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Early growth response protein-1 mediates lipotoxicity-associated placental inflammation: role in maternal obesity

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Saben J, Zhong Y, Gomez-Acevedo H, Thakali KM, Borengasser SJ, Andres A, Shankar K. Early growth response protein-1 mediates lipotoxicity-associated placental inflammation: role in maternal obesity. Am J Physiol Endocrinol Metab 305:E1–E14, 2013. First published April 30, 2013; doi:10.1152/ajpendo.00076.2013.—Obesity is associated with low-grade chronic inflammation, which contributes to cellular dysfunction promoting metabolic disease. Obesity during pregnancy leads to a proinflammatory milieu in the placenta; however, the underlying causes for obesity-induced placental inflammation remain unclear. Here, we examine the mechanisms by which saturated fatty acids and inflammatory cytokines induce inflammation in placental trophoblasts. We conducted global transcriptomic profiling in BeWo cells following palmitate and/or TNFα treatment and gene/protein expression analyses of MAPK pathways and characterized downstream transcription factors directly regulating inflammatory cytokines. Microarray analysis revealed increased expression of genes regulating inflammation, stress response, and immediate early response in cytotrophoblasts in response to palmitic acid (PA), TNFα, or a combination of both (PA + TNFα). Both gene ontology and gene set enrichment analysis revealed MAPK and EGR-1 signaling to be upregulated in BeWo cells, which was confirmed via immunoblotting. Importantly, activation of JNK signaling was necessary for increased proinflammatory cytokine (IL-6, TNFα, and IL-8) and EGR1 mRNA. Consistent with the requirement of JNK signaling, ChIP analysis confirmed the recruitment of c-Jun and other MAPK-responsive immediate early factors on the EGR1 promoter. Moreover, recruitment of EGR-1 on cytokine promoters (IL-6, TNFα, and IL-8) and an impaired proinflammatory response following knockdown of EGR-1 suggested it as a central component of the mechanism facilitating inflammatory gene expression. Finally, akin to in vitro findings, term placenta from obese women also had both increased JNK and p38 signaling and greater EGR1 protein relative to lean women. Our results demonstrate that lipotoxic insults induce inflammation in placental cells via activation of JNK/EGR1 signaling.

developmental programming; early growth response protein-1; fatty acids; gestational obesity; mitogen-activated protein kinase

AT PRESENT, MORE THAN 60% OF ALL PREGNANCIES in the US are in women who are either overweight or obese at conception (45, 52). Pregravid obesity significantly increases the risk of preclampsia, gestational diabetes, and other labor-related complications in the mother. As to the offspring, maternal obesity elevates the risk of being born large for gestational age and with birth defects (34). Moreover, early exposure to an obese intrauterine environment increases the offspring’s risk for developing obesity, cardiovascular disease, and diabetes in adulthood (5, 6, 45, 71, 75). As the only fetal tissue in direct contact with the maternal environment, the placenta serves as the singular interface between mother and the developing fetus. The placenta also mediates changes in the in utero environment by altering maternal metabolism and by directly regulating the transport of nutrients to the fetus (8). Therefore, alterations in maternal diet, nutritional status, and disease are communicated to the fetus through the placenta. Hence, the placenta plays a central role in how maternal obesity influences programming of health outcomes in the offspring (72).

Maternal obesity during pregnancy has been associated with a proinflammatory milieu during embryogenesis and in the placenta during mid- to late gestation (14, 54, 80). To date, placental inflammation has been characterized by increased macrophage infiltration (7, 14, 80) and increased cytokine production within the placenta (7, 54, 80). Although inflammation of the placenta and pregravid obesity appear to be unambiguously linked, two important issues remain unresolved: 1) the nature of maternal signals associated with obesity that initiate inflammation and 2) the precise molecular mechanisms orchestrating proinflammatory responses in placenta. Increased inflammation may alter placental development and/or function (54) and in turn promote long-term changes in fetal and offspring metabolism. Furthermore, obesity is associated with numerous physiological changes (hyperinsulinemia, elevated acute-phase proteins, subclinical endotoxemia, etc.) that could potentially contribute to placental inflammation. Most notably, fatty acids and inflammatory cytokines have been shown to induce proinflammatory responses in variety of cellular contexts (11, 47, 66, 69) and are elevated in obese pregnancies (50–52, 54), suggesting lipotoxicity as a causal mechanism of placental inflammation.

Mitogen-activated kinases (MAPK) are central mediators of proinflammatory signaling. The three major MAPK cascades, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinases (JNK), and p38 MAPK (p38), signal independently or interdependently with overlapping substrates to regulate gene expression (30, 37). Inflammatory cytokines, saturated fatty acids, and bacterial products (such as LPS) elicit proinflammatory responses by binding to cognate cytokine or Toll-like receptors (TLR), activating JNK and p38 MAPK signaling and downstream transcription factors (30, 37, 38). Likewise in hepatocytes, adipocytes, muscle, and immune cells, saturated fatty acids signal via TLR4 to activate MAPKs (JNK and NF-κB) to regulate the transcription of inflammatory cytokines (IL-1, IL-6, IL-8, and TNFα) (3, 23, 24, 27). However, very little is known regarding the mechanisms underlying lipotoxicity-induced inflammation in placenta cells. Specifically, the role of immediate early response transcription factors
associated with regulation of inflammatory cytokines remains unknown in the placenta.

In the present report, we tested the hypothesis that obesity-related factors (saturated fatty acids and/or inflammatory cytokines) would lead directly to inflammation in placental cytotrophoblasts (BeWo cells). Specifically, using microarrays, we analyzed gene expression profiles in the trophoblasts following lipotoxic challenge with palmitate and TNFα. Second, we examined the roles of MAPKs (ERK1/2, JNK, and p38) and immediate early response transcription factors in regulating cytokine gene expression. Utilizing loss-of-function approaches, we determined a role for MAPK/early growth response protein-1 (EGR-1) signaling in mediating the proinflammatory response to fatty acids. Finally, we examined the status of MAPK and EGR-1 pathways in term placenta from obese women with uncomplicated pregnancies. Our data indicate that placental trophoblasts are responsive to lipotoxicity and demonstrate a role for EGR-1 in regulation of obesity-induced placenta inflammation.

METHODS

Cell culture and experimental conditions. BeWo cells (American Type Culture Collection, Manassas, VA) were maintained in Ham’s F-12K medium (Kaighn’s modification) supplemented with 15% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Carlsbad, CA) under standard cell culture conditions. Following overnight serum deprivation, cells were treated with either palmitic acid (PA; 500 μM), TNFα (10 ng/ml), a combination of PA and TNFα (500 μM and 10 ng/ml, respectively), or fatty acid-free BSA alone (CON) for the amount of time specified in the individual figures. A molar ratio of 3:1 (PA/BSA) was used at a pH of 7.3. In some experiments, cells were treated with one of the following specific MAPK inhibitors: 10 μM SP-600125 (JNK/SAPK1/2 inhibitor; AG Scientific, San Diego, CA), 50 μM PD-98059 (ERK1/2 inhibitor, Millipore, Billerica, MA), or 10 μM SB203580 (p38 inhibitor; Sigma-Aldrich) for 2 h prior to the treatments with PA, TNFα, or the combination.

Collection of term placental samples. Placenta were collected at the University of Arkansas for Medical Sciences (UAMS) after informed consent was obtained from mothers at term. The protocol was approved by the Institutional Review Board at UAMS (NCT01104454). Only nonsmoking mothers without gestational diabetes, preeclampsia, or other complications were included. Both vaginal and cesarean deliveries were included. No information was collected on maternal race or ethnicity. Maternal prepregnancy weight and height were self-reported, whereas birth weight, sex, fetal heart rate, and Apgar score (1 and 5 min) of the baby were obtained from medical history. Subjects were dichotomized on the basis of BMI into lean (BMI 18–24.9 kg/m²; n = 11) and obese groups (BMI 30–45 kg/m²; n = 11). Placenta were processed within 20 min after collection. Placental weight was obtained after the umbilical cord and fetal membranes were trimmed. Samples were collected for protein analysis from six separate locations covering the entire placenta. Tissue lysates from nine samples were used for protein analysis from six samples/group were represented over two microarrays. Briefly, 0.5 μg of purified RNA was used to synthesize cDNA. Biotin-labeled aRNA was synthesized from double-stranded cDNA using the GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA). The probe array was scanned after hybridization, wash, and staining protocols using a GeneChip Scanner 3000 according to the manufacturer’s instructions (58, 60, 67).

Microarray data normalization and analysis. Microarray data analysis was carried out using GeneSpring version 11 software (Agilent Technologies, Santa Clara, CA) (58, 61, 62). CEL files containing the probe level intensities were processed using the robust multiarray analysis algorithm for background adjustment, normalization, and log2 transformation of perfect match values (28). Subsequently, the data were subjected to normalization by setting measurements ≤0.01 to 0.01 and by per-chip and per-gene normalization using GeneSpring normalization algorithms. Raw data are accessible in the National Center for Biotechnology Information GEO database (GSE43685). The normalized data were used to generate lists of differentially expressed genes between PA, TNFα, and combination treatment groups relative to CON. Genes were filtered based on minimum ±1.5-fold change (treatment vs. CON) and a P value ≤0.05 using Student’s t-test. A list of transcripts that were differentially expressed as a function of treatment (PA, TNFα, and PA + TNFα) was generated, and correlation-based hierarchical clustering between treatment groups was performed. Known biological functions of genes were queried using Affymetrix NetAffx and gene ontology (GO) analyses for biological processes performed using GeneSpring (58, 67). Finally, gene set enrichment analysis (GSEA) was utilized to identify molecular functions and transcription factors enriched by PA and combination treatments. GSEA does not rely on an arbitrary cutoff (such as fold change between groups) and is a computational method that determines whether an a priori-defined set of genes shows statistically significant and concordant differences between two biological states (62, 68).

Real-time RT-PCR. Total RNA (1 μg) was reverse transcribed using the iScript cDNA synthesis kit, and subsequent real-time PCR analysis was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Gene-specific primers were designed using Primer Express Software (Applied Biosystems). The relative amounts of mRNA were quantified using a standard curve and normalized to the expression of cyclophilin A mRNA (60).

Immunoblotting. Total cell and tissue lysates were prepared in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1.0% NP-40, 1.0% deoxycholic acid, 0.1% SDS, and 2 mM EDTA) containing 1 mM PMSF and protease inhibitor cocktail. Nuclear proteins were isolated using NE-PER reagents according to manufacturer’s instructions (Thermo Fisher Scientific, Rockford, IL). Proteins were resolved by SDS-PAGE, and immunoblotting was carried out using standard procedures (59, 62). Membranes were incubated with primary antibodies against EGR-1, p-ERK1/2, ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA), serum response factor (SRF), ERK1/2, p38, JNK/SAPK, p-JNK/SAPK, c-Jun (Cell Signaling Technology, Danvers, MA), p-p38 (New England BioLabs, Ipswich, MA), TATA-binding protein (Abcam, Cambridge, MA), GAPDH (Sigma-Aldrich), and α-tubulin (Active Motif, Carlsbad, CA) for 16 h at 4°C. HRP-conjugated secondary antibodies against rabbit and mouse IgG (Santa
Cruz Biotechnology) were used for protein detection. Quantitation of immunoblots was performed using Quantity One software (Bio-Rad).

**ChIP.** Chromatin immunoprecipitation. Recruitment of transcription factors on promoters of target genes was monitored using chromatin immunoprecipitation (ChIP) assay, using previously described procedures (57). Following respective treatments, cells were cross-linked with 1% formaldehyde for 10 min, and nuclei were isolated in ChIP cell lysis buffer (5 mM PIPES, 85 mM KCl, and 0.5% NP-40). Cells from three to four separate dishes per group were pooled to create three biological replicates. Nuclei were resuspended in ChIP sonication buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), and chromatin was sheared using a Covaris S220 ultrasonicator (Covaris, Woburn, MA). Prior to immunoprecipitation (IP), a 50-μl aliquot of sheared chromatin was removed from each sample and stored at −70°C to be utilized as DNA input. Aliquots of sheared chromatin were incubated with antibody-bound magnetic protein A/G beads (Millipore). All antibodies were ChIP grade. Antibody-chromatin complexes were washed serially in low-salt, high-salt, and LiCl-containing wash buffer and eluted in elution buffer (1% SDS, 0.1 M NaHCO₃). Immunoprecipitated DNA was purified using standard phenol-chloroform-IAA following overnight reverse cross-linking and treatment with RNaseA and proteinase K (Life Technologies). The yield of specific promoter target sequences was analyzed by PCR with input or IP DNA using GoTaq Green master mix (Promega, Madison, WI). Primer sequences are provided in Table 1. PCR amplicons were separated via agarose gel electrophoresis and visualized by ethidium bromide staining.

**H3K4me3 ChIP-seq and data analysis.** The genome-wide localization of the activating histone mark H3K4me3 was analyzed by ChIP-seq in BeWo cells after treatment with or without PA for 24 h. ChIP was performed as described above using ChIP-validated H3K4me3 antibody (Abcam). Purified immunoprecipitated and input DNA were utilized to prepare libraries for sequencing using NEBNext reagents. Briefly, 150 ng of DNA was used for end filling, dA tailing, and ligation with Illumina’s paired-end adapters according to the manufacturer’s recommendations. Adapter-ligated DNA was amplified using PCR, and products ~200–350 bp were used for cluster generation. Single-read 36-bp sequencing of libraries was performed with a Genome Analyzer IIx (Illumina, San Diego, CA). Image analysis, including base calls, was performed by Real-Time Analysis software (RTA version 2.6; Illumina), and preprocessing, including demultiplexing, was performed using scripts in the CASAVA version 1.7 pipeline (Illumina). Alignment to the human hg19 genome was carried out using Bowtie and exported into SAM format. Identification of H3K4me3 peaks was performed using MACS (79), and data were exported in .BED format. Two comparisons were made: control vs. input to localize regions of active transcription in BeWo cells and PA vs. CON to identify regions that undergo epigenomic modification following PA exposure. Subsequent data analysis, including annotation of peaks with the closest transcription start site (TSS), genomic localization, and visualization of coverage maps, was carried out using SeqMonk and PeakAnalyzer software packages (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/; Babraham Bioinformatics, Cambridge, UK). Enrichment of GO terms for biological processes was carried out using GoRilla, and P values were corrected using the FDR method.

**Statistical analysis.** Data are expressed as means ± SE. Real-time RT-PCR data are expressed as mean fold change from control ± SE, and Western blot data are expressed as means ± SE. Statistical differences between CON and PA or CON and PA + TNFα were determined using a two-tailed Student t-test. In cases where four groups (CON, PA, TNFα, and PA + TNFα) were compared, a two-way ANOVA followed by all pairwise comparison by the Student-Neuman-Keuls method was performed. P ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SigmaStat 3.3 software (Systat Software, San Jose, CA).

**RESULTS**

Palmitate and TNFα have distinct effects on the BeWo cell transcriptome. Hierarchical clustering of microarray data revealed significant treatment effects on global gene expression. Compared with the control group, PA, TNFα, and the combination altered the expression of 724, 167, and 952 transcripts, respectively (±1.5 fold, P ≤ 0.05; Fig. 1A). Table 2 depicts the top 10 genes that were either up- or downregulated by each treatment. EGR1 had the greatest fold increase following both PA and combination treatments and the greatest fold decrease following TNFα treatment (Table 2). Hierarchical clustering of the union of differentially expressed genes revealed that PA and PA + TNFα clustered together, suggesting a stronger influence of PA on gene expression changes compared with TNFα (Fig. 1B). Approximately one-half of the genes altered by the combination were unique (i.e., not altered with either PA or TNFα alone; Fig. 1A), indicating an interaction between the two treatments. Analysis of biological processes revealed that genes involved in response to extracellular stimuli (organic substance, chemical stimulus, and cytokine stimulus), lipotoxicity (lipid localization and storage, apoptosis, cellular stress, and unfolded protein response), and inflammation (cytokine signaling, MAPK signaling, and immune response) were significantly altered following the combination treatment (Fig. 1C). Consistent with GO term enrichment, genes involved in the response to chemical stimulus (Fig. 1D) regulating immediate early response (FOS, JUN, and EGR1) and lipid storage (PLIN2, ANGPTL4, APOA2) were similarly increased in the PA and the PA + TNFα treatments relative to CON. Moreover, genes regulating inflammation (SAA1, TNFα, IL6, and CXCL16) were induced mainly with the combination. Similarly, GSEA of molecular functions altered by PA or the combination identified MAPK signaling (Fig. 1E), cytokine binding, and nuclear cytoplasmic transport (data not shown). Enrichment

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**Table 1. DNA oligonucleotides for ChIP**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>Human EGR-1 promoter</td>
<td>5′-GGGCGCTTGTAGTACAGACAGGATAGAAGG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
</tr>
<tr>
<td></td>
<td>5′-TCAGGGCGCTTCTCTAGCTCCGTAGAAGA-3′</td>
</tr>
<tr>
<td>2 × SRE and CRE-1 sites</td>
<td>5′-TACGTGACAGATTCAGGTGGG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
</tr>
<tr>
<td></td>
<td>5′-ATTGTGAAAGTGTCAGAAGGCGG-3′</td>
</tr>
<tr>
<td>3 × SRE site</td>
<td>5′-AGGGAGAACCCAGCAGCTCCAAGG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
</tr>
<tr>
<td></td>
<td>5′-TTCGGCCTCCGCTCCCTTCTAA-3′</td>
</tr>
<tr>
<td>Human TNFα promoter</td>
<td>5′-CAGAATCGCTATGGAAGAGGCAGAAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
</tr>
<tr>
<td></td>
<td>5′-CTCGTCTGGGAGAAAGACACAG-3′</td>
</tr>
<tr>
<td>Human IL-6 promoter</td>
<td>5′-TCGGTCTCATCAGCTGTCGTCAG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
</tr>
<tr>
<td></td>
<td>5′-AGTCATTGCTGATGCTGTCGTCAG-3′</td>
</tr>
<tr>
<td>Human IL-8 promoter</td>
<td>5′-AGTCCCTGTGAGCTGTCGTCGTCAG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
</tr>
<tr>
<td></td>
<td>5′-AGGGTGGCTGACAGGCTGTCGTCGTCAG-3′</td>
</tr>
</tbody>
</table>

ChIP, chromatin immunoprecipitation; EGR-1, early growth response protein-1; AP-1, activating protein-1; CRE-1 and -2, cAMP response element-1 and -2, respectively; SRE, response elements from serum response factor.
of transcription factor motifs revealed genes regulated by immediate early response transcription factors downstream of MAPKs (SRF, ATF3, and CREB/c-Jun) to be increased significantly with both PA and PA + TNFα treatment (Fig. 1F). Real-time PCR confirmed that genes involved in proinflammatory processes were increased primarily in the TNFα and the combination, whereas immediate early transcription factors and genes involved in lipid metabolism...
were increased following treatment with PA or PA + TNFα (Fig. 1G).

PA and PA + TNFα treatment is associated with MAPK activation. To determine the temporal changes in MAPK with either PA or PA + TNFα, we assessed total and phosphorylated protein levels of p38, JNK, and ERK1/2 MAPKs over 24 h (Fig. 2). Levels of phosphorylated JNK increased ~1.5-fold (P < 0.02) at 6 h following PA treatment, whereas p38 or ERK1/2 phosphorylation (relative to total protein) was not changed (Fig. 2, A and C). Similarly, JNK activation was evident at 3 h following PA + TNFα treatment (P < 0.01) and remained higher through 24 h of treatment (P < 0.01; Fig. 2, B and D). In contrast to PA alone, the combination treatment induced phosphorylation of ERK1/2 by two- (P < 0.003) and 3.5-fold (P < 0.001) at 60 min and 6 h, respectively. There was no change in p38 activation following treatment with PA + TNFα. Together, these data indicate that lipotoxic stimulus activates JNK signaling in trophoblast cells, which may promote downstream regulation of inflammatory cytokine expression.

Specific MAPK inhibitors were used to further examine the importance of JNK signaling in the regulation of cytokine gene expression (Fig. 2E). Pretreatment with both JNK and ERK1/2 inhibitor was sufficient to prevent the induction of TNFα, IL-6, and IL-8 by PA (Fig. 2E). p38 MAPK inhibition also blocked increases in TNFα and IL-8 mRNA following exposure to PA; however, it did not impair the induction of IL-6. All three inhibitors were unable to completely abolish the induction of inflammatory cytokines TNFα or IL-6 mRNA (Fig. 2E) by the combination treatment, suggesting that mechanisms outside of MAPK signaling, such as MAPK-independent activation of NF-kB, are likely involved in regulating cytokine production in response to PA + TNFα. However, JNK inhibition decreased the induction of TNFα and IL-6 mRNA by 75 and 25%, respectively, suggesting that JNK activation contributes significantly to the proinflammatory response. Collectively, these data support the conclusion that JNK signaling is necessary for a proinflammatory response to lipotoxicity; however, it appears that other MAPK signaling may also be important for this response.

MAPKs regulate ATF3, c-Jun, and SRF in response to PA and PA + TNFα. BeWo cells exposed to either PA or the combination showed a twofold increase in the mRNA expression of immediate early transcription factors ATF3 and c-Jun and a 1.2-fold increase of SRF by 6 h (Fig. 3A). Moreover, ATF3, c-Jun, and SRF nuclear proteins were significantly greater by 8 h of PA (P < 0.02) and PA + TNFα (P < 0.002) treatments (Fig. 3B). These temporal changes are consistent with the activity downstream of JNK activation observed at 6 h (Fig. 2). Accordingly, inhibition of JNK signaling was sufficient to not only inhibit the induction of ATF3, c-Jun, and SRF expression in response to PA and PA + TNFα but also significantly reduce the expression of these genes below levels in CON cells (P < 0.05; Fig. 3C).

Immediate early response transcription factors are recruited to EGR1 promoter, in turn regulating cytokine expression. EGR1 mRNA was maximally induced by 12 h following treatments with PA or the combination (P < 0.05; Fig. 4A). Active gene transcription is associated with the H3K4me3 (trimethyl) chromatin modification (2, 35). Using ChIP-seq, we examined genome-wide localization of H3K4me3 in control and PA-treated cells. Untreated cells showed ~3,000 peaks (5% FDR, P < 0.00001, M-fold = 10–30), ~90% of which were ≥500 bp from TSS. PA treatment resulted in ~800 differential H3K4me3 marks that were annotated to the closest TSS using SeqMonk. GO biological process analysis of genes that gained K4 trimethylation after PA treatment clearly showed enrichment of pathways regulating apoptosis, response to stimulus, and proliferation (Fig. 4B). Of these genes, H3K4me3 of EGR1 promoter was also increased (Fig. 4C), indicating acquisition of a permissive chromatin profile following PA treatment. The EGR1 promoter contains two CRE sites (ATF3/CREB), one activating protein-1 (AP-1) (c-Jun) site, and five CRE sites (SRF) that are central in gene regulation (Fig. 4D). ChIP analysis for ATF3, c-Jun, and SRF showed 1.7-fold greater recruitment of ATF3 to the EGR1 promoter in PA-treated cells (Fig. 4E), whereas the recruitment of c-Jun and SRF were increased 1.8- and 1.4-fold, respectively, following the combination treatment (Fig. 4E). Together, these data indicate that MAPK-responsive immediate early transcription factors regulate EGR1 mRNA expression. Consistent with the increase in EGR1 mRNA, PA and PA + TNFα treatments increased EGR-1 protein in the nucleus by 20 and 30% (P < 0.05), respectively (Fig. 5A). Furthermore, ChIP for EGR-1 showed increased recruitment of EGR-1 to the promoter regions of IL-6 (6.4- and 8.5-fold), TNFα (3.0- and 3.4-fold), and IL-8 (2.1- and 3.0-fold) after PA and combination treatment (Fig. 5B). Additionally, PA + TNFα treatment led to a 30 and 50% increase in recruitment of EGR-1 to the IL-6 and IL-8 promoters, respectively, over that of PA alone (Fig. 5B), suggesting that EGR-1 may also contribute to the additional inflammatory response observed in combination-treated cells.

To assess the role of EGR-1 in regulating cytokine expression, we generated BeWo cells transduced with shRNA targeting EGR1 (shEGR1). Quantitative RT-PCR confirmed a 62% decrease in EGR1 mRNA relative to cells expressing scram-
bled shRNA control (Fig. 5C). Furthermore, knockdown of EGR1 prevented the normal induction of EGR1 following treatments with PA or PA + TNFα (Fig. 5C). EGR1 knockdown also abrogated the increase in TNFα, IL-6, and IL-8 mRNA expression in response to PA treatment. Following treatment with PA + TNFα, knockdown of EGR1 completely inhibited the induction of IL-6 and IL-8 and reduced the induction of TNFα by 42% (P < 0.05; Fig. 5D). Together, these data indicate that the induction of EGR-1 is necessary for increased cytokine expression in response to PA and the combination in placental cells.

EGR-1 protein and JNK signaling is increased with pregravid obesity. To examine the translational significance of MAPK/EGR-1 signaling in human obesity, we utilized term

Fig. 2. Temporal analysis of MAPK activation and cytokine gene expression in response to PA or PA + TNFα. A and B: immunoblot analysis for total and phosphorylated p38, JNK1/2, and ERK1/2 over a 24-h time course following treatment with PA (A) or PA + TNF-α (B). C and D: densitometric quantitation of immunoblots (normalized to α-tubulin) for phosphorylated protein (∗), total protein (gray circle), or the ratio of phosphorylated to total protein (■) for PA (C) or PA + TNF-α treated cells (D). Values are expressed as mean fold change over 0 h. *Significant difference for the ratio of phosphorylated to total protein (P ≤ 0.05) compared with 0-h time point. E: mRNA expression of cytokine genes at 24 h following exposure to PA or PA + TNF-α with and without preexposure to MAPK inhibitors SP-600125 (JNK inhibitor), PD-98059 (ERK1/2 inhibitor), and SB-203580 (p38 inhibitor). Gene expression was normalized to cyclophillin mRNA (n = 6/group). Statistical differences were determined using Student’s t-test. *Significance, P ≤ 0.05.
placental samples from lean and obese women (n = 11/group). Maternal and neonatal characteristics of the subjects are presented in Table 3. Maternal age, height, and parity were not significantly different between lean and obese groups, whereas maternal prepregnancy weight and BMI were significantly greater among the obese women (P < 0.05). In addition, birth weight and infant sex were not different between groups. Consistent with our in vitro data, obese placenta had significantly more p-JNK and p-P38 protein compared with lean controls (Fig. 6, A and B). Downstream of MAPKs, EGR-1 protein was also significantly increased by 33% (P < 0.05) in obese term placenta compared with lean controls (Fig. 6C).

Although too few subjects were studied to statistically address sex differences, placenta from both male and female sexes showed comparable increases in Egr-1 and pJNK (data not presented).

DISCUSSION

An increasing body of both experimental and clinical evidence suggests that maternal obesity prior to and during gestation promotes a proinflammatory intrauterine environment (7, 54, 56, 62, 80). This is significant because placental and embryonic inflammation has been linked to adverse outcomes
in fetal development (22, 25, 53, 78) and has been hypothesized to be a causal pathway for long-term metabolic programming in the offspring. In the present work, we utilized a widely studied placental trophoblast model to evaluate genome-wide responses to lipotoxic insults and to mechanistically identify pathways leading to inflammation. Several novel observations are evident from the present work. Exposure to lipotoxic stimuli led to 1) a distinct upregulation of proinflammatory genes, including activation of MAPKs, which are necessary mediators of placental inflammation; 2) recruitment of immediate early-response transcription factors ATF3 c-Jun and SRF downstream of JNK activation, leading to a transcriptionally permissive chromatin profile of the EGR-1 promoter; and 3) an obligatory requirement of JNK and EGR-1 for induction of inflammatory cytokine expression. Together, these results define a mechanistic pathway through which lipotoxicity induces inflammation in placenta cells. Most importantly, our findings show that JNK/EGR-1 signaling is activated in term placenta from obese women, highlighting the translational relevance of these findings.

Obesity in general, is characterized by a state of subclinical chronic inflammation (1, 10, 41), as is the maternal-fetal
Table 3. Maternal and neonatal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Lean (n = 11)</th>
<th>Obese (n = 11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>28.1 ± 2.1</td>
<td>31.1 ± 1.8</td>
<td>0.32</td>
</tr>
<tr>
<td>Prepregnancy weight, lbs</td>
<td>129.8 ± 3.7</td>
<td>213.7 ± 6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height, in.</td>
<td>64.4 ± 0.7</td>
<td>65.1 ± 1.1</td>
<td>0.55</td>
</tr>
<tr>
<td>BMI</td>
<td>22.1 ± 0.4</td>
<td>35.9 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parity</td>
<td>1.8 ± 0.4</td>
<td>2.5 ± 0.5</td>
<td>0.28</td>
</tr>
<tr>
<td>Birth weight</td>
<td>3,256.8 ± 105</td>
<td>3,312.7 ± 135</td>
<td>0.77</td>
</tr>
<tr>
<td>Apgar</td>
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<td></td>
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</tr>
<tr>
<td>1 min</td>
<td>8.8 ± 0.1</td>
<td>8.6 ± 0.3</td>
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</tr>
<tr>
<td>5 min</td>
<td>8.9 ± 0.1</td>
<td>9.3 ± 0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Fetal HR, beats/min</td>
<td>138.9 ± 2.5</td>
<td>138.4 ± 4.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Sex of baby</td>
<td>4 females, 7</td>
<td>4 females, 7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. HR, heart rate.

interface in obese pregnant women (7, 54, 56, 80). During pregnancy, maternal obesity has been associated with higher circulating inflammatory cytokines (IL-6 and CRP) (1, 7, 54), elevated IL-6 and TNFα mRNA expression in peripheral blood mononuclear cells (7, 14), and an exaggerated proinflammatory milieu of the placenta (7, 14, 54, 80). More specifically, inflammation in the obese placenta has been characterized by elevated numbers of proinflammatory immune cells and TNFα mRNA expression, which suggest that TNFα can exquisitely alter the expression of proinflammatory cytokines or LPS (7, 80). Elevated numbers of proinflammatory immune cells are also associated with increased cytokine production (IL-1, IL-6, IL-8, and TNFα) in both midgestation and term placenta (7, 54, 80). Although these cytokines primarily promote the recruitment of additional immune cells, they may also stimulate signaling pathways within other placental cells (trophoblasts, endothelial cells, and stromal cells), leading to cellular stress and dysfunction. Our data clearly show that trophoblasts are responsive to extracellular TNFα and can exquisitely alter the expression of proinflammatory cytokines in response to a lipotoxic environment. Therefore, trophoblast cells are likely to contribute to excess inflammation associated with maternal obesity.

The placenta serves as the only portal for nutrients to the developing fetus and is key in regulating the utero environment (4). Lipotoxicity and inflammation have been shown to induce cellular stress, resulting in altered tissue function (9, 16, 48, 76). Such changes in placental development and/or function may have detrimental consequences on fetal metabolism. Accordingly, alterations in both amino acid transport (14, 15) and lipid transport (40, 74, 81) have been associated with maternal obesity and increased placental inflammation. Likewise, in a nonhuman primate model of maternal obesity, increased fetal BMI and ponderal index were observed along with placental changes such as elevated proinflammatory signature, decreased system A amino acid transport, and increased placental thickness (14). Additionally, impaired system A amino acid transport has been observed in placenta from human subjects (15). Previous studies have also shown a strong association between maternal obesity-associated inflammation with augmented placental lipid (40), elevated fetal circulating lipids (81), and increased fetal adiposity (49). Specifically, in a comprehensive study of lean and obese pregnant women, Radaelli et al. (49) showed a strong positive relationship between increased maternal IL-6 and fetal adiposity. Similarly, increased neonatal adiposity was associated with elevated placental TNFα, leptin, phospholipase expression, and ω-3 polyunsaturated fatty acids (74). Activation of phospholipases PLAG2 and PLAG5 in placenta cells occurred following exposure to TNFα and leptin (74), demonstrating a potential link between maternal inflammation and altered placental lipid transport. Although the current study did not focus on the effects of lipotoxicity and trophoblast nutrient transport, exposure to palmitate and TNFα increased expression of APOA2 and PLIN2, an observation that is consistent with previous reports.

Although the precise mechanisms regulating increased cytokine production in placenta from obese women remain yet to be described, the present work suggests that JNK signaling is a central factor. These findings are consistent with a previous report (80) showing increased TLR2 and -4 mRNA and JNK and NF-κB signaling in placenta of overnourished sheep. Downstream of TLR4 activation, two main pathways exist to regulate proinflammatory cytokine gene expression. First is the NF-κB pathway, which includes activation of IKK, leading to degradation of IκB, freeing NF-κB from the cytoplasm and allowing translocation to the nucleus to regulate gene expression (19, 31, 44). Second, TLRs can activate MAPKs, p38, and JNK, which in turn activate immediate early-response transcription factors that regulate proinflammatory cytokine expression (23, 37). Specifically, heterodimetric transcription factor I (AP-1), consisting of transcription factor dimers from the Jun (c-Jun or JunD) transcription factor group, fos group (c-Fos), or activating transcription factors (ATFs), is fundamental to initiating a proinflammatory response (37). However, the immediate early transcription factor EGR-1 is also important for regulating the expression of inflammatory cytokines IL-6, IL-8, TNFα, and CCL2 (11, 26, 64). EGR-1, a Cys2/His2-type zinc finger transcription factor, regulates a number of cellular processes, including cellular growth, proliferation, apoptosis, and differentiation in response to an expansive range of extracellular stimuli (12, 46). Importantly, EGR-1 has also been shown to regulate both inflammation (11, 26, 64) and insulin signaling (32, 63) and provide a central link between metabolic disease and inflammation. EGR1 expression is regulated downstream of MAPKs through transactivation by other immediate early transcription factors (26). The specific upstream factors regulating EGR1 vary depending on the extracellular stimuli and the cellular context. Our data indicate that JNK-dependent activation of c-Jun and ATF3 is critical for the transactivation of EGR1 in response to a lipotoxic environment, a mechanism that is consistent with a previous report in immune cells (26). Moreover, the observed association between increased JNK signaling and EGR-1 protein in obese placenta may suggest that EGR-1 can mediate obesity-induced placental inflammation and warrants further investigation.

DNA binding sites for NF-κB, AP-1, and EGR-1 have been described in the promoters of a number of inflammatory cytokine genes including TNFα (73), IL-6 (13, 26, 35) and IL-8 (26, 33), indicating direct regulation of cytokine expression by these transcription factors. Importantly, studies have shown that combinations of transcription factor binding are required for transactivation of proinflammatory cytokine expression in an inducer-specific manner (11, 13, 21, 29, 73, 77). For example, EGR-1 is essential for IL-8 production in prostate cancer cells but works synergistically with NF-κB (42). In macrophages and monocytes, lipopolysaccharide-induced TNFα
expression requires transactivation by EGR-1, NF-κB, and AP-1 (73, 77), suggesting that cooperation between transcription factors is necessary for complete induction of inflammatory cytokine expression. Our results suggest that in trophoblasts EGR-1 is important for the induction of TNFα, IL-6, and IL-8 expression in response to a lipotoxic environment. However, it is likely that other transcription factors are also necessary to form a complete activating complex. This is consistent with evidence in macrophages, where saturated fatty acid-mediated TNFα expression was associated with increased EGR1 and CREB but decreased NF-κB activation (11).

Upstream of TLRs, increased circulating fatty acids commonly observed in obesity are the probable link between obesity and placental inflammation since they induce TLR4 activation of JNK signaling (18, 39, 80). Although our clinical study was not designed to measure serum lipids, activated JNK and increased EGR-1 observed in obese placentas suggests a potential role for fatty acid signaling. Importantly, our data in BeWo cells show a direct link between elevated saturated fatty acids and increased inflammatory cytokine production, consistent with a previous study in human primary trophoblast cells (47). Interestingly, we observed a decrease in TLR2 and TLR4 mRNA expression following fatty acid and TNFα exposure, suggesting probable negative feedback regulation. ATF3 has been shown to induce a negative feedback mechanism that attenuates saturated fatty acid induction of TLR4 signaling (20,
70), consistent with the gene expression observed in our study. However, a parallel increase in cytokine expression and JNK activation suggests that TLR4-signaling cascade was activated earlier during the fatty acid exposure. These data suggest that negative feedback mechanisms are in place in trophoblast cells to help regulate fatty acid-induced inflammation and that chronic exposure to a lipotoxic environment may activate alternative pathways to inflammation.

Recent evidence has emerged from studies on the independent effects of maternal high-fat feeding that support the hypothesis that increased circulating lipids, as opposed to maternal obesity per se, are the mediator of fetal programming (36, 43, 65). Importantly, gestational exposure to high-fat diets has been associated with epigenetic changes (histone acetylation and methylation) in adipokine genes (43) and genes regulating glucose metabolism (65) in offspring tissues. Additionally, maternal high-fat diet has been associated with expression changes in genes encoding enzymes that regulate histone and DNA methylation in the placenta (17), suggesting a potential mechanism for fatty acid regulation of gene expression. Although placental inflammation has not been identified specifically as the link between high-fat feeding and fetal programming, it is likely to contribute. Accordingly, our data show a direct link between saturated fatty acid exposure and changes in chromatin structure within placenta cells that is permissive to increased gene expression of EGR1 and subsequent increases in inflammatory cytokine gene expression. Taken together, these findings suggest that a lipotoxic maternal environment is conducive to epigenetic adaptations, which may alter placental regulation of the fetal domain.

In conclusion, we present a genome-wide transcriptomic view of the response of trophoblast cells following lipotoxic challenge. Gene expression analysis revealed a distinctive role for MAPK activation, specifically JNK signaling, and subsequent immediate early transcription factors in regulation of EGR-1 levels. Furthermore, our studies identified EGR1 as the essential mediator for lipotoxicity-induced cytokine gene expression in placenta cells. Most importantly, our findings show that analogous changes in JNK/EGR-1 signaling occur in term placenta from obese women, highlighting the translational relevance of these findings. These findings provide novel insights into the mechanisms underlying obesity-induced placental inflammation and provide further clues into the nexus between maternal obesity, inflammation, and metabolic programming in the offspring.

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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

J.S., A.A., and K.S. contributed to the conception and design of the research; J.S., Y.Z., K.M.T., and S.J.B. performed the experiments; J.S., Y.Z., H.G.-A., K.M.T., S.I.B., and K.S. analyzed the data; J.S. interpreted the results of the experiments; J.S. prepared the figures; J.S. and K.S. drafted the manuscript; J.S., H.G.-A., K.M.T., A.A., and K.S. edited and revised the manuscript; A.A. and K.S. approved the final version of the manuscript.

REFERENCES


