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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

Objective: Adipose tissue plays a central role in the balance of metabolic homeostasis. An obese adipocyte is challenged by many insults such as surplus energy, inflammation, insulin resistance and considerable stress to endoplasmic reticulum. ER stress has been casually linked as one of the contributing factors for increased inflammation and insulin resistance in adipocytes. Our aims were to examine: 1) what are the inducers of ER stress in primary human adipocytes and 2) whether salicylate can alleviate the ER stress induced by these inducers.

Research Design and Methods: The primary human preadipocytes were cultured and differentiated. The differentiated adipocytes were then treated with lipopolysaccharide (LPS), high glucose (HG), tunicamycin (Tun) and saturated fatty acids (SFA) either alone or in combination with sodium salicylate (Sal). The ER stress pathways activated were studied.

Results: First we show that there is increased ER stress in obese human adipose tissue by measuring ER stress proteins and mRNA. The differentiated primary human adipocytes treated with LPS, Tun, HG and SFA showed activation of PERK and ATF6 ER stress pathways. This activation was alleviated when adipocytes were treated with LPS, Tun, HG and SFA in combination with Sal.

Conclusions: Here we show that: 1) there is increased ER stress in obese human adipose tissue; 2) LPS, HG and SFA induce significant ER stress in primary human adipocytes and 3) The ER stress induced by LPS, Tun, HG and SFA is alleviated by Sal in primary human adipocytes.

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 between obesity-induced inflammation and insulin resistance is increased stress of the endoplasmic reticulum (ER) (1; 2). The ER is a highly dynamic organelle with a central role in lipid and protein biosynthesis (3). The ER is exquisitely sensitive to alterations in homeostasis, and proteins formed in the ER may fail to attain correct conformation during pathological nutrient excess. Accumulation of misfolded proteins in the ER causes ER stress and activation of a signal called the Unfolded Protein Response (UPR) (4). The aim of the UPR is to alleviate ER stress, restore ER homeostasis, and prevent cell death. To achieve these goals, the UPR induces several coordinated responses, including: 1) a decrease in the arrival of new proteins into the ER by translational inhibition; 2) an increase in the amount of ER chaperones; 3) extrusion of misfolded proteins and 4) if everything fails then apoptosis is triggered. Factors acknowledged to elicit cellular stresses are hyperglycaemia, hyperlipidaemia, viral infections and increased protein synthesis; the majority of which are features of obesity and T2D(5; 6).

The UPR signals through three ER transmembrane sensors: PKR-like ER-regulated kinase (PERK), inositol requiring enzyme1α (IRE1α) and activating transcription factor6 (ATF6) (7-9). These then activate an adaptive response that results in inhibition of protein translation and increase in transcription of protein-folding chaperones and ER-associated degradation genes (3; 10). In severe stress, UPR induces apoptosis through several different mechanisms (11). ATF6 induces X-box binding protein 1 (XBP1) transcription and IRE1α upon activation initiates splicing of XBP1 (XBP1s) mRNA which encodes a transcriptional activator that modulates the UPR through regulation of transcription of ER chaperones (12). PERK

phosphorylates the eukayotic translation initiation factor 2α (eIF2α) (13). Phosphor-eIF2α (p-eIF2α) then attenuates protein synthesis and reduces ER protein overload(14; 15). This also results in increased alternative translation of activation transcription factor4 (ATF4), which induces expression of many genes, including those involved in apoptosis: C/EBP homologous protein (CHOP) (14; 16-18).Upon activation during UPR, the cytoplasmic domain of ATF6 is cleaved and the cleaved N-terminal fragment translocates to nucleus and activates the transcription of ER chaperones such as glucose regulated protein (Grp)78/Bip, protein disulfide isomerase (PDI), Ero1-Lα and calnexin to augment the ER protein folding capacity (1; 2; 9; 10; 19).

An enhanced level of the UPR is specifically prominent in obese, insulin-resistant human adipose tissues (11; 20; 21). ER stress and the UPR are linked to major inflammatory and stress-signalling networks, including the activation of JNK and IKK-NFκB pathways and the production of reactive oxygen species (ROS) (22). Intriguingly, these are also the pathways and mechanisms that play a central role in obesity-induced inflammation and metabolic abnormalities (2). High doses of salicylates have been shown to lower blood glucose concentrations (23). Yuan et al. (24) have investigated potential mechanisms of these hypoglycaemic effects in order to identify potential mediators of insulin resistance and molecular targets for intervention. In their study severe obese rodents treated with salicylates demonstrated reduced signalling through IKKβ pathway, either by salicylate inhibition or decreased IKKβ expression. This was accompanied by improved insulin sensitivity *in vivo*. By inhibiting IKKβ activity salicylates have been shown to inhibit the activation of nuclear factor-kB (NF-kB) via inhibition of phosphorylation and degradation of IkBα (25; 26). This inhibition of NF-kB may explain some of the clinically documented anti-inflammatory and insulin sensitising effects of salicylates.

Although ER stress, increased adipose tissue inflammation 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 is not known. Therefore, the objective of the present study was to firstly show the existence of ER stress in obese human adipose tissue, secondly identify the originators of this stress and thirdly demonstrate the role of anti-inflammatory agent, sodium salicylate (sal) on ER stress components in primary human adipocytes. The stromal fractions from human abdominal subcutaneous (AbSc) adipose tissue (AT) were cultured and fully differentiated. The differentiated adipocytes were then treated with most probable ER stress inducers: lipopolysaccharides (LPS), high glucose (HG), tunicamycin (Tun) and saturated fatty acids (SFA) with and without sodium salicylate (Sal). The ER stress markers were measured either by immunoblotting or RT-PCR.

Research Design and Methods

Subjects. Human Abdominal Subcutaneous (AbSc) adipose tissue (AT) was collected from patients (age: 40.75 (mean ± SD) ±4.99yrs; Lean BMI: 22.04±2.56kg/m^2 and obese BMI 30±3.5kg/m^2) undergoing elective or liposuction surgery with informed consent obtained in accordance with LREC guidelines and approval. All tissue samples were flash frozen and/or utilized for isolation of stromal fraction used for culturing primary human adipocytes as detailed (27). In total, 20 human non-diabetic adipose tissue samples were analyzed, which were sub-divided into: Lean AbSc (n=10) and Obese AbSc (n=10) based on BMI.

Cell Culture

Abdominal subcutaneous adipose tissue was digested with collagenase (Worthington Biochemical, Reading, USA) as previously described to isolate mature adipocytes and pre-adipocytes (28). Firstly, stromal fractions of Human AbdSc AT (BMI 25.04±0.56kg/m^2; n = 3-6) were cultured into tissue culture flasks to confluence and then trypsinised to get enough

cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates to confluence in DMEM/Ham's F-12 medium containing 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and transferrin (5 µg/ml). At confluence, preadipocytes were differentiated in preadipocyte differentiation media (Promocell, Germany) containing biotin (8 µg/ml), insulin (500 ng/ml), Dexamethasone (400 ng/ml), IBMX (44 µg/ml), L-Thyroxin (9ng/ml) and Ciglitazone (3µg/ml) for 72 hours. After 72 hours the differentiating cells were grown in nutrition media containing DMEM/Ham's F-12 phenol-free medium (Invitrogen, UK), 3% FCS, d-biotin (8 µg/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until the cells were fully differentiated (14-18 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method as previously documented (Sigma-Aldrich Corp., Poole, UK) (29).

Treatments:

For treatments, fully differentiated adipocytes (day 15) were grown in normal DMEM/Ham's F-12 phenol-free medium containing only 2% serum (detoxification media) for 24 hours to remove any effects of growth factors and other components used in nutrition media. The treatments were then placed in the fresh detoxification media for 24 hrs. The cells were treated with LPS (100ng/ml), tunicamycin (750ng/ml), glucose (25mM): referred as high glucose (HG) (sigma-aldrich), saturated fatty acids (SFA) (2mM) and sodium salicylate (Sal) (20mM) for 24 hours. SFA was prepared as 40mM stocks by dissolving Stearic Palmitic acid Mixture (Fluka) in absolute ethanol and then lyophilising it. The lyophilised SFA was then re-constituted in 1 ml 3% BSA (Free-fatty acid free) in Geys Buffer by vortexing and then sonicating. The dissolving buffer without SFA was used as control whenever SFA was used as treatment. All the data shown in this paper is from 24 hour treatments only.

Lipid staining of differentiated adipocytes

Lipid staining was performed using a modified method described elsewhere (2). Briefly, on d06, d10, d15 and d18 differentiated adipocytes were washed with PBS, fixed with 10% formalin and stained with 2.5% Oil Red O (Gurr Ltd., London, UK) prepared in isopropanol for 1 hour at room temperature. Cells were washed with distilled water and viewed with a light microscope. Differentiation of preadipocytes was determined by photographic assessment of the accumulation of lipid over time.

Immunoblotting

Cells were washed in PBS and harvested in 250 µl of lysis buffer (20 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1mM EGTA, pH 8; 1% Triton X-100; 10% glycerol; 1.5mM MgCl2) containing protease and phosphatase inhibitors (10 mM NaF; 1 mM PMSF; 1 mM sodium metavanadate; 5 µg/ml aprotinin; 10 µg/ml leupeptin) and stored at -80˚C until use. Homogenized human adipose tissue was extracted using RIPA buffer method (28). Total protein was determined by Bradford assay (BioRad, UK). 10-20 µg of protein from cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, UK) by electroblotting. The membranes were blocked with 0.2% I-BlockTM (Applied Biosystems, UK); 0.1% Tween20 in PBS and probed with primary and secondary antibodies. Primary antibodies were: phospho and total Akt, phospho and total eIF2α, Bip/GRP78, Calnexin, Protein disulfide isomerase (PDI), Ero1-Lα, phospho-PERK (p-PERK), IRE1α and β-actin (Cell Signalling Technologies). Antigen-antibody complexes were visualized using ECL reagents (Amersham, UK). Scanned 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 various proteins (for phosphor proteins) and were then converted to fold change compared to the controls.

Extraction of RNA and Quantitative RT-PCR:

To characterize gene expression, RNA was extracted from (RNeasy Lipid Tissue Mini Kit, Qiagen) adipocytes, 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 (30). Pre-optimized quantitative primer and probe sequences for genes were utilized (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene r18S, provided as a pre-optimized control probe (Applera), enabling data to be expressed in relation to an internal reference to allow for differences in relative threshold efficiency. Data was obtained as cycle threshold (Ct) values according to the manufacturer's guidelines (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt=Ct of 18S housekeeping gene subtracted from Ct of gene of interest). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging, data has been transformed through the Power equation $2^{-\Delta\Delta Ct}$, all statistics were performed at this stage.

***Statistical analysis*.**

Data in the text and figures are presented as mean ± standard error of mean (SEM) of at least three independent experiments performed in triplicates 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.

RESULTS

ER stress markers are up-regulated in obese human AbSc AT compared to lean

Protein expression of the ER stress markers was measured in 4 obese and 4 lean human AbSc AT. We first examined the p-PERK and IRE1α protein levels which are the regulators of two

important ER stress pathways. The p-PERK expression was increased in obese subjects but was not significant while the expression of IRE-1α was significantly increased in obese compared to lean (Fig. 1A). Then we examined the mRNA expression of the ATF6 which is the regulator of third ER stress pathway. The ATF6 protein was difficult to detect because the antibodies tried against ATF6 failed to detect either of the two bands (cleaved and uncleaved) and were not claen. Previously, it has been shown by Namba *et al*. (31) that up-regulation of ATF6 mRNA expression is involved in enhancing ER stress response and is a good marker for ATF6 ER stress pathway. Therefore, we looked at the mRNA expression of ATF6 from AbSc AT of 10 lean and 10 obese subjects. Indeed the mRNA expression of ATF6 was significantly higher (8 fold) in obese subjects ($\Delta Ct=07.36\pm1.47$) compared to lean ($\Delta Ct=10.57\pm1.13$) ($p<0.001$) (Fig. 1B). Therefore, all the three know UPR pathways are up-regulated in AbSc AT of obese compared to lean as shown by others(11; 20; 21).

Then we measured the expression of down-stream targets of the above described pathways. The expression of chaperone proteins Grp78/Bip1, Calnexin, PDI and Ero1-Lα were all significantly increased in AbSc AT of obese subjects (Fig. 1A). The protein expression was measured by normalising against β-actin. Even though the sample number is very less, the data presented is very consistent and also the up-regulation of ER stress markers in obese AbSc AT has been shown by 3 different groups with higher sample sizes (11; 20; 21). This observation is only a prelude to the rest of the study.

Sal down-regulates PERK and ATF6 pathways up-regulated by LPS, Tun, HG and SFA in fully differentiated primary human adipocytes

Proximal events for the UPR include the activation of ER-resident signaling molecules such as PERK and ATF6 in order to initiate transcriptional and translational programs that alleviate ER stress. To determine the origins of ER stress in human adipocytes, stromal

fractions from human AbSc AT were cultured and differentiated into adipocytes (Fig. 2A) and these primary adipocytes were then treated with most probable factors elevated in obesity such as LPS, HG and SFA for 24 hours. Tun which is a known inducer of ER stress in almost all the cellular systems studied so far was used as a positive control. First we examined the activation () of down-stream-target of p-PERK, *i.e.*, p-eIF2α. Interestingly, LPS, Tun, HG and SFA all of them significantly induced the p-eIF2α expression compared to cells treated just with the solvent of these substrates (controls) (Fig. 2B and C). We were also interested in looking at the effect of Sal on this pathway. Sal significantly down-regulated the expression of p-eIF2α in adipocytes treated with LPS, Tun, HG and SFA in combination with Sal (Fig. 2B and C). This provides first strong evidence that LPS, HG and SFA activate PERK pathway while Sal alleviates this activation in cultured primary human adipocytes.

Then we examined the effect of these treatments on ATF6 mRNA expression levels. The ATF6 mRNA level was also significantly increased in the cells treated with LPS, HG and Tun (Fig. 2D). It was not significant in SFA treated cells. Sal again significantly down-regulated ATF6 mRNA levels in adipocytes treated with LPS, HG and Tun in combination with Sal (Fig. 2D). ATF6 mRNA expression was significantly down-regulated in cells treated with Sal alone. Sal also down-regulated ATF6 mRNA expression in SFA treated cells but it wasn't significant (Fig. 2D).

Down-stream targets of PERK and ATF6 pathway are up-regulated by LPS, Tun, HG and SFA: Sal alleviates ER stress response

Protein chaperones and down-stream targets of PERK and ATF6 pathways either protein or mRNA expression levels were measured in the adipocytes treated with LPS, Tun, HG, SFA alone or in combination with Sal. ER resident proteins such as Grp78/BiP, calnexin, PDI and Ero1-Lα act as molecular chaperones to promote proper folding and/or prevent aggregation

of folding intermediates. UPR is induced when Grp78/Bip expression is induced and the PERK, ATF6 and IRE1α which are bound to Grp78/Bip, dissociate and are activated (1). Therefore, we first examined the protein expression of Grp78/Bip which is a down-stream target of ATF6. Grp78/Bip was significantly increased in Tun and SFA treated cells only (Fig. 3A and B). There was an increase in LPS treated cells but the increase wasn't significant. HG didn't have any effect on Grp78/Bip expression. Sal significantly down-regulated the expression of Grp78/Bip in Tun and SFA treated cells (Fig. 3A and B). Another protein chaperone calnexin was significantly up-regulated by LPS, Tun, HG and SFA (Fig. 3 C and D). Sal significantly down-regulated the calnexin expression in the adipocytes treated with LPS, Tun, HG and SFA (Fig. 3 C and D). Sal alone also significantly suppressed the calnexin expression compared to the controls.

mRNA expression of other protein chaperones PDI and Ero1-Lα was measured. mRNA expression of both these ER stress markers were significantly increased by LPS, Tun, HG and SFA (Fig. 3 E and F). Sal again significantly down-regulated the expressions of these chaperones in the adipocytes treated in combination with Sal. Only PDI wasn't significantly suppressed by sal in HG treated cells. Sal alone significantly down-regulated PDI mRNA expression levels (Fig. 3E).

Signalling through PERK, ATF6 and IRE1α can induce pro-apoptotic signals during extreme ER stress. They do this by activating down-stream targets such as CHOP. Therefore, we examined the mRNA expression of ER stress-induced apoptotic transcription factor CHOP or GADD153 in LPS, Tun, HG and SFA treated primary adipocytes. All these treatments significantly increased the CHOP expression (at least 2-folds) (Fig. 3G). Interestingly, Sal significantly down-regulated CHOP expression induced by LPS, tun, HG and SFA (Fig. 3G).

Salicylate induces p-AktSer473

Hu et al. (32) have reported transient activation of Akt in ER-induced apoptosis and p-Akt (Ser473), is also an important member of insulin signalling pathway and helps in increasing glucose transport and overall insulin sensitivity of the cell. Therefore, we examined Akt activation by measuring p-AktSer473 levels in the adipocytes treated with LPS, Tun, HG and SFA and also in combination with Sal. Interestingly, LPS, Tun and HG significantly induced p-AktSer473 levels compared to controls. There was further significant induction by Sal in adipocytes treated with LPS, Tun, HG and SFA (Fig. 4 A and B). Sal alone also significantly increased p-AktSer473 levels (Fig. 4 A and B).

DISCUSSION

Adipose tissue plays a central role in the balance of metabolic homeostasis. An obese adipocyte is challenged by many insults such as surplus energy, inflammation, insulin resistance and considerable stress to various organelles. ER is one such organelle which shows significant signs of stress and has been casually linked as one of the contributing factors for increased inflammation which could then lead to insulin resistance. Our first aim was to show the existence of ER stress in human adipose tissue, specifically, obese adipose tissue and then to look for the factors which might be responsible for origin of this stress in cultured fully differentiated primary human adipocytes (Fig. 2A). Also, salicylates have been shown to reduce inflammation and induce insulin sensitivity, therefore, here we also show that salicylates have an effect on ER stress signalling. The results of the present study demonstrate that firstly, there is increased ER stress in obese AbSc AT, secondly, the factors inducing this response could be LPS, hyperglycaemia and SFA, thirdly this stress response is alleviated by salicylates and could contribute to increased insulin sensitivity in adipocytes.

We first examined the ER stress protein markers in lean and obese human AbSc AT and found that most of the ER stress markers were significantly elevated in obese AbSc AT. The

IRE1α and ATF6 expression levels were significantly increased in obese AbSc AT. The p-PERK was also increased but the increase was not significant. This observation confirms the three studies carried out on human adipose tissue (11; 20; 21). PERK, ATF6 and IRE1α play a central role in UPR signalling. Upon activation these induce the expression of protein chaperones for the proper folding of the protein and protein complexes (33) (34). The protein chaperones Grp78/Bip, Calnexin, Ero1-Lα and PDI were all significantly up-regulated in obese human AbSc AT compared to lean AbSc AT (Fig. 1A). Increased expression of PDI and calnexin confirms the earlier observation made by Boden et al.(20).

To identify the factors responsible for inducing ER stress in human adipose tissue we cultured stromal fraction isolated from human adipose tissue and then differentiated them. The differentiated adipocytes were then treated with LPS, Tun, HG and SFA with and without salicylate. LPS (or endotoxin) (35), hyperglycaemia (36)and free fatty acids (FFA)(37; 38) have all been shown to be elevated in blood during obesity and have been linked to increased inflammation and insulin resistance and therefore, were investigated as possible factors for inducing ER stress in human adipocytes. Tunicamycin is a well known ER stress inducer and therefore was used as a positive control. Our data clearly demonstrates that LPS, Tun, HG and SFA activate the PERK pathway. This activation was measured by looking at the expression levels of p-eIF2α, a down-stream target of PERK which was significantly increased in LPS, Tun, HG and SFA treated adipocytes. PERK is activated in response to accumulation of misfolded proteins in the ER, reducing the rate of protein synthesis through eIF2α phosphorylation at ser51 to assure proper protein folding (14; 15). This also induces the transcription of protein chaperones. P-eIF2α activation was totally eliminated when the above treatments were given in combination with salicylate. This observation contradicts another study in promonocytic cell line THP-1 where salicylate and aspirin have been shown to induce eIF2α phosphorylation and hence protein synthesis

attenuation (39).One explanation could be, in that study the cells were exposed to salicylates or aspirin for a very short time, a maximum of 3 hours for salicylate and 6 hrs for aspirin while our observations are based on 24 hrs exposure or it could be a cell specific response. Our observation is further supported by showing the significant increase in expression of protein chaperones, specifically CHOP by LPS, Tun, HG and SFA and this induction is significantly down-regulated by Sal. Other studies have shown that to up-regulate CHOP transcription the PERK-eIF2α-ATF4 branch of the UPR is essential (17).

Modification of ATF6 protein is important for the ER stress response. ER stressors stimulate the cleavage of ATF6 by Site-1 protease (S1P) and Site-2 protease (S2P) into p50-ATF6, which acts as a transcription factor. Namba et al. (31) have shown that all of the ER stressors they tested (such as thapsigargin and tunicamycin) up-regulated ATF6 mRNA expression and the cells over-expressing ATF6 mRNA showed enhanced ER stress response. Indeed the adipocytes treated with tunicamycin, in this study showed highly significant induction of ATF6 mRNA expression compared to the controls. Therefore, up-regulation of ATF6 mRNA expression could be used as an indicator for activation of ATF6 pathway. LPS and HG also induced ATF6 mRNA expression significantly while SFA did induce some expression but it was not significant. Salicylate significantly down-regulated the ATF6 mRNA expression induced by LPS, Tun and HG. Induction of ATF6 pathway is supported by the increase in expression of protein chaperones up-regulated by ATF6. The protein chaperones calnexin, PDI, Ero1-Lα and CHOP expression were induced in adipocytes treated with LPS, Tun, HG and SFA. Sal again helps in alleviating ER stress by down-regulating both the PERK as well as ATF6 pathways.

The most interesting observation was of Grp78/Bip expression with these treatments. The Grp78/Bip expression was significantly increased only by Tun and SFA which was down-regulated by Sal. LPS did show activation but it wasn’t significant. Despite considerable

activation of the UPR, HG did not induce Bip/Grp78 expression. Zhang et al. (40) have made the same observations in INS-1 pancreatic β-cells treated with high glucose (30mM). The reason for this is not yet known and would be interesting to investigate further, even more so because Bertolotti et al. (41) have shown that PERK is found in a complex with Bip/Grp78 in cells without ER stress conditions and is inactive. In order to activate eIF2α, it must dissociate from Bip/Grp78 under UPR condition and we have clearly shown that the p-eIF2α and its down-stream target CHOP are activated by HG. It is quite possible that another mechanism exists for PERK activation under hyperglycaemic condition.

We were also interested in investigating whether these UPR inducing factors would have effect on p-AktSer473 levels. Interestingly, p-AktSer473 was induced by LPS, Tun and HG while SFA had no effect. This is not a unique observation, Hu et al. (32) have reported transient activation of Akt during ER stress, induced by the thapsigargin and tunicamycin in MCF-7 cells. They have also shown blocking Akt activity sensitised MCF-7 cells to ER stress-induced apoptosis, suggesting that Akt activation is a pro-survival pathway activated during ER stress. Ho et al. (42) have also shown Akt activation under hyperglycaemic condition (33mM Glucose) in human umbilical vein endothelial cells (HUVECs) within 24 hrs. (31)Similar observation has been made in LPS treated THP-1 cells (43). The adpocytes treated with salicylate alone or in combination induced p-AktSer473 significantly, at least two fold higher than LPS or HG alone. This could be because of the anti-inflammatory and insulin sensitising effect of salicylates where it inhibits the activation of nuclear factor-kB (NF-kB) via inhibition of phosphorylation and degradation of IkBα (25; 26).

Salicylate is an interesting molecule and in our study we have demonstrated that it successfully alleviates ER stress induced by LPS, HG and SFA. From this study we could also deduce that it has alleviating effect on at least two of the three ER stress pathways, namely, the PERK and the ATF6.We haven’t investigated the IRE1α pathway yet and is the

objective of our future study. The mechanism by which salicylate ameliorates ER stress is unknown. One of the possible mechanisms could be the one demonstrated by Yuan et al. (24) in liver and skeletal muscle tissues of rodents. They and others have shown that sodium salicylate and its acetylated form aspirin can inhibit the activation of NF-kB by preventing the phosphorylation and subsequent degradation of IkBα by down-regulating IkB kinase β (IKKβ) (24; 44). This mechanism will only be true if the ER stress induced by LPS, HG and SFA was the result of increased inflammation at the first place. We still don't understand whether ER stress is the result of increased inflammation or *vice versa*. Probably, investigation of IRE1α pathway will shed some more light for a possible mechanism.

In conclusion this manuscript answers two main issues: 1) what are the inducers of ER stress in human adipocytes and 2) whether salicylate can alleviate the ER stress induced by these inducers. We have clearly demonstrated that LPS, HG and SFA induce significant ER stress in primary human adipocytes specifically the PERK and ATF6 pathways and salicylate fully alleviates this stress. In addition, salicylate also induces insulin sensitivity by activating Akt. Future studies should look at the role of IRE1α pathway by these inducers and also the effect of salicylates on this pathway. It would also be interesting to understand the mechanism involving Bip/Grp78 under hyperglycaemic condition. Role of salicylates as a suppressor of ER stress also needs further investigation.

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Figure Legends

Fig.1: Expression of ER stress markers. (A) Protein expression levels of ER stress markers: p-PERK, IRE1α, Grp78/Bip, Calnexin, PDI, Ero1-Lα and β-actin(loading control) in lean (n=4) vs. Obese (n=4) human AbSc AT. The protein expression was determined from whole AT lysate by western blot. (B) mRNA expression ATF6 in human AbSc AT, Lean vs Obese: both n=10. mRNA was determined by qRT-PCR. In the bar figure, values are the mean±SEM (n=4). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ by Student's *t-test*.

Fig.2: Primary human adipocytes culture and ER stress pathway expression studies from treated cells treated with LPS, Tun, HG and SFA either alone or in combination with Sal. (A) Oil red o stained lipid droplets in fully differentiated primary human adipocytes. (B) p-eIF2α in LPS and Tun treated cells either alone or in combination with Sal. (C) p-eIF2α in HG and SFA treated cells either alone or in combination with Sal. B and C were measured by western blot. (D) mRNA expression ATF6 in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. mRNA was determined by qRT-PCR. In the bar figure, values are the mean±SEM (n=3). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ by Student's *t-test*.

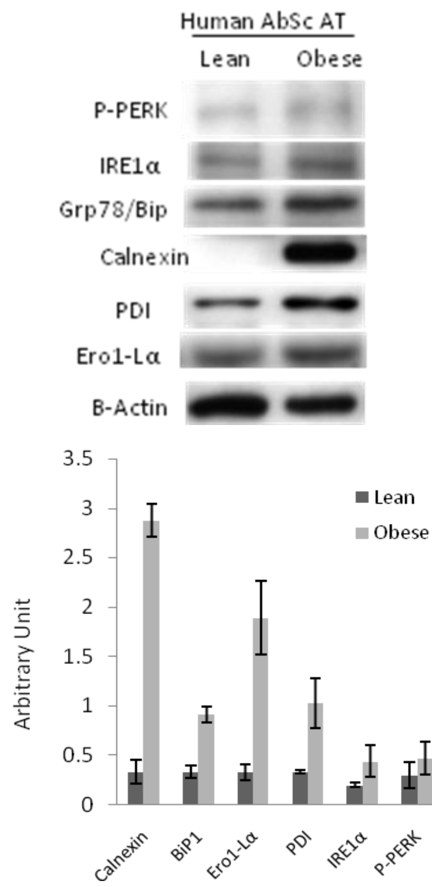
Fig. 3: Expression of ER stress Chaperones. (A) Protein expression levels of Grp78/Bip in LPS and Tun treated cells either alone or in combination with Sal. (B) Protein expression levels of Grp78/Bip in HG and SFA treated cells either alone or in combination with Sal. (C) Protein expression levels of Calnexin in LPS and Tun treated cells either alone or in combination with Sal. (D) Protein expression levels of Calnexin in HG and SFA treated cells either alone or in combination with Sal. (E) mRNA expression PDI in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. (F) mRNA expression Ero1-Lα in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. (G) mRNA expression CHOP in adipocytes treated with LPS, Tun, HG and SFA either alone

or in combination with Sal. mRNA was determined by qRT-PCR. In the bar figure, values are the mean±SEM (n=3). *p<0.05, **p<0.01 and ***p<0.001 by Student's *t-test*.

Fig.4: Expression of p-AktSer473. (A) Protein expression levels of p-AktSer473 in LPS and Tun treated cells either alone or in combination with Sal. (B) Protein expression levels of p-AktSer473 in HG and SFA treated cells either alone or in combination with Sal. Protein expression was measured by western blot. In the bar figure, values are the mean±SEM (n=3). *p<0.05, **p<0.01 and ***p<0.001 by Student's *t-test*.

Fig. 1

A



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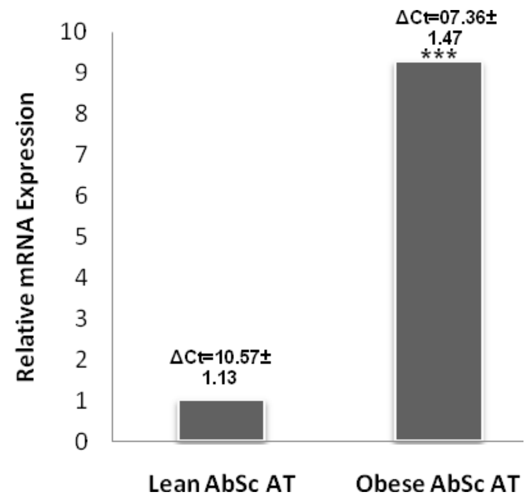
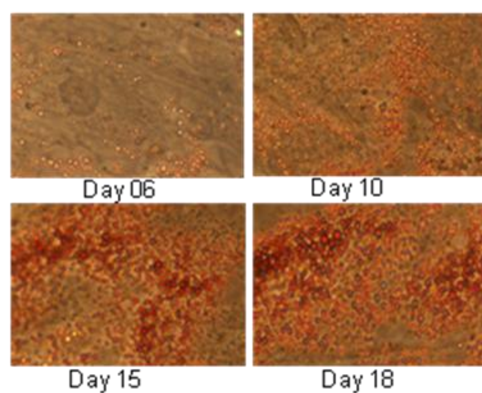
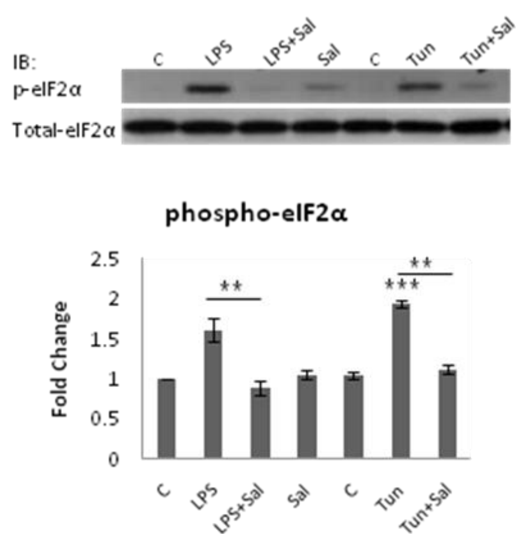
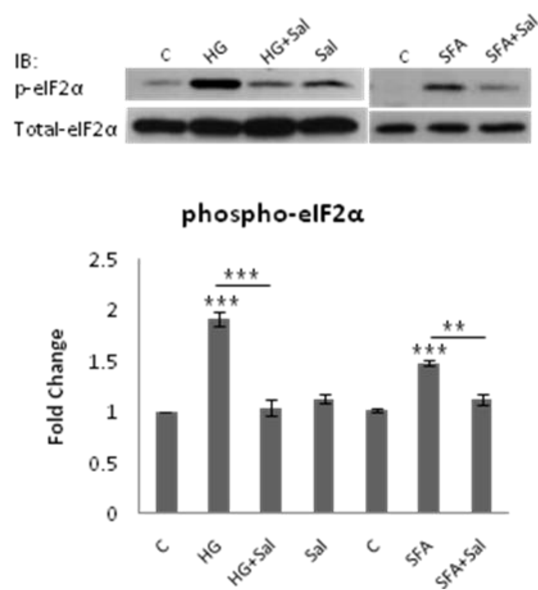


Fig. 2







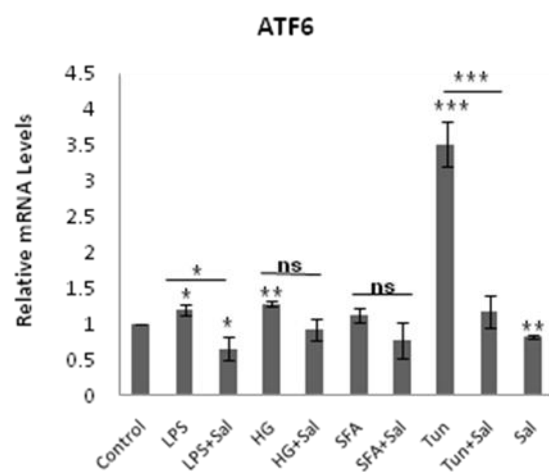
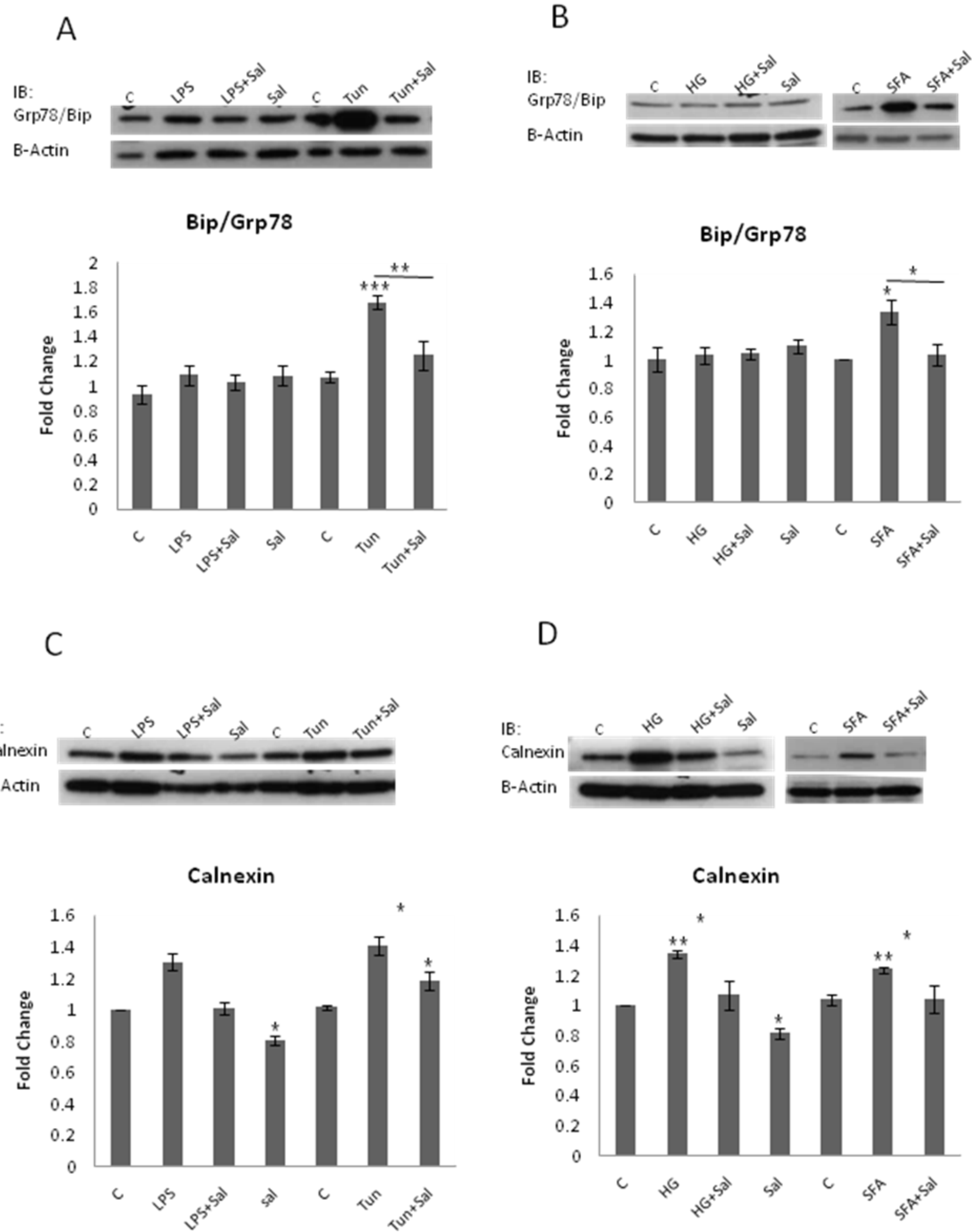
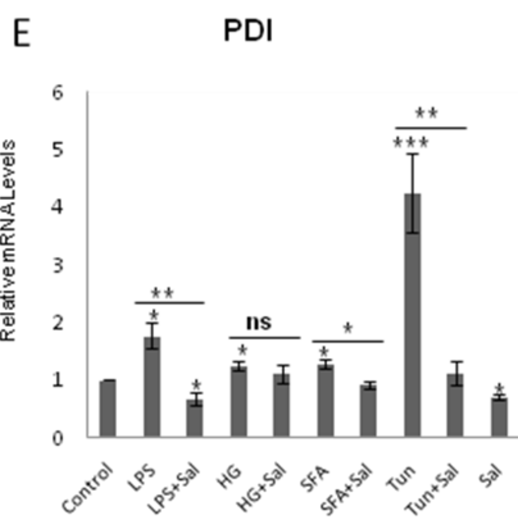


Fig. 3

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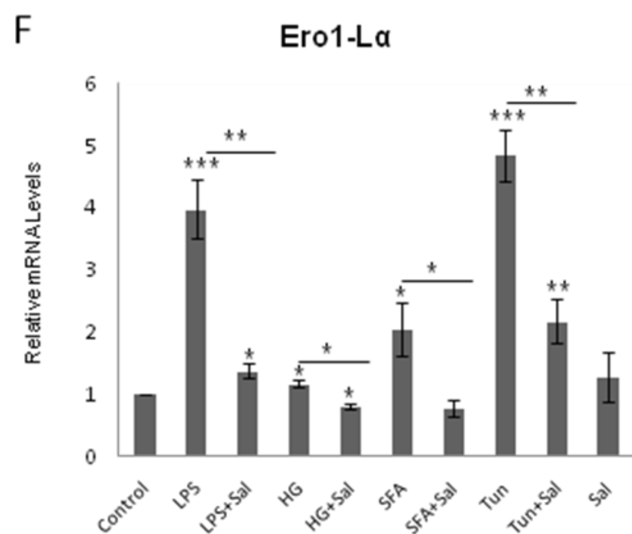
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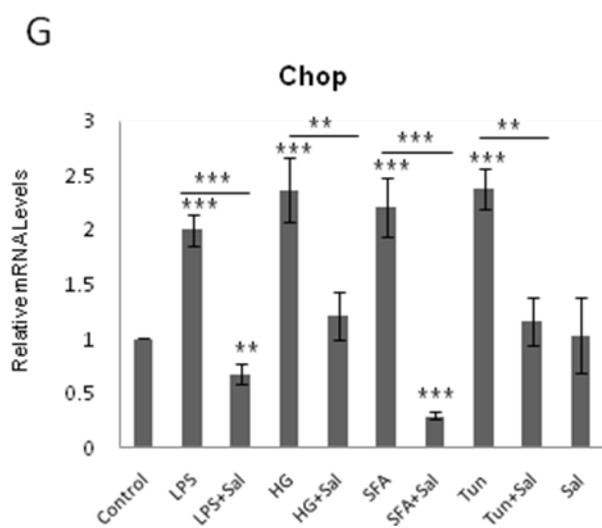
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HG+Sal
SFA
SFA+Sal
Tun
Tun+Sal
Sal

Fig. 4