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Lipopolysaccharide, high glucose and saturated fatty acids induce endoplasmic reticulum stress in cultured primary human adipocytes: Salicylate alleviates this stress

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ABSTRACT

Recent findings indicate that endoplasmic reticulum (ER) stress is significantly increased in adipose tissue of obese human subjects and is critical to the initiation and integration of pathways of inflammation and insulin action. But the factors inducing ER stress in human adipose tissue are unknown. The common factors increased in obesity and linked to insulin resistance are hyperglycaemia, hyperlipidemia and also endotoxemia. Therefore, our aims were to investigate: (1) the role of lipopolysaccharide (LPS), high glucose (HG) and saturated fatty acids (SFA) as inducers of ER stress in primary human adipocytes and (2) whether salicylate, a known anti-inflammatory compound, can alleviate this effect. Components of the ER stress pathways were studied in human abdominal subcutaneous (AbSc) adipose tissue (AT) from obese and lean. Following the culture and differentiation of primary human preadipocytes, these adipocytes were treated with LPS, HG, tunicamycin (Tun) and SFA either alone or in combination with sodium salicylate (Sal). Markers of ER stress were significantly increased in AbSc AT of obese. Differentiated human adipocytes treated with LPS, Tun, HG and SFA showed significant activation of eukaryotic translation initiation factor 2α (eIF2α) and activating transcription factor 6 (ATF6) and their down-stream targets. Sal alleviated this effect and activated AktSer473 phosphorylation. This study presents important evidence that: (1) there is increased ER stress in adipose tissue of obese individuals, (2) LPS, hyperglycaemia and saturated fatty acids induce significant ER stress in primary human adipocytes and (3) this induction is alleviated by salicylate.

1. Introduction

Obesity-associated inflammation is a key contributory factor in the pathogenesis of type 2 diabetes mellitus (T2D) and cardiovascular disease (CVD) but the fundamental mechanisms responsible for activating innate immune inflammatory pathways and insulin resistance are currently unclear. Murine studies have revealed that one key link is increased endoplasmic reticulum (ER) stress [1]. The ER has a central role in lipid and protein biosynthesis. During pathological nutrient excess, proteins formed in the ER may fail to attain correct conformation and accumulation of misfolded proteins in the ER causes stress and activates the Unfolded Protein Response (UPR) signal [2].

The UPR signals through three ER transmembrane sensors: PKR-like ER-regulated kinase (PERK), inositol requiring enzyme1α (IRE1α) and activating transcription factor 6 (ATF6) [3]. These activate an adaptive response that results in inhibition of protein translation and increase in transcription of protein-folding chaperones and ER-associated degradation genes [2,4]. PERK phosphorylates the eukaryotic translation initiation factor 2α (p-eIF2α) [5]. p-eIF2α then attenuates protein synthesis and reduces ER protein overload and also activates activation transcription factor4 (ATF4), which induces expression of many genes, including those involved in apoptosis: C/EBP homologous protein (CHOP) [6]. ATF6 activates transcription of ER chaperones: glucose regulated protein (Grp)78/Bip, protein disulfide isomerase (PDI), Ero1-Lα and calnexin to augment the protein folding capacity [1].

An enhanced level of the UPR has been demonstrated in obese, insulin-resistant human adipose tissues [7–9]. ER stress and the UPR are linked to major inflammatory and stress-signalling networks, including the activation of JNK and IKK-NFκB pathways which play a central role in obesity-induced inflammation and metabolic abnormalities [10]. High doses of salicylates have been shown to lower blood glucose concentrations [11]. Severely obese

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rodents when treated with salicylates demonstrated reduced signalling through IKKβ pathway and this was accompanied by improved insulin sensitivity *in vivo* [12,13].

Although ER stress and metabolic dysfunction is associated with obesity in rodent models, the importance of ER stress and the potential inducers of ER stress in human adipocytes are not known. Therefore, the objective of the present study was to show the existence of ER stress in obese human adipose tissue, identify the potential originators of this stress and also demonstrate the role of anti-inflammatory agent, sodium salicylate on ER stress in primary human adipocytes. The primary human preadipocytes were cultured and fully differentiated adipocytes were treated with most probable factors: lipopolysaccharides (LPS), high glucose (HG), tunicamycin (Tun) and saturated fatty acids (SFA) with and without salicylate (Sal). ER stress pathways and Akt activation were studied.

2. Materials and methods

2.1. Subjects

Human Abdominal Subcutaneous (AbSc) adipose tissue (AT) was collected from patients (age: 40.8 (mean ± SD) ± 5yrs; Lean BMI: 22.04 ± 2.6 kg/m^2 and obese BMI 30 ± 3.5 kg/m^2) undergoing liposuction surgery with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. All tissue samples were flash frozen and/or utilized for isolation of human preadipocytes as detailed [14].

2.2. Cell culture

Human AbSc AT (BMI 25.04 ± 0.6 kg/m^2; n = 3–6) were digested with collagenase to isolate preadipocytes as described by Zuk et al. [14]. Preadipocytes were then cultured into tissue culture flasks until confluent and then trypsinized to obtain cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates (10^4 cells/well in 2 ml media) to confluence in DMEM/Ham's F-12 phenol-free medium (Invitrogen, UK) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and transferrin (5 μg/ml). At confluence, preadipocytes were differentiated in differentiation media (Promocell, Germany) containing biotin (8 μg/ml), insulin (500 ng/ml), Dexamethasone (400 ng/ml), IBMX (44 μg/ml), L-Thyroxin (9 ng/ml) and Ciglitazone (3 μg/ml) for 72 h. After this period, the differentiating cells were grown in nutrition media containing (NM) DMEM/Ham's F-12, 3% FCS, D-biotin (8 μg/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (14–18 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method (Sigma–Aldrich) [15].

2.3. Treatments

Differentiated adipocytes (day 15) 24 h prior to treatments were switched to detoxification media (DMEM/Ham's F-12 phenol-free medium containing only 2% serum) to remove effects of growth factors and other components in NM. The treatments were then placed in the fresh detoxification media. The cells were treated with lower or already published concentrations of LPS (100 ng/ml) [16], Tun (750 ng/ml) [2], glucose (HG) (25 mM), SFA (2 mM) and Sal (20 mM) for a maximum of 24 h. SFA was prepared as 40 mM stocks by dissolving Stearic Palmitic acid Mixture (Fluka, UK) in absolute ethanol and then lyophilised and was re-constituted in 1 ml 3% BSA (Free-fatty acid free) in Gey's balanced salt solution (Sigma–Aldrich). The dissolving buffers or solvents were used as controls. All the data shown in this study is from 24 h treatments only, even though for LPS and Tun, lower time points of 6 and 12 h were also studied.

2.4. Immunoblotting

Cells were washed with cold PBS and harvested in lysis buffer (20 mM Tris–HCl, pH 7.5; 137 mM NaCl; 1 mM EGTA, pH 8; 1% Triton X-100; 10% glycerol; 1.5 mM MgCl2) containing protease and phosphatase inhibitors (10 mM NaF; 1 mM PMSF; 1 mM sodium metavanadate; 5 μg/ml aprotinin; 10 μg/ml leupeptin) (Sigma–Aldrich, UK) and stored at −80 °C until required. SDS–PAGE and Western blot analysis were performed as described [16]. Primary antibodies were: phospho and total Akt, phospho and total eIF2α, Bip/GRP78, Calnexin, PDI, Ero1-Lα, phospho-PERK (p-PERK), IRE1α and β-actin (Cell Signalling Technologies). Antigen–antibody complexes were visualized using ECL reagents (Amersham, UK). Autoradiographs were semi-quantified using 2D densitometry software (GeneTools, UK). The bands were first normalised as a function of the loading control (protein of interest/β-actin) or total expression of the proteins (for phosphor proteins), then converted to fold change compared with controls.

2.5. Extraction of RNA and quantitative RT-PCR

RNA was extracted from adipocytes (RNeasy Lipid Tissue Mini Kit, Qiagen), according to manufacturers' instructions. Following DNase treatment and reverse transcription, mRNA expression levels were determined using an ABI 7500 Real-time PCR Sequence Detection system [17]. Pre-optimized quantitative primer and probe sequences for genes ATF6, Ero1-Lα, CHOP and PDI were utilized (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene r18S (Applera). Data were obtained as cycle threshold (Ct) values and used to determine ΔCt values (ΔCt = Ct of gene of interest − Ct of r18s). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging, data was transformed through the Power equation $2^{-\Delta\Delta Ct}$.

2.6. Statistical analysis

Data is presented as mean ± standard error of the mean (SEM) for at least three independent experiments to ensure reproducibility. Students t test was used to compare values between two groups unless stated otherwise. p Values <0.05 were considered to represent statistically significant differences.

3. Results

3.1. ER stress markers are up-regulated in obese human AbSc AT

Protein expression of the ER stress markers was measured in four obese and four lean human AbSc AT. The p-PERK and IRE1α proteins were increased in obese subjects – although this was only significant for IRE1α expression compared with lean (Fig. 1A). ATF6, which regulates the third ER stress pathway, was investigated by examining mRNA expression via real-time PCR, as this proved a more reliable method [18]. ATF6 mRNA expression from AbSc AT of 10 lean and 10 obese subjects showed to be significantly higher (8-fold) in obese subjects (ΔCt = 07.36 ± 1.47) compared with lean (ΔCt = 10.57 ± 1.13) (p < 0.001) (Fig. 1B). Furthermore, the protein expression of down-stream targets of the ER stress pathways, Grp78/Bip1, Calnexin, PDI and Ero1-Lα, were all significantly increased in AbSc AT of obese subjects (Fig. 1A).

3.2. LPS, Tun, HG and SFA up-regulate PERK and ATF6 pathways in primary human adipocytes: Sal down-regulates them

To determine the origins of ER stress fully differentiated human adipocytes were treated with most probable factors elevated in