Cellular inhibitors of apoptosis (cIAP) 1 and 2 are increased in placenta from obese pregnant women

Short Title: cIAP and placental inflammation

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ABSTRACT

Introduction: Independent of their role in apoptosis, cellular inhibitors of apoptosis (cIAP) 1 and 2, have emerged as regulators of inflammation. Obesity in pregnancy is characterised by maternal and placental inflammation. Thus, the aim of this study was to determine the effect of maternal obesity and pro-inflammatory mediators on cIAP expression in human placenta.

Methods: The expression of cIAP was assessed in human placenta from lean (n=15) and obese (n=14) patients by qRT-PCR and Western blotting. Primary trophoblast cells were used to determine the effect of pro-inflammatory cytokines on cIAP expression, and the effect of cIAP siRNA on pro-inflammatory cytokines.

Results: cIAP1 and cIAP2, gene and protein expression were significantly higher in placenta from women with pre-existing maternal obesity compared to placenta from lean women. Additionally, bacterial endotoxin LPS and the pro-inflammatory cytokines tumour necrosis factor (TNF)- α and interleukin (IL)-1 β significantly increased the expression of both cIAP1 and cIAP2 in primary trophoblast cells isolated from human term placenta. Knockdown of cIAP1 or cIAP2 in human primary trophoblast cells significantly decreased TNF- α induced expression and secretion of pro-inflammatory cytokines IL-6 and IL-8 and of matrix metalloproteinase (MMP)-9.

Discussion: cIAP1 and cIAP2 expression is increased in placenta from women with pre-existing maternal obesity and in response to treatment with pro-inflammatory cytokines. Functional studies in placental trophoblast cells revealed that cIAPs are involved in TNF- α induced-expression of pro-inflammatory cytokines. Given the central role of pro-inflammatory cytokines in placental nutrient transport, this data suggest that cIAP1 and cIAP2 may play a role in fetal growth and development.

INTRODUCTION

Obesity has been greatly increasing in various regions across the globe [1]. Similarly, the number of pregnancies complicated by obesity has also increased over the past 20 years [2]. Obesity in pregnancy is associated with both short and long term risks for the mother and her infant [3, 4].

Obese pregnant women are at a higher risk of developing GDM, preeclampsia, thrombosis, sleep apnoea, and pregnancy loss while neonatal outcomes include macrosomia, jaundice, birth defects, stillbirth and perinatal death. In the long-term, infants are at increased risk of developing obesity, diabetes and cardiovascular disease. The societal and financial costs of obesity in pregnancy are substantial [4, 5].

It is now well established that obesity [6] and pregnancy [7] are two conditions associated low-grade inflammation. However, inflammation is intensified in pregnancies complicated by obesity. Obese pregnancies are associated with increased levels of pro-inflammatory cytokines in the placenta [8-16]. Obese pregnancies are associated with increased levels of pro-inflammatory cytokines in both the maternal circulation and in placental tissues [8-12, 17, 18]. Recent studies have also shown that the inflammatory signalling proteins mitogen activated protein kinase (MAPK) p38 and signal transducer and activator of transcription 3 (STAT3) [8, 12] and nuclear factor- κ B (NF- κ B) [19] are activated in the obese placenta. These signalling pathways may activated be due to elevated maternal circulating cytokines; TNF- α has been shown to activate p38-MAPK and STAT3 phosphorylation in cultured trophoblast cells [8] and NF- κ B in trophoblast JEG-3 cells [20]. It is hypothesised that inflammatory processes associated with elevated maternal body mass index (BMI) may influence fetal growth by altering placental function [8]. Thus, understanding how the inflammatory milieu in which the fetus develops is regulated may aid in therapies to improve fetal growth and development.

Cellular inhibitor of apoptosis protein-1 (cIAP1/*birc2*) and cIAP1 (*birc3*) belong to the IAP family of anti-apoptotic proteins, which is characterised by the presence of baculoviral IAP repeat (BIR) motifs [21]. As their name suggests, IAPs, especially XIAP (*birc4*), have a well characterised role in regulating cell survival [22-24]. However, several studies now show that cIAPs have a much wider spectrum of action, including regulating inflammatory pathways that are independent of their role in apoptosis [25-31]. For example, knockdown experiments revealed that cIAP2 was required for optimal TNF- α -induced cytokine and chemokine production in HeLa cells [29] and cIAP antagonism modulates TNF- α induced inflammatory effects *in vivo* [29]. cIAPs are also transcriptionally inducible in response to pro-inflammatory cytokines including TNF- α [30, 32-36].

Both cIAP1 and cIAP2 are expressed in human term placenta where they have been immunolocalised to the cytotrophoblasts and syncytiotrophoblasts of term placentas [37]. There is, however, no data on the effect of pre-existing maternal obesity on the expression of cIAPs in human placenta, or on their role in modulating TNF- α induced inflammation. Thus, the aims of this study were to determine the effect of pre-existing maternal obesity and pro-inflammatory mediators on the expression of cIAP1 and cIAP2 in human placenta. In addition, the effect of cIAP1 and cIAP2 knockdown by siRNA in primary trophoblast cells on markers of inflammation was also determined.

MATERIALS AND METHODS

Tissue collection

Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and written informed consent was obtained from all participating subjects.

Women were invited to provide samples on the day of admission for surgery. All tissues were obtained at the time of term Caesarean section in the absence of labour to ensure there were no effects of labour on cIAP expression. Indications for Caesarean section included repeat Caesarean section or breech presentation. Women fulfilling any of following criteria were excluded; vascular/renal complication, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute fetal distress and women with any other adverse underlying medical conditions.

Placenta was collected for two studies; expression studies using placenta villous tissue and cell culture studies using freshly isolated trophoblast cells. Placenta was processed within 15 min of delivery. Placental lobules (cotyledons) were obtained from various locations of the placenta; the basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Tissues were washed extensively with PBS, and (i) immediately snap frozen in liquid nitrogen and immediately stored at -80°C for analysis of gene and protein expression by qRT-PCR and Western blotting as detailed below; or (ii) used for isolation of trophoblast cells as detailed below.

For the expression study, placenta was collected from normal glucose tolerant (NGT) women who entered pregnancy lean (BMI between 18-25 kg/m²; n=15 patients) or obese (BMI $\geq$ 30 kg/m²; n=14 patients). The relevant clinical details of the subjects are detailed in Table 1.

Isolation and purification of primary trophoblast cells from term placentas for culture

Isolation and purification of primary trophoblast cells was performed from placenta from NGT non-obese pregnant women. These cells were used for the cell culture experiments and siRNA studies detailed below. Placental villous cytotrophoblasts were isolated as previously described [38] by DNase/trypsin digestion and purified by separation on a Percoll gradient. Briefly, placental villous tissue (~25 g) was dissected and washed in saline and then digested three times in a HEPES-buffered salt solution containing 0.25% trypsin and 0.2 mg/ml DNase. Tissue was shaken at 37°C for 30 min. The cytotrophoblast cells were separated on a Percoll gradient and resuspended in standard cell culture medium (5.5 mM glucose, 44.5% DMEM, 44.5% Ham's-F12, and 10% fetal calf serum supplemented with antibiotics). The cells were plated on 24-well plates at a density of 5×10^5 cells per well. The cells were cultured for a total of 90 h at 37°C in 8% air, 5% CO₂ atmosphere and the cell culture media was changed daily. Trophoblast cells purity was confirmed by high protein expression of cytokeratin-7 (epithelial cell marker), absence of vimentin (fibroblast cell marker) expression, and secretion of hCG (measure of biochemical differentiation).

Trophoblast cell culture treatments

Sixty-six hours after plating, to allow for syncytialisation, cells were treated in the absence or presence of 1 µg/ml lipopolysaccharide (LPS; derived from Escherichia coli 026:B6 (Sigma-Aldrich; St. Louis, MO, USA)), 10 ng/ml TNF- α (PeproTech; Rocky Hill, NJ, USA) and 1 ng/ml IL-1 β (PeproTech; Rocky Hill, NJ, USA) for 24 h. Cells were collected and stored at -80°C until assayed for cIAP1 or cIAP2 expression by qRT-PCR as detailed below. MTT assay was performed

on all treatments to determine any significant effects on cell viability. Experiments were performed on placenta obtained from 6 patients.

Gene silencing of cIAP1 and cIAP2 with siRNA

Primary trophoblast cells were also used to investigate the effect of siRNA-mediated gene silencing of cIAP1 and cIAP2 on inflammation and oxidative stress. cIAP1 (HSC.RNAI.N001166.12.1) and cIAP2 (HSC.RNAI.N001165.12.1) were obtained from Integrated DNA Technologies (IDT; Coralville, IA, USA), and the negative control (NC) siRNA was obtained from Sigma (Sigma-Aldrich; St. Louis, MO, USA). Twenty hours after plating, the trophoblast cells were transfected with cell culture media containing 50 nM NC siRNA, 50 nM cIAP1 siRNA or 50 nM cIAP2 siRNA and 0.3% Lipofectamine 2000 (Life Technologies; Mulgrave, Vic, Australia), incubated for 24 h, and removed, and fresh medium was added to wells. After 66 h (total culture time), trophoblast cells were treated with or without 1 ng/ml TNF- α , and the cells were incubated at 37°C for an additional 24 h. Cells were collected and stored at -80°C until assayed for mRNA expression by qRT-PCR as detailed below. Media was collected and stored at -80°C until assayed for cytokine release as detailed below. MTT assay was performed on all treatments to determine any significant effects on cell viability. Experiments were performed on placenta obtained from 5 patients.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells and tissues using TRIsure reagent according to manufacturer's instructions (Bioline; Alexandria, NSW, Australia), as previously described [39]. RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). RNA (0.5 μ g for tissue and 0.15 μ g for cells) was converted to cDNA using the Tetro cDNA synthesis kit (Bioline; Alexandria, NSW, Australia) according to the manufacturer's instructions. The cDNA was diluted fifty-fold, and 4 μ l of this was used to perform RT-PCR using Sensimix Plus SYBR green (Bioline; Alexandria, NSW, Australia)

and 200 nM of pre-designed and validated QuantiTect primer assays (Qiagen; Chadstone Centre, Vic, Australia). The RT-PCR was performed using a CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia). Average gene CT values were normalised to the average 18S Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method. For the tissue expression, fold change was calculated relative to the lean group. For the trophoblast cells studies, fold change was calculated relative to basal. For the trophoblast cells siRNA studies, fold change was calculated relative to TNF- α -stimulated NC siRNA transfected cells, which was set at 1.

Western blotting

Western blotting was performed as we have previously described [39]. Forty micrograms of protein was separated onto 10% polyacrylamide gels (Bio-Rad Laboratories; Gladesville, NSW, Australia) and transferred to nitrocellulose. The blots were then cut at ~50 kD. The top part of the blots (>50 kD) were incubated with 1 μ g/ml rabbit polyclonal anti-cIAP1 (#sc-7943, Santa Cruz Biotechnology; Santa Cruz, CA, USA) or 1 μ g/ml rabbit polyclonal anti-cIAP2 (#sc-7944, Santa Cruz Biotechnology; Santa Cruz, CA, USA) while the bottom of the blots (<50 kD) were probed with mouse monoclonal anti- β -actin (A5316; Sigma-Aldrich; St. Louis, MO) at 1/20,000 prepared in blocking buffer (5% skim milk/TBS-T (0.05%)) and for 16 h at 4°C. Membranes were viewed and analysed using the ChemiDoc XRS system (Bio-Rad Laboratories; Gladesville, NSW, Australia). Semi-quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories). The protein expression of cIAP1 or cIAP2 was normalised to β -actin expression and the fold change was calculated relative to the lean group.

Enzyme immunoassays

The release of IL-6 and IL-8 was performed using CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies; Mulgrave, Victoria, Australia). The limit of detection for the IL-6 and IL-8 assays was 16 and 12 pg/ml, respectively. The inter- and intra-assay coefficients of variation (CV) for all assays were less than 10%.

Gelatin zymography

Secreted pro MMP-2 and pro MMP-9 expression in conditioned media was analysed by gelatin zymography as previously described [40]. Proteolytic activity was visualised as clear zones of lysis on a blue background of undigested gelatin. Gels were scanned using the ChemiDoc XRS system (Bio-Rad Laboratories; Gladesville, NSW, Australia), inverted, and densitometry performed using Quantity One image analysis software (Bio-Rad Laboratories; Gladesville, NSW, Australia). Fold change was calculated relative to TNF- α -stimulated NC siRNA transfected cells, which was set at 1.

Statistical analysis

Statistics was performed on the normalised data unless otherwise specified. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Two sample comparisons, either a paired or unpaired Student's t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons, the homogeneity of data was assessed by the Bartlett's test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (with LSD post-hoc testing to discriminate among the means). Statistical significance was ascribed to P value <0.05 . Data were expressed as mean $\pm$ standard error of the mean (SEM).

RESULTS

Effect of pre-existing maternal obesity on placental cIAP1 and cIAP2 expression

Tissues were obtained at the time of term Caesarean section (in the absence of labour) from lean (n=15 patients) and obese (n=14 patients) women. Demographic data of the participants involved in this investigation are summarised in Table 1. The women were stratified according to their pre-pregnancy BMI as either lean or obese. There were no significant differences in maternal age, gestational age at delivery, and gestational weight gain between the two groups. As expected, maternal BMI (both pre-pregnancy and at delivery) was significantly higher in the lean patients compared to the obese patients. Neonate birthweight, ponderal index and placenta weight were also higher in the obese pregnant group.

The gene expression of cIAP1 and cIAP2 was assessed by qRT-PCR and protein expression was assessed by Western blotting and the data presented in Figure 1. The gene and protein expression of cIAP1 and cIAP2 was significantly higher in placenta from obese pregnant women when compared to placenta from lean pregnant women.

Effect of pro-inflammatory mediators on trophoblast cIAP expression

Obesity in pregnancy is associated with endotoxemia and inflammation [17]. Thus, the next aim was to determine the effect of bacterial endotoxin LPS and the pro-inflammatory cytokines IL-1 β and TNF- α on cIAP1 and cIAP2 expression in trophoblast cells. As shown in Figure 2, LPS, IL-1 β and TNF- α all significantly increased cIAP1 and cIAP2 gene expression.

Effect of cIAP1 and cIAP2 gene silencing in primary trophoblast cells on inflammation

In non-gestational tissues, cIAPs are required for TNF- α signalling [30, 41]. Thus, the next part of this study was to determine the effect of cIAP1 and cIAP2 knockdown on inflammation in the

1 presence of TNF- α . For these studies, primary trophoblast cells were used and knockdown of cIAPs
2 performed by siRNA. The efficacy of transfection was analysed by qRT-PCR, with a 75%
3 knockdown observed for cIAP1 and 72% knockdown for cIAP2. There was no effect of cIAP1
4 siRNA knockdown on cIAP2 expression, and there was no effect of cIAP2 siRNA knockdown on
5 cIAP1 expression.

6
7 The effect of cIAP1 or cIAP2 siRNA on TNF- α -induced expression and release of the pro-
8 inflammatory cytokines IL-6 and IL-8 in primary trophoblast cells is demonstrated in Figure 4.
9 When compared to basal NC siRNA transfected cells, TNF- α caused a significant increase in both
10 the mRNA expression (Figures 3A,B) and release (Figures 3C,D) of IL-6 and IL-8. This increase in
11 cytokine mRNA expression and release was significantly decreased in cells deficient in cIAP1 or
12 cIAP2.

13
14 The effect of cIAP1 or cIAP2 siRNA on MMP-2 and MMP-9 gene expression and release is
15 demonstrated in Figure 4. TNF- α treatment significantly increased MMP-9 mRNA expression
16 (Figures 4B) and pro MMP-9 expression (Figure 4D). The effect of cIAP1 and cIAP2 silencing was
17 a decrease in TNF- α -induced MMP-9 gene expression and pro MMP-9 levels. On the other hand,
18 there was no effect of TNF- α or cIAP siRNA knockdown on MMP-2 mRNA expression and
19 activity (Figures 4A,C).

DISCUSSION

The novel findings of this study are that in placenta from women with pre-existing maternal obesity, cIAP1 and cIAP2 expression are higher when compared to placenta from lean women. Obesity in pregnancy is associated with endotoxemia and elevated circulating levels of pro-inflammatory cytokines [9, 10, 12, 17, 18]. Additionally, placental expression and secretion of pro-inflammatory cytokines is also higher in obese pregnancies [8-12, 17, 18]. In keeping with the increase in cIAPs in the obese placenta, the endotoxin LPS and the pro-inflammatory cytokines TNF- α and IL-1 β increased cIAP1 and cIAP2 expression in human primary trophoblast cells isolated from term placenta. There are no studies detailing cIAP expression in obesity; however, the findings of this study are in support of studies in macrophages [27], glioma cells [33], rat hepatocytes [34], human neutrophils [42], human endothelial cells [32, 43] and Jurkat T cells [35] whereby LPS and/or pro-inflammatory cytokines have been shown to increase the expression of cIAP1 and or cIAP2.

All three IAPs can bind to caspases, however, only XIAP is a direct inhibitor of caspases that carry out the cell-death program [44, 45]. More recently, cIAPs have been identified as important modulators of inflammation. Indeed, there is now increasing evidence that cIAPs regulate pro-inflammatory cytokines and chemokines in response to TNF- α [27, 29-31, 41, 46]. With respect to obesity in pregnancy, TNF- α is an important cytokine whose expression is increased in both placenta [9] and adipose tissue [47]. Placental derived TNF- α or TNF- α secreted from adipose tissue may contribute to placental inflammation by inducing the synthesis of other pro-inflammatory mediators [48]. The biological activities of TNF- α are mediated by two distinct TNF receptors, p55 type 1 receptor (TNFR1) and p75 type 2 receptor (TNFR2). Recent evidence suggests that TNFR1 is critical for pro-inflammatory gene transcription via the classical NF- κ B pathway [49]. TNF- α binding to the TNFR1 recruits a number of adaptor and signalling molecules such as TNF receptor type 1-associated death domain protein (TRADD), receptor-interacting

protein (RIP), TNF receptor-associated factor 2 (TRAF2), and cIAP1 and cIAP2. The cIAP proteins ubiquitinate RIP1 which eventually leads to the proteasomal degradation of inhibitor of κ B (I κ B)- α . This liberates NF- κ B proteins to translocate from the cytosol into the nucleus to stimulate transcription of NF- κ B target genes.

Notably, NF- κ B is increased in placenta of obese pregnant women [19] and TNF- α has been shown to activate the classical NF- κ B signalling pathway in trophoblast JEG-3 cells [20]. Therefore, in this study, loss of function studies utilising siRNA were performed to determine the role of cIAPs in shaping TNF- α dependent inflammatory signalling in primary trophoblast cells isolated from human term placenta. Using siRNA, the data obtained in this study support a role for cIAP1 and cIAP2 in inflammation in human placenta. Specifically, knockdown of cIAP1 or cIAP2 was associated with a decrease in TNF- α induced IL-6 and IL-8 expression and secretion. It is thus possible that the increase in cIAP1 and cIAP2 in the obese placenta may be responsible for the increase in pro-inflammatory cytokines observed in obese pregnancies [8-12, 17, 18].

MMPs are proteolytic enzymes that can act as modulators of inflammation [50, 51]; they have been shown activate the expression of pro-inflammatory cytokines and chemokines [52, 53] Although there are no studies that have investigated placental MMPs with respect to obesity, they are increased in the placenta of patients with type 2 diabetes [54] and are activated by inflammation in the placenta [55]. In this study, knockdown of cIAP1 or cIAP2 in primary human trophoblast cells was associated with a decrease in TNF- α induced MMP-9 gene expression and pro MMP-9 secretion.

In placenta, pro-inflammatory cytokines might play a role in insulin resistance evident in this condition [56]. For example, IL-6 stimulates trophoblast fatty acid accumulation [57] and increases trophoblast System A amino acid transporter activity and expression [58], which could contribute to

an excessive nutrient transfer associated with obesity. Collectively, this data suggests that cIAP1 and cIAP2 may also be important metabolic regulators in human placenta. Future studies to determine the role of cIAPs in nutrient transport in the placenta are warranted.

It has recently been shown that cIAP1 and cIAP2 can regulate inflammation and innate immunity through the molecular regulation of the inflammasome [25]. Specifically, macrophages from cIAP2 knockout mice are deficient in caspase-1 activation and subsequent production of IL-1 β in response to various inflammasome agonists [25]. Moreover, there was dampened neutrophil infiltration and cytokine production in response to exposure to inflammasome stimuli in cIAP1 or cIAP2 knockout mice or mice administered with an IAP antagonist [25]. Notably, increased activation of the inflammasome has been reported in human placenta from obese pregnant women [8]. The inflammasome, and in particular its generation of active caspase-1, is required to process pro IL-1 β to an active, secreted molecule [59]. In primary human trophoblast cells, IL-1 β inhibits the insulin signalling pathway by inhibiting IRS-1 signalling and preventing insulin-stimulated amino acid transport [11]. It is thus possible that cIAPs may also regulate the activation of IL-1 β and subsequent insulin signalling and amino acid transport [11] in human placenta.

In this study, a well-defined study population was studied. Patients were excluded for clinical factors which may influence the maternal inflammatory profile such as asthma, preeclampsia, diabetes and hypertension. In addition, placentas were obtained from non-smokers and from women at the time of term Caesarean section (non-labouring). Patients were also matched for fetal gender, gestational age and importantly gestational weight gain. A limitation of this study is that maternal and placental inflammation was not assessed in this cohort of samples. Nevertheless, previous studies have demonstrated that obese pregnancies are associated with increased levels of pro-inflammatory cytokines in both the maternal circulation and in placental tissues [8-12, 17, 18].

1 In conclusion, this study, for the first time, reports increased expression of cIAP1 and cIAP2 in
2 human placenta from women with pre-existing maternal obesity. In addition, the pro-inflammatory
3 mediators LPS, TNF- α and IL-1 β increased expression of cIAP1 and cIAP2 in primary placental
4 trophoblast cells. It is thus possible, that increased placental expression of cIAPs in obesity may be
5 due to the elevated maternal or placental inflammation associated with this condition [8-12, 17, 18].
6 Knockdown of cIAP1 or cIAP2 were associated with a decrease in pro-inflammatory cytokine
7 expression in response to TNF- α . Given the central role of pro-inflammatory cytokines in regulating
8 placental nutrient transport [11, 57, 58], it is possible that cIAPs may be important metabolic
9 regulators in human placenta. Future studies are warranted to further elucidate the role cIAPs in the
10 placenta.

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DISCLOSURE SUMMARY

The author has nothing to declare.

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Table 1. Relevant characteristics of the patients in the study group

	Lean	Obese
	(n=15)	(n=14)
Maternal Age (years)	33.3 ± 1.3	31.4 ± 1.5
Pre-pregnancy BMI (kg/m²)	23.1 ± 0.5	37.3 ± 1.1*
Maternal BMI at delivery (kg/m²)	26.7 ± 0.6	40.5 ± 1.0*
Gestational weight gain (kg)	9.0 ± 0.6	8.3 ± 0.6
Gestational age at birth (weeks)	38.3 ± 0.2	38.9 ± 0.1
Fetal Gender	7 Male; 8 Female	7 Male; 7 Female
Placental weight	550 ± 29	700 ± 33*
Neonate birthweight (g)	3078 ± 119	3708 ± 101*
Ponderal Index	2.5 ± 0.2	2.7 ± 0.1*
Parity	2.7 ± 0.3	2.4 ± 0.5
Gravida	1.9 ± 0.2	2.2 ± 0.2

Values represent mean ± SEM

* $P < 0.05$ vs. lean (Student's t-test)

FIGURE LEGENDS**Figure 1. Effect of pre-existing maternal obesity on cIAP1 and cIAP2 expression in human placenta**

Human placenta was obtained from lean pregnant women (n=15 patients) and obese pregnant women (n=14 patients) at the time of term Caesarean section. **(A,B)** cIAP1 and cIAP2 gene expression was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to the lean group. Data is displayed as mean $\pm$ SEM. * P <0.05 vs. lean (Student's t-test) or ** P <0.0 vs. lean (Mann-Whitney test). **(C,D)** cIAP1 and cIAP2 protein expression was assessed by Western blot. Protein expression was normalised to β -actin expression and the fold change was calculated relative to the lean group. Data is displayed as mean $\pm$ SEM. * P <0.05 vs. lean (Student's t-test) or ** P <0.0 vs. lean (Mann-Whitney test). **(E)** Representative Western blot from 12 patients (6 lean and 6 obese) is also shown.

Figure 2. Effect of pro-inflammatory cytokines on cIAP1 and cIAP2 expression in human primary trophoblast cells

Human primary trophoblast cells was incubated in the absence or presence of 10 ng/ml TNF- α , 1 ng/ml IL-1 β or 10 μ g/ml LPS for 24 h (n=6 patients). **(A)** cIAP1 and **(B)** cIAP2 gene expression was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to basal. Data is displayed as mean $\pm$ SEM. * P <0.05 vs. basal (paired-sample comparison).

Figure 3. Effect of cIAP1 and cIAP2 siRNA on pro-inflammatory cytokine and chemokine expression

Human primary trophoblast cells were transfected with or without 200 nM cIAP1, cIAP2 or NC siRNA for 48 h then treated with 1 ng/ml TNF- α for an additional 24 h (n=5 patients). **(A,B)** Gene

expression for IL-6 and IL-8 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to TNF- α -stimulated NC siRNA transfected cells. Data displayed as mean $\pm$ SEM. * P <0.05 vs. TNF- α -stimulated NC siRNA transfected cells (one-way ANOVA). **(C,D)** The incubation medium was assayed for concentration of IL-6 and IL-8 by ELISA. The fold change was calculated relative to TNF- α -stimulated NC siRNA transfected cells. Data displayed as mean $\pm$ SEM. * P <0.05 vs. TNF- α -stimulated NC siRNA transfected cells (one-way ANOVA).

Figure 4. Effect of cIAP1 and cIAP2 knockdown by siRNA on MMP-2 and MMP-9 expression

Human primary trophoblast cells were transfected with or without 200 nM cIAP1, cIAP2 or NC siRNA for 48 h then treated with 1 ng/ml TNF- α for an additional 24 h (n=5 patients). **(A,B)** Gene expression for MMP-2 and MMP-9 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to TNF- α -stimulated NC siRNA transfected cells. Data displayed as mean $\pm$ SEM. * P <0.05 vs. TNF- α -stimulated NC siRNA transfected cells (one-way ANOVA). **(C-E)** The incubation medium was assayed for pro MMP-2 and pro MMP-9 expression by gelatin zymography. Pro MMP-2 and pro MMP-9 levels were confirmed by densitometry, and the fold change was calculated to TNF- α -stimulated NC siRNA transfected cells. Data is displayed as mean $\pm$ SEM. * P <0.05 vs. TNF- α -stimulated NC siRNA transfected cells (one-way ANOVA). **(E)** Representative zymography from one patient is shown.

Figure 1

Figure 2

Figure 3

Figure 4

Highlights

- Cellular inhibitors of apoptosis (cIAP) 1 and 2 are increased in placenta from obese women
- LPS, TNF- α and IL- β increased the expression of cIAP1 and cIAP2 in human trophoblast cells
- Knockdown of cIAP1 decreased TNF- α induced cytokine expression from primary trophoblast cells
- Knockdown of cIAP2 decreased TNF- α induced cytokine expression from primary trophoblast cells

Conflict of Interest

The author has nothing to declare.

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