RX5) of 1.5E3 and PE fluorescence (ASC-1) of 6.9E3. These data indicate that the studied cells are brown preadipocytes, and that human brown preadipocytes express the marker for white preadipocytes Ribosomal Protein L3 ASC-1 on their surface in addition to purinergic receptor P2RX5.

B. Brown Adipocytes

Twenty-four hours after preadipocytes reached 100% confluence, cells were differentiated by 72 hours of treatment in preadipocyte differentiation medium. Beginning from day four after the cells were incubated in brown adipocyte maintenance medium with or without fructose, cells were visually inspected, media were collected, and mRNA was extracted every second or third day. The first hints of tolerance to fructose were identified by observing the cells microscopically. Multilocular fat droplets (Fig. 2a) within the cells developed to a comparable extent in cells incubated with both sugars as compared to those incubated with glucose alone.

Glucose consumption was measured with an automated analyzer and fructose consumption (at 1 g/L baseline) was measured with a biochemical assay to determine the utilization of the different hexoses by the cells. Because media were changed at intervals of 2-3 days, sugar ingestion was calculated by dividing the total decrease of the respective sugar in the media by the number of days the cells were in contact with the respective media. Thus, hexose utilization per day could be inferred: the combined consumption of fructose and glucose in the fructose-containing medium was at a comparable level to that of glucose consumption in medium containing glucose alone. Thus, cells in media containing both hexoses utilized amounts of glucose and fructose that was approximately equal to utilization of glucose in media with glucose only (Table 1). These data imply that the same amount of cells (all wells were confluent) utilize the same amount of energy independent of the energy source, which is in agreement with previous results reporting consumption of energy by white adipocytes [4].

TABLE 1 HEXOSE CONSUMPTION OF HUMAN BROWN ADIPOCYTES IN MEDIA WITH AND WITHOUT ADDED FRUCTOSE

	Glucose only	0.1 g/L Fructose	1 g/L Fructose
Glucose intake (μmol/d/10cm²)	3,24	3,38	2,68
Fructose intake (μmol/d/10cm²)	0	*	0,70

*: Fructose decline was not measurable with the ELISA assay.

The utilization of the different hexoses by the cells over five weeks was analyzed. Because media were changed at intervals of 2 to 3 days, sugar ingestion was calculated by dividing the total decrease of the respective sugar in the media by the number of days the cells were in contact with the respective media.

Furthermore, data regarding different utilization of fructose versus glucose were generated by mRNA expression analysis. The amounts of actin mRNA served as the quantitation standard for the other monitored genes. P2RX5, UCP-1, GLUT5, and ASC-1 mRNAs showed a transient increase during the first days in culture, clearly induced by fructose.

The relative amount of the respective sugar transporter mRNA was quantified. Cells with only glucose in the growth medium showed relatively constant low GLUT4 (insulin dependent glucose receptor) mRNA levels (approximately 100-fold less than GLUT5 mRNA levels) over five weeks in the medium, as well as cells in the parallel presence of fructose in the medium (0.1 and 1 g/L), as shown in Fig. 2c. GLUT5 (the major fructose transporter) levels, however, after a transient peak, increased steadily up to a similar extent in all three media (Fig. 2d), whereas with glucose only, levels did not peak during the first days in medium.

The kinetics of GPAM mRNA levels, in addition to the development of lipid droplets (40% of the cells were filled with lipid droplets after three weeks) in fructose-containing media showed steady increases comparable to the levels observed in media with glucose only. FASN mRNA expression exhibited more complex dynamics with initial increases in all three media. After a decline, the levels increased in all three media until the end of the experiment. Both genes are involved in de novo lipogenesis. The expression of these genes did not reach maximum levels until the end of the experiment (Fig. 2e and 2f).

The differentiated brown adipocytes expressed adiponectin mRNA that increased toward the end of the experiment. Leptin mRNA levels, however, showed a steady incremental increase in all three media from the very beginning until the end of the experiment (Fig. 2g and 2h).

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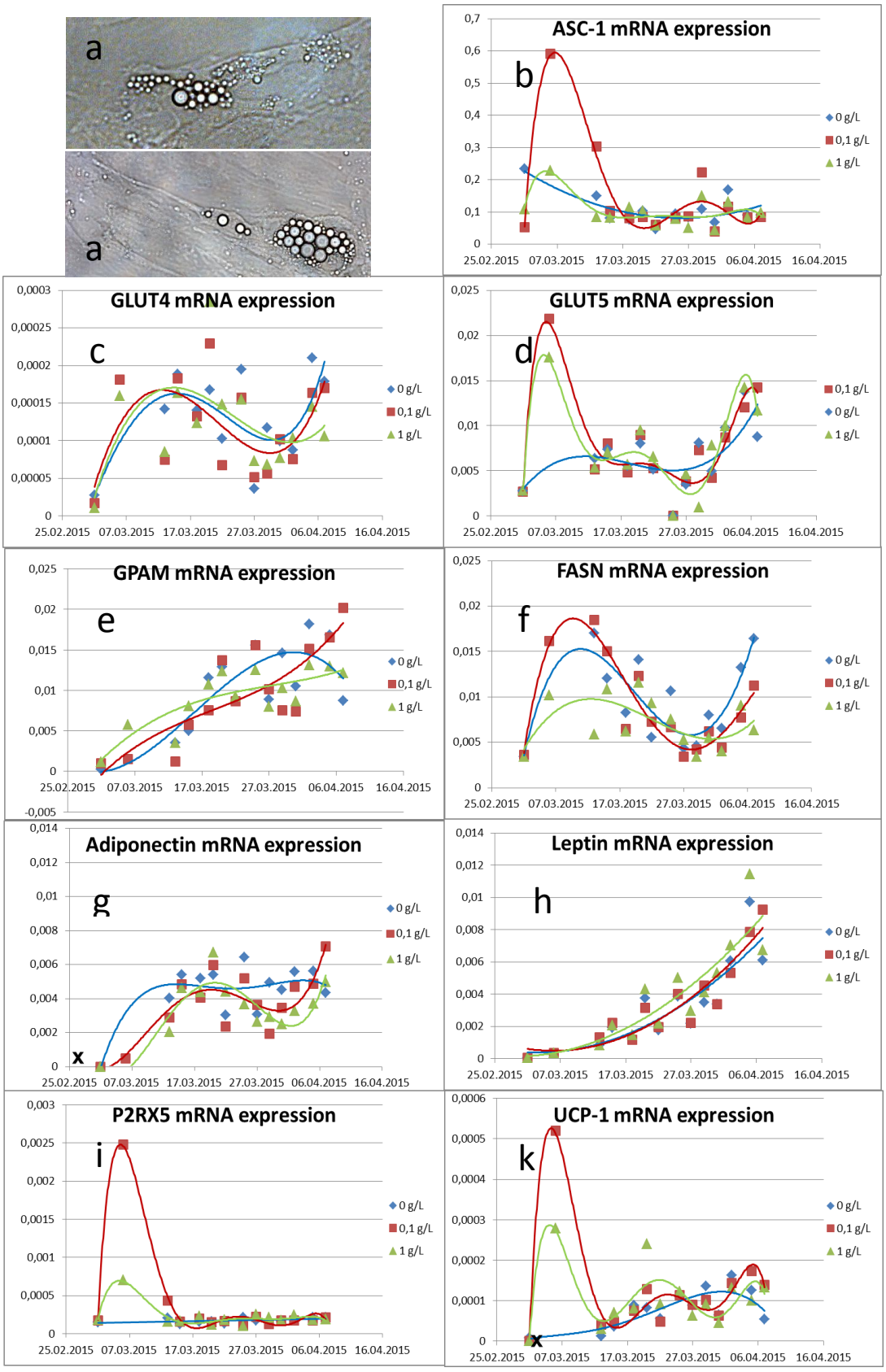

Fig. 2 Fructose transiently induces genes of human brown adipocytes in in vitro cultures

(a) Multilocular lipid droplets in a human brown adipocyte after two weeks in culture medium without (a1) and with (a2) additional 1 g/L fructose, original magnification: 400x; Expression of the respective mRNA levels in the presence (red squares= 0.1 g/L, green triangle = 1 g/L) and the absence of fructose (blue diamonds) is shown. Y-axis: relative expression compared to expression of actin mRNA. The data represent the means of two qPCRs of two pooled dishes of one representative experiment. Trend curves are polynomials generated by Excel (Microsoft Office Standard 2010). x (in Fig. 2g and k): represents that mRNA levels are not measurable (Ct > 40). Ct was set to 40 to generate the trend curve.

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Similar to the preadipocytes, the brown adipocytes expressed steady amounts of ASC-1 mRNA after two weeks and an initial peak in media containing fructose that was notably higher than P2RX5 mRNA levels (Fig. 2b and 2i). UCP-1 mRNA expression exhibited similar kinetics to P2RX5 mRNA expression, albeit at lower levels (Fig. 2k).

In summary, the data clearly show that brown adipocytes thrive in the presence of high amounts of glucose and fructose; they develop multilocular lipid droplets and produce increasing amounts of leptin mRNA over the course of three weeks. Fructose in the media influences the metabolism of the cells at least transiently, as demonstrated by the mRNA expression levels of various genes.

IV. DISCUSSION AND CONCLUSION

Currently, the number of obese people exceeds the number of malnourished people by thirty percent worldwide. The increase is strongly linked to rapid increases in a number of serious conditions such as type-2-diabetes, hypertension, cardiovascular disease, dyslipidemia, sleep apnea, and certain types of cancer [8, 9] The rapid rise of obesity is accompanied by increasing fructose consumption, among many other factors. Consumption of sugar-sweetened beverages that contain high amounts of fructose alone has risen by more than 100% in the United States between 1980 and 2006 [10].

Fructose is primarily metabolized by the liver. Readily-available fructose from sugar-sweetened drinks and highly-processed foods is absorbed quickly and exhibits a strong first-pass effect [11]. The liver eliminates a high portion of the fructose from the blood stream during its first passage. This is largely achieved by the action of ketohexokinase, which rapidly converts fructose to fructose-1-phosphate in the hepatocytes [12]. Most deleterious effects of fructose are attributed to this rapid conversion and the subsequent effects of fructose-1-phosphate and its secondary metabolites [11]. Ketohexokinase knock-out mice have been protected from the harmful metabolic effects of fructose, whereas in tissue specific knock-outs, which express ketohexokinase only in the liver and in no other tissues, the deleterious effects are exacerbated [13].

Adipocytes do express the fructose transporter GLUT5, the major specific fructose transporter [14], but they typically express only minute amounts of ketohexokinase. Fructose may be alternatively metabolized in these cells after being phosphorylated by hexokinase to fructose-6-phosphate, albeit at a much slower rate as compared to hepatocytes.

A high-fructose diet has also been shown to inhibit insulin-induced repression of lipolysis in adipocytes [15]. The authors were not able to elucidate the exact mechanisms of this effect, but they speculate that a fraction of the fructose may reach systemic circulation and act directly on adipose tissue. In a previous study [4], the authors determined that this is the mechanism happening in vivo and that, moreover, fructose induces human adipocytes to create a rapid rise in fat content by inducing several key metabolic genes.

This study also investigated the claims that brown adipocytes can mitigate the effects of high caloric intake via uncoupled respiration. In the cultures of human brown adipocytes, fructose affects the metabolism of the cells by temporarily increasing the expression levels of several measured genes. It certainly will be worthwhile to study the underlying pathways [16] that activate particular genes of the studied panel. The effects of fructose in in vitro cultures are transient; after several days, the metabolism of the cells seems to be altered and differences in the patterns of gene expression are alleviated. Understandably, fructose increases expression of GLUT5 (the main fructose transporter) mRNA during the first days in culture, whereas GLUT4 (the major insulin-dependent glucose transporter in adipocytes) [17] mRNA was not affected. This contrasts with cultures of white adipocytes [4], though the brown adipocytes absorbed glucose in high amounts. Adipocyte medium is basically composed of DMEM and additives, and typically contains 1 g/L glucose. Because the media was analyzed for the uptake of hexoses after the experiments, the high amount of glucose (3 g/L) in the medium supplied by the manufacturer was surprising. This quantity rather simulates a derailed diabetic condition; nevertheless, cells grew well in this medium. An increase in uptake of either hexose in the presence of the other was not observed, but this may be due to the constant insulin levels in the media. The situation may vary in vivo, particularly under conditions of metabolic syndrome and insulin resistance.

Adiponectin is an abundant anti-inflammatory cytokine which is secreted by adipose tissue and has been implicated in insulin sensitivity and obesity [18]. The overall expression level of adiponectin mRNA in the cultured cells is low and changes very little. However, in white adipose tissue (WAT), the adiponectin transcript levels exhibit approximately identical high abundance as actin does, and brown adipose tissue was shown to behave similarly [19]. Most likely, the employed cell culture system fails to emulate physiological conditions in regard to adiponectin, possibly due to the lack of specific cytokines and hormones. Leptin [20] mRNA expression, however, increased to the end of the experiment, an outcome that, again, was not observed in cultures of white adipocytes [21]. Thus, brown adipocytes exhibit different characteristics in in vitro cultures than white adipocytes.

Obesity is characterized by a state of mild inflammation, and the expression and release of inflammation-related adipokines generally rises as adipose tissue expands. One exception is adiponectin; with its anti-inflammatory action, its levels decrease. WAT may be the primary site of inflammation in obesity; increased circulating levels of inflammatory markers reflect spillover from “inflamed” tissue, leading to the obesity-associated pathologies of type 2 diabetes and metabolic syndrome [20]). This metabolic state may not be shared by in vitro cell cultures.

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To monitor lipid synthesis in the cells, FASN (a key enzyme in the synthesis of fatty acids from acetyl–CoA) was employed [22], in addition to GPAM, which catalyzes the first rate-limiting step in the synthesis of glycerolipids [23], and analyzed by mRNA expression. Both FASN and GPAM mRNA levels combined with the generation of lipid vesicles of brown adipocytes incubated with fructose and glucose increased with similar kinetics compared to cells incubated with glucose only. Thus, the brown adipocytes thrive well with high amounts of hexoses in the medium, and the likely marker of uncoupled energy metabolism, UCP1 [24] mRNA, after a transient peak, shows only marginal expression levels that increase just slightly over the course of the experiment.

It has been reported that white, beige, and brown adipocytes can be separated by a variety of surface markers [25]. The initial experiments to measure mRNA amounts of ASC-1 and P2RX5, to distinguish white and brown adipocytes, respectively, showed a two-hundred-fold higher expression of ASC-1 mRNA than P2RX5 mRNA. Flow cytometry analysis revealed that all analyzed cells co-express both markers on their surfaces. Interestingly, both gene expression levels were influenced transitorily by fructose in the medium.

In summary, added fructose to in vitro culture systems is taken up by human brown adipocytes without impediment. This happens under medium conditions that provide cells with plenty of nutrients (3g/L glucose), of which only minor amounts are metabolized between medium renewals. From these data, it can be speculated that similar effects of fructose containing diets also happen in vivo. Apart from toxic action on liver cells, human adipocytes, including brown adipocytes, might be stimulated to take up extra fructose and generate new lipid vesicles, further deregulating energy homeostasis. A tight therapeutical control of brown adipose tissue [5] would be necessary in order to eventually achieve effects on feeding and body weight. This, too, will be the focus of further work because, as shown in this work, human brown adipocytes do not behave appreciably differently in regard to lipogenesis in in vitro cultures than white adipocytes. Uncoupled respiration in cultures of pure brown adipocytes does not independently occur to a remarkable extend in the presence of prominent amounts of hexoses, and would certainly have to be triggered externally.

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