NOD1 expression is increased in the adipose tissue of women with gestational diabetes

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Abstract

Maternal peripheral insulin resistance and increased inflammation are two features of pregnancies, complicated by gestational diabetes mellitus (GDM). The nucleotide-binding oligomerisation domain (NOD) intracellular molecules recognise a wide range of microbial products, as well as other intracellular danger signals, thereby initiating inflammation through activation of nuclear factor κ B (NF κ B). The aim of this study was to determine whether levels of NOD1 and NOD2 are increased in adipose tissue of women with GDM. The effect of NOD1 and NOD2 activation on inflammation and the insulin signalling pathway was also assessed. NOD1, but not NOD2, expression was higher in omental and subcutaneous adipose tissues obtained from women with GDM when compared with those from women with normal glucose tolerance (NGT). In both omental and subcutaneous adipose tissues from NGT and GDM women, the NOD1 ligand *g*-D-glutamyl-meso-diaminopimelic acid (iE-DAP) significantly induced the expression and secretion of the pro-inflammatory cytokine interleukin 6 (IL6) and chemokine IL8; *COX2* (*PTGS2*) gene expression and subsequent prostaglandin production; the expression and secretion of the extracellular matrix remodelling enzyme matrix metalloproteinase 9 (MMP9) and the gene expression and secretion of the adhesion molecules *ICAM1* and *VCAM1*. There was no effect of the NOD2 ligand muramyl dipeptide on any of the endpoints tested. The effects of the NOD1 ligand iE-DAP were mediated via NF κ B, as the NF κ B inhibitor BAY 11-7082 significantly attenuated iE-DAP-induced expression and secretion of pro-inflammatory cytokines, *COX2* gene expression and subsequent prostaglandin production, *MMP9* expression and secretion and *ICAM1* and *VCAM1* gene expression and secretion. In conclusion, the present findings describe an important role for NOD1 in the development of insulin resistance and inflammation in pregnancies complicated by GDM.

Key Words

- ▶ adipose tissue
- ▶ gestational diabetes
- ▶ NOD1
- ▶ inflammation
- ▶ insulin resistance

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Introduction

Gestational diabetes mellitus (GDM) is characterised as any degree of glucose intolerance with first recognition during pregnancy. It is the most common type of diabetes found in pregnancy, affecting up to 14% of all pregnancies (Kim *et al.* 2007); the prevalence of GDM is increasing

worldwide, in part due to the increased rates of obesity (Ferrara 2007). Of clinical significance, there are short- and long-term consequences for both mother and infant (Dabelea *et al.* 2000, Sobngwi *et al.* 2003), enhancing the susceptibility to a number of chronic diseases including

obesity, diabetes, cardiovascular disease and certain cancers later in life.

There is now increasing evidence that the obese and diabetic environment may induce a number of changes in maternal adipose tissue, which may play an important role in the growth and development of the foetus. For example, maternal adipose tissue's insulin resistance, an important adaptation that occurs in the later part of pregnancy, is even more pronounced in women with GDM (Catalano *et al.* 2002, Colomiere *et al.* 2010), resulting in greater foetal substrate availability and thus enhanced adiposity (Lain & Catalano 2007). Maternal adipose tissue also synthesises and secretes a number of inflammatory mediators, which are dysregulated in obese and GDM pregnancies, that have been shown to correlate with foetal adiposity (Kautzky-Willer *et al.* 1997, Krauss *et al.* 2002, Radaelli *et al.* 2006).

Toll-like receptors and nucleotide-binding oligomerisation domain containing protein-like receptor (NLR) are the two families of pattern recognition receptors (PRRs) that play critical roles in inflammation. The two prominent members of the NLRs are the cytosolic PRRs NOD1 and NOD2. Their activation, by bacterial peptidoglycans, results in nuclear factor κ B (NF κ B)-induced gene transcription. Recently, NOD1 and NOD2 have been shown to play an important role in inflammation and insulin resistance in adipocytes (Winzer *et al.* 2004, Yi-Jun *et al.* 2012, Zhao *et al.* 2011, Zhou *et al.* 2012, Purohit *et al.* 2013). The levels of *NOD1* and *NOD2* mRNA are markedly increased in differentiated murine 3T3-L1 adipocytes and human primary adipocyte cultures upon adipocyte conversion. Moreover, *Nod1* mRNA is markedly increased only in the fat tissues of diet-induced obese mice (Zhao *et al.* 2011). The *Nod1/2* knockout mice are protected from high-fat-diet-induced inflammation, lipid accumulation and peripheral insulin intolerance (Schertzer *et al.* 2011). Conversely, direct activation of NOD1 protein causes insulin resistance (Schertzer *et al.* 2011, Zhao *et al.* 2011, Zhou *et al.* 2012). Activation of NOD1 also induces the expression and/or secretion of a number of pro-inflammatory mediators from adipocytes (Garvey *et al.* 1993, Lappas 2013a, Purohit *et al.* 2013).

Given that obesity and GDM share many common features, it is possible that in GDM, the activation of NOD1 and NOD2 in adipose tissue may enhance inflammation and induce insulin resistance; two key features of this disease. However, the expression and regulation of NOD1 and NOD2 in adipose tissues of pregnant women have not yet been investigated. Thus, the aim of this study was to determine the effect of pre-existing maternal obesity and

GDM on *NOD1* and *NOD2* gene expression in subcutaneous and omental adipose tissues. To determine whether NOD1 and NOD2 induce markers of inflammation and interfere with the insulin signalling pathway, the effect of the NOD1 ligand *g*-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and the NOD2 ligand muramyl dipeptide (MDP) was examined in subcutaneous and omental adipose tissues of pregnant women.

Materials and methods

Tissue collection and preparation

Human subcutaneous and omental adipose tissues were obtained (with the approval of the Research Ethics Committee of Mercy Health) from consenting women who delivered healthy, singleton infants at term (>37 weeks gestation).

Human subcutaneous (from the anterior abdominal wall) and omental adipose tissues were obtained from a total of 46 pregnant women (28 normal glucose tolerance (NGT) and 18 GDM). The tissues were obtained within 15 min of delivery and the dissected fragments were thoroughly washed in ice-cold PBS to remove any blood, snap frozen in liquid nitrogen and either stored at -80°C until required for RNA extraction for gene expression analysis by quantitative RT-PCR (qRT-PCR), or immediately used for explant studies as detailed below. All tissues were obtained at the time of term Caesarean section before the onset of labour. Indications for Caesarean section included repeat Caesarean section or breech presentation. Women with any adverse underlying medical condition (i.e. including asthma, preeclampsia and pregestational diabetes) were excluded. Samples were collected from lean (BMI <24.9 kg/m²), overweight (BMI between 25 and 29.9 kg/m²) and obese (BMI >30 kg/m²) subjects. The women were classified as lean, overweight or obese based on their BMI, calculated at their first antenatal visit at approximately 12 weeks gestation. Women with GDM were diagnosed according to the criteria of the Australasian Diabetes in Pregnancy Society (ADIPS) by either a fasting venous plasma glucose level of ≥ 5.5 mmol/l glucose and/or ≥ 8.0 mmol/l glucose 2 h after a 75 g oral glucose load at approximately 26–28 weeks gestation. All women with GDM were prescribed insulin in addition to dietary management. All pregnant women were screened for GDM, and women in the NGT group had a negative screening result. The relevant clinical details of the subjects are detailed elsewhere (Lappas 2014a).

Tissue explant culture

Subcutaneous and omental adipose tissue explants were also examined to determine the effect of the NOD1 ligand iE-DAP and the NOD2 ligand MDP on inflammation. For these studies, subcutaneous and omental adipose tissues were obtained from NGT pregnant women ($n=6$ patients) and women with GDM ($n=6$ patients). Adipose tissue explants were produced as described previously (Lappas 2013a). Tissue explants were incubated, for 20 h, in the absence or presence of 5 $\mu\text{g/ml}$ iE-DAP or 5 $\mu\text{g/ml}$ MDP (purchased from Life Research, Scoresby, VIC, Australia). The concentrations of iE-DAP and MDP were based on results from past studies (Lappas 2013b). In order to determine whether iE-DAP and MDP regulate inflammation via NF κ B, additional experiments were also carried out whereby tissues from NGT pregnant women were pre-treated with 50 μM BAY 11-7082 for 60 min before the addition of iE-DAP and MDP for 20 h. The concentration of BAY 11-7082 was based on results from previously published studies (Lappas 2013b). At this dose, there was no effect of BAY 11-7082 on basal cytokine secretion (data not shown). After final incubation, tissue and media were collected separately and stored at -80°C for further analysis as detailed below.

Glucose uptake

Subcutaneous and omental adipose tissue explants from NGT pregnant women were generated as detailed earlier and glucose uptake in adipose tissue was measured as described previously (Lappas *et al.* 2012). Briefly, after final incubation with treatment agents, tissues were pre-incubated in the absence or presence of 20 μM cytochalasin B in Krebs buffer for 5 min. 2-Deoxy-D-glucose (2DG) uptake was measured by adding 3 $\mu\text{Ci/ml}$ [^{14}C]-2DG (PerkinElmer, Glen Waverley, VIC, Australia) and 1 mM 2DG to Krebs buffer containing 0.1% BSA (fatty acid free) and 0.1 μM insulin for 20 min. Tissues were immediately collected and washed in ice-cold PBS three times and solubilised for 4 h in 1 M NaOH at 60°C . Tissues were neutralised with 1 M HCl and then centrifuged at 15 000 g for 5 min to pellet insoluble material. The supernatant was transferred to a vial containing 3 ml of liquid scintillation fluid. All samples were counted for radioactivity in a liquid scintillation counter. GLUT-specific glucose uptake was measured by subtracting values for [^{14}C]-2DG uptake in the presence of 20 μM cytochalasin B. The rate of [^{14}C]-2DG transport was expressed in nanomoles per minute per milligram protein. Fold change was calculated relative to basal levels, which were set at 1.

RNA extraction and qRT-PCR

Total RNA was extracted from tissues using TRIsure according to the manufacturer's instructions (Bioline, Alexandria, NSW, Australia). RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). RNA was converted to cDNA using the SuperScript VILO cDNA synthesis Kit (Invitrogen) according to the manufacturer's instructions. The cDNA was diluted to 50-fold, and 4 μl of this was used to perform RT-PCR using SensiFAST SYBR (Bioline) and 100 nM of pre-designed and validated primers. Primers were purchased from either Qiagen (QuantiTect primer assays) or Integrated DNA Technologies (PrimeTime qPCR Assays). The RT-PCR was carried out using a CFX384 real-time PCR detection system from Bio-Rad Laboratories. Average gene threshold cycle (Ct) values were normalised to the average GAPDH Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method and shown as mean $\pm$ S.E.M. of relative gene expression. For the explant studies, there was a large variability of baseline values, which is normal for tissues derived from different patients. Thus, fold change was calculated relative to either basal levels or levels with iE-DAP, which were set at 1.

Western blotting analysis

Tissue lysates were prepared and western blotting was performed as described previously (Lappas *et al.* 2005a). Rabbit polyclonal anti-phosphorylated IRS-1 (Tyr1229), rabbit polyclonal anti-IRS-1 (A19), and rabbit polyclonal anti-GLUT4 (H61) were purchased from Santa Cruz Biotechnology. Samples (40 μg) of protein were separated on polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose. Protein expression was identified by comparison with the mobility of a protein standard. The membranes were viewed and analysed using the Chemi-Doc system (Bio-Rad). 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). Data were corrected for background, normalised to β -actin (mouse monoclonal anti- β -actin (A5316; Sigma) and fold change was calculated relative to basal levels, which were set at 1.

Enzyme immunoassays

The release of interleukin 6 (IL6) and IL8 was examined by sandwich ELISA according to the manufacturer's

Figure 1

NOD1 is increased in adipose tissues of women with GDM. (A and B) Omental and subcutaneous adipose tissues were obtained from lean ($n=10$ subjects), overweight ($n=8$ subjects) and obese ($n=10$ subjects) pregnant women at the time of term Caesarean section. *NOD1* and *NOD2* mRNA expression was normalised to actin mRNA expression. Fold changes were calculated relative to the level for the lean group, and data are displayed as mean $\pm$ S.E.M. (C and D) Omental and subcutaneous adipose

tissues were obtained from NGT ($n=18$ subjects; eight overweight and ten obese) and GDM ($n=18$ subjects; eight overweight and ten obese) pregnant women at the time of term Caesarean section. *NOD1* and *NOD2* mRNA expression were normalised to actin mRNA expression. Fold changes were calculated relative to NGT, and data are displayed as mean $\pm$ S.E.M. * $P<0.05$ vs NGT mRNA expression (t-test).

instructions (Invitrogen). The release of PGE2 and PGF2 α into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, Waterloo, NSW, Australia). The concentration of sICAM1 and sVCAM1 in the media was measured by sandwich ELISA according to the manufacturer's instructions (R&D

Systems Inc., Minneapolis, MN, USA). All data were corrected for total protein and expressed as either picograms or nanograms per milligram protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, IL, USA), using BSA as a reference standard, as described previously (Lappas *et al.* 2005b). The calculated interassay and intraassay coefficients of variation were all $<10\%$.

Figure 2

The NOD1 ligand iE-DAP induces pro-inflammatory cytokines in adipose tissues of pregnant women. (A, B, C and D) Omental and (E, F, G and H) subcutaneous adipose tissues were incubated in the absence or presence of 5 μ g/ml iE-DAP or 5 μ g/ml MDP for 20 h ($n=6$ NGT patients and $n=6$ GDM patients). (A, C, E and G) Expression of *IL6* and *IL8* was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and the fold

changes were calculated relative to basal levels. Data are displayed as mean $\pm$ S.E.M. * $P<0.05$ vs basal (paired-sample comparison). (B, D, F and H) The incubation medium was assayed by ELISA for the concentration of *IL6* and *IL8*. Each bar represents the mean $\pm$ S.E.M. * $P<0.05$ vs basal (paired-sample comparison).

Figure 3

The NOD1 ligand iE-DAP induces COX2 and subsequent prostaglandin production in adipose tissues of pregnant women. (A, B and C) Omental and (D, E and F) subcutaneous adipose tissues were incubated in the absence or presence of 5 µg/ml iE-DAP or 5 µg/ml MDP for 20 h ($n=6$ NGT patients and $n=6$ GDM patients). (A and D) COX2 gene expression was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and the

fold changes were calculated relative to basal levels. Data are displayed as mean $\pm$ S.E.M. * $P<0.05$ vs basal (paired-sample comparison). (B, C, E and F) The incubation medium was assayed by EIA for concentration of PGE2 and PGF2α. Each bar represents the mean $\pm$ S.E.M. * $P<0.05$ vs basal (paired-sample comparison).

Gelatin zymography

Matrix metalloproteinase 9 (MMP9) activity was assessed by gelatin zymography as described previously (Lappas *et al.* 2006) on conditioned media collected from tissue explants. Proteolytic activity was visualised as clear zones of lysis on a blue background of undigested gelatin. Gels were scanned (ChemiDoc; Bio-Rad Laboratories), inverted, and densitometry was performed using Quantity One image analysis software (Bio-Rad Laboratories). Fold change was calculated relative to either basal levels or levels with iE-DAP, which was set at 1.

Statistical analysis

Statistical analysis 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, were 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 test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (using least significant difference correction to discriminate among the means). Statistical significance was ascribed to *P* value <0.05 . Data were expressed as mean $\pm$ S.E.M.

Results

NOD1 expression is increased in adipose tissue of women with GDM

Demographic data of all participants involved in the investigation are detailed elsewhere (Lappas 2014a).

Figure 4

The NOD1 ligand iE-DAP induces ECM matrix remodelling enzymes in adipose tissues of pregnant women. (A and B) Omental and (C and D) subcutaneous adipose tissues were incubated in the absence or presence of 5 µg/ml iE-DAP or 5 µg/ml MDP for 20 h ($n=6$ NGT patients and $n=6$ GDM patients). (A and C) *MMP9* expression was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and the fold changes were calculated relative to basal levels. Data are displayed as

mean $\pm$ S.E.M. * $P < 0.05$ vs basal (paired-sample comparison). (B and D) Representative zymography demonstrating pro-MMP9 expression from omental and subcutaneous adipose tissues from one patient. *MMP9* expression levels were confirmed by densitometry. The fold changes were calculated relative to basal levels. Each bar represents the mean $\pm$ S.E.M. * $P < 0.05$ vs basal (paired-sample comparison).

In the lean, overweight and obese cohort, there were no significant differences in maternal age, gestational age and foetal birth weight between any of the groups studied. For the NGT vs GDM cohort, all samples were obtained from women who were either overweight or obese (the NGT and the GDM cohorts consisted of eight overweight and ten obese patients each). Again, there were no significant differences in maternal age, maternal BMI, gestational age and foetal birth weight between the GDM and NGT women.

There was no effect of pre-existing maternal obesity on *NOD1* or *NOD2* expression in omental or subcutaneous adipose tissue (Fig. 1A and B). mRNA expression of *NOD1* was significantly higher in omental and subcutaneous adipose tissues obtained from women with GDM (Fig. 1C). On the other hand, adipose tissue *NOD2* expression was not different between NGT and GDM women (Fig. 1D).

Effect of NOD1 ligand iE-DAP and NOD2 ligand MDP on markers of inflammation in pregnant adipose tissue

The next aim was to determine whether NOD1 and NOD2 regulate pro-inflammatory mediators in subcutaneous and omental adipose tissues. This was done by incubating

tissues with the NOD1 agonist iE-DAP and the NOD2 agonist MDP for 20 h. Adipose tissue was obtained from women with NGT and women with GDM. Enzyme immunoassays were used to determine the release of the pro-inflammatory cytokine IL6; the chemokine IL8; the prostaglandins PGE2, PGF2 α and PGI2; and the adhesion molecule sICAM1 and sVCAM1. The expression of secretory pro MMP2 and pro MMP9 was measured by gelatin zymography. Gene expression for these analytes was assessed by qRT-PCR.

As shown in Fig. 2A, B, C, D, E and F, iE-DAP significantly increased the gene expression of the pro-inflammatory cytokine *IL6* and the chemokine *IL8* (*CXCL8*). This was associated with significantly increased release of IL6 and IL8 from omental and subcutaneous adipose tissues. On the other hand, there was no effect of the NOD2 ligand on cytokine expression or secretion.

Expression of *COX2* gene and subsequent PGE2 and PGF2 α release from omental and subcutaneous adipose tissues were also significantly increased by treatment with the NOD1 ligand iE-DAP (Fig. 3A, B, C, D, E and F). Again, *COX2* expression and prostaglandin release were not affected by treatment with MDP.

MMPs are involved in the remodelling and/or degradation of the extracellular matrix (ECM). In this study,

Figure 5

The NOD1 ligand iE-DAP induces the expression and secretion of adhesion molecules in adipose tissues of pregnant women. (A, B, C and D) Omental and (E, F, G and H) subcutaneous adipose tissues were incubated in the absence or presence of 5 µg/ml iE-DAP or 5 µg/ml MDP for 20 h ($n=6$ patients). (A, C, E and G) Expression of *ICAM1* and *VCAM1* was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and

the fold changes were calculated relative to basal levels. Data are displayed as mean $\pm$ s.e.m. * $P<0.05$ vs basal (paired-sample comparison). (B, D, F and H) The incubation medium was assayed by ELISA for the concentration of sICAM1 and sVCAM1. Each bar represents the mean $\pm$ s.e.m. * $P<0.05$ vs basal (paired-sample comparison).

adipose tissue *MMP9* gene expression and the release of pro *MMP9* enzyme activity were significantly augmented by iE-DAP (Fig. 4A, B, C and D). On the other hand, NOD2 activation by MDP had no effect on *MMP9* expression or release. There was no effect of iE-DAP or MDP on *MMP9* gene expression and pro *MMP2* secretion (data not shown).

The expression of the genes encoding the adhesion molecules *ICAM1* and *VCAM1* was significantly amplified in the presence of the NOD1 ligand iE-DAP (Fig. 5A, C, E and G). This was associated with significantly increased secretion of sICAM1 and sVCAM1 (Fig. 5B, D, F and H). Again, there was no effect of the NOD2 ligand MDP.

Figure 6

The NOD1 ligand iE-DAP increases pro-inflammatory cytokines via the NF κ B pathway. Omental and subcutaneous adipose tissues from NGT pregnant women were pre-incubated in the absence or presence of 50 µM BAY 11-7082 for 60 min and then treated with 5 µg/ml iE-DAP for 20 h ($n=6$ patients). (A and C) Expression of *IL6* and *IL8* genes was analysed by qRT-PCR.

Gene expression was normalised to *GAPDH* mRNA expression and the fold changes were calculated relative to iE-DAP. Data are displayed as mean $\pm$ s.e.m. * $P<0.05$ vs iE-DAP (one-way ANOVA). (B and D) The incubation medium was assayed by ELISA for the concentration of *IL6* and *IL8*. Each bar represents the mean $\pm$ s.e.m. * $P<0.05$ vs iE-DAP (one-way ANOVA).

Figure 7

The NOD1 ligand iE-DAP increases the COX2 prostaglandin pathway via the NFκB pathway. Omental and subcutaneous adipose tissues from NGT pregnant women were pre-incubated in the absence or presence of 50 μM BAY 11-7082 for 60 min and then treated with 5 μg/ml iE-DAP for 20 h ($n=6$ patients). (A) COX2 gene expression was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and the fold changes were calculated relative to iE-DAP. Data are displayed as mean $\pm$ S.E.M. * $P < 0.05$ vs iE-DAP (one-way ANOVA). (B and C) The incubation medium was assayed by EIA for concentration of PGE2 and PGF2α. Each bar represents the mean $\pm$ S.E.M. * $P < 0.05$ vs iE-DAP (one-way ANOVA).

The NOD1 ligand iE-DAP regulates inflammation via NFκB

The next aim was to explore whether the NOD1 agonist iE-DAP regulates pro-inflammatory mediators in adipose tissue or pregnant women via NFκB. To address this, experiments were conducted in which omental or subcutaneous adipose tissues were pre-incubated for 60 min with the NFκB inhibitor BAY 11-7082 before stimulation with iE-DAP for 20 h. In both omental and subcutaneous adipose tissues, BAY 11-7082 significantly attenuated iE-DAP-induced cytokine gene expression (Fig. 6A and C) and release (Fig. 6B and D) COX2 mRNA expression (Fig. 7A) and the resultant PGE2 and PGF2α release (Fig. 7B and C); *MMP9* gene expression (Fig. 8A) and enzyme activity (Fig. 8B); and *ICAM1* and *VCAM1* mRNA expression (Fig. 9A and C) and sICAM1 and sVCAM1 secretion (Fig. 9B and D).

The NOD1 ligand iE-DAP impairs insulin signalling in pregnant omental adipose tissue

In women with GDM, expression of *GLUT4* (*SLC2A4*) gene and subsequent glucose uptake are lower in adipose tissue (Garvey *et al.* 1993, Colomiere *et al.* 2010). Given that *NOD1* expression was higher in adipose tissue obtained from women with GDM, the next aim of this study was to determine the effect of the NOD1 ligand iE-DAP on insulin signalling in adipose tissue from pregnant women. For these studies, only omental adipose tissue was used, as glucose uptake was very low in subcutaneous adipose tissue. The data depicted in Fig. 10 indicate that iE-DAP significantly decreased the expression of phosphorylated IRS1 protein, *GLUT4* mRNA, *GLUT4* protein and 2DG uptake.

Discussion

This study, for the first time, to my knowledge, demonstrates that *NOD1* expression is increased in both subcutaneous and omental adipose tissues obtained from women with GDM when compared with BMI matched NGT women. Further to this, treatment of subcutaneous and omental adipose tissues from NGT and GDM pregnant women with the NOD1 ligand iE-DAP significantly increased the expression of a number of inflammatory markers. Specifically, there was an increase in the expression and secretion of the pro-inflammatory cytokine IL6, the pro-inflammatory chemokine IL8, COX2 and subsequent prostaglandin production, the ECM remodelling/degrading enzyme MMP9, and the

Figure 8

The NOD1 ligand iE-DAP regulates ECM matrix remodelling enzymes via the NF κ B pathway. Omental and subcutaneous adipose tissues from NGT pregnant women were pre-incubated in the absence or presence of 50 μ M BAY 11-7082 for 60 min and then treated with 5 μ g/ml iE-DAP for 20 h ($n=6$ patients). (A) *MMP9* gene expression was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and the fold change was calculated relative to iE-DAP. Data are displayed as mean $\pm$ S.E.M. * $P<0.05$ vs iE-DAP (one-way ANOVA). (B) Representative zymography from one patient. *MMP9* expression levels were confirmed by densitometry. The fold changes were calculated relative to iE-DAP. Each bar represents the mean $\pm$ S.E.M. * $P<0.05$ vs iE-DAP (one-way ANOVA).

gene expression and secretion of the adhesion molecules *ICAM1* and *VCAM1*. These effects of NOD1 appear to be mediated via the pro-inflammatory transcription factor NF κ B, as BAY 11-7082 ameliorated iE-DAP-induced inflammatory proteins. In addition, NOD1 activation of

adipose tissue from pregnant women resulted in a decrease in insulin-stimulated glucose uptake. Collectively, these data indicate that NOD1 may contribute to the pathophysiology of GDM. Notably, there was no effect of GDM on the expression of *NOD2* in both subcutaneous and omental adipose tissues. In addition, there was no effect of the *NOD2* ligand on any of the inflammatory mediators or on the insulin signalling pathway.

When compared with NGT controls, *NOD1* expression was increased in both omental and subcutaneous adipose tissues obtained from women with GDM. *NOD2* expression was similar in the adipose tissues of NGT and GDM women. Although *NOD1* and *NOD2* mRNA are markedly increased during adipocyte differentiation (Zhao *et al.* 2011, Purohit *et al.* 2013), only *NOD1* mRNA is markedly increased in adipose tissue in diet-induced obese mice (Zhao *et al.* 2011). Moreover, and in keeping with the findings of this study, only activation of *NOD1*, but not *NOD2*, dose-dependently suppresses 3T3-L1 adipocyte differentiation (Zhao *et al.* 2011, Purohit *et al.* 2013). Collectively, the findings of this study indicate that *NOD2* is not active in adipose tissue of pregnant women. Indeed, *NOD1* and *NOD2* detect distinct structures of bacterial peptidoglycan. *NOD1* recognises bacterial peptidoglycan-derived peptides iE-DAP derived primarily from Gram-negative bacilli and particular Gram-positive bacteria (Chamaillard *et al.* 2003), whereas *NOD2* recognises the minimal peptidoglycan MDP, the largest peptidoglycan motif common to all bacteria (Girardin *et al.* 2003). There is now some evidence that metabolic endotoxemia originating from the diet or the environment disrupts the balance between the immune and the metabolic systems (Cani *et al.* 2007) which may lead to GDM. Thus, it is possible that this increase in bacteria may activate *NOD1*, leading to the increased inflammation and insulin resistance observed in women with GDM (Garvey *et al.* 1993, Colomiere *et al.* 2010, Lappas 2013a, Oliva *et al.* 2013). Indeed, it has recently been shown that diet-induced modification of the gut flora with probiotics can ameliorate the insulin resistance and glucose homeostasis of pregnant women (Laitinen *et al.* 2009).

Human adipose tissue is divided into two depots: the subcutaneous and the omental fat depot. They exhibit differences in their cellular composition, and metabolic and biochemical properties. For example, due to its direct access to the liver through the portal vein, omental adipose tissue is strongly associated with insulin resistance and type 2 diabetes (Fox *et al.* 2007). This study demonstrates that the NOD1 ligand iE-DAP significantly inhibits the insulin signalling pathway in omental adipose

Figure 9

The NOD1 ligand iE-DAP increases expression of adhesion molecules via the NF κ B pathway. Omental and subcutaneous adipose tissues from NGT pregnant women were pre-incubated in the absence or presence of 50 μ M BAY 11-7082 for 60 min and then treated with 5 μ g/ml iE-DAP for 20 h ($n=6$ patients). (A and C) *ICAM1* and *VCAM1* gene expression was analysed by qRT-PCR. Gene expression was normalised to *GAPDH* mRNA expression and

the fold changes were calculated relative to iE-DAP. Data are displayed as mean $\pm$ s.e.m. * $P < 0.05$ vs iE-DAP (one-way ANOVA). (B and D) The incubation medium was assayed by ELISA for concentration of siCAM1 and sVCAM1. Each bar represents the mean $\pm$ s.e.m. * $P < 0.05$ vs iE-DAP (one-way ANOVA).

tissue of pregnant women, as evidenced by decreased phosphorylated IRS1, GLUT4 expression and glucose uptake. Thus, NOD1 may be one of the mechanisms causing the peripheral insulin resistance observed in GDM (Garvey *et al.* 1993, Colomiere *et al.* 2010). In agreement, other studies have also shown an important role for NOD1 in insulin action. In 3T3-L1 adipocytes and mature adipocytes, NOD1 activation suppresses insulin signalling by attenuating tyrosine phosphorylation and increases inhibitory serine phosphorylation of IRS1, resulting in decreased insulin-induced glucose uptake (Zhao *et al.* 2011, Purohit *et al.* 2013). These studies have been

supported by results from *in vivo* studies. In mice, the absence of NOD1 and NOD2 attenuates diet-induced insulin intolerance, and NOD1 ligands induce whole-body insulin resistance (Schertzer *et al.* 2011).

IL6 is one of several pro-inflammatory cytokines that have been associated with insulin resistance and type 2 diabetes (Kern *et al.* 2001, Pradhan *et al.* 2001, Senn *et al.* 2002, Nieto-Vazquez *et al.* 2008). High levels of IL6 are also associated with GDM (Yu *et al.* 2007, Kuzmicki *et al.* 2009, Morisset *et al.* 2011). CXC chemokines, such as IL8 (CXCL8), are among the most critical inflammatory mediators for the recruitment of neutrophils. Women with

Figure 10

The NOD1 ligand iE-DAP inhibits the insulin signalling pathway. Omental adipose tissue from NGT pregnant women was incubated in the absence or presence of 5 μ g/ml iE-DAP or 5 μ g/ml MDP for 20 h followed by a 30 min incubation with 0.1 μ M insulin ($n=4$ patients). (A) Phosphorylated IRS1 (pIRS1) protein expression was normalised to total IRS1, and the fold changes were calculated relative to basal levels. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$ vs basal (paired sample comparison). A representative Western blot from two patients is also shown. (B) *GLUT4* gene

expression was normalised to *GAPDH*, and the fold changes were calculated relative to basal levels. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$ vs basal (paired sample comparison). (C) GLUT4 protein expression was normalised to β -actin, and the fold changes were calculated relative to basal levels. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$ vs basal (paired sample comparison). A representative Western blot from two patients is also shown. (D) 2DG uptake data are displayed as mean $\pm$ s.e.m. * $P < 0.05$ vs basal 2DG uptake (paired sample comparison).

GDM have significantly higher *IL8* mRNA expression in visceral adipose tissue compared with NGT women (Kuzmicki *et al.* 2012). Similarly, in this study, IL8 release was higher in omental adipose tissue from women with GDM compared with NGT women. In this study, ligand activation of NOD1, but not NOD2, resulted in increased expression and secretion of IL6 and IL8 from adipose tissue. Similarly, stimulation of NOD1 with a synthetic ligand Tri-DAP induces the pro-inflammatory chemokine MCP1 and RANTES and the pro-inflammatory cytokines tumour necrosis factor α , MIP2 (human IL8 homologue) and IL6 mRNA expression in 3T3-L1 adipocytes and human primary adipocytes (Zhao *et al.* 2011, Zhou *et al.* 2012). In addition, NOD1 activation provokes adipose tissue inflammation *in vivo* (Schertzer *et al.* 2011). Collectively, these results indicate that NOD1 activation may facilitate the infiltration of leukocytes into adipose tissue, leading to adipose inflammation, a key feature of GDM pregnancies (Lappas 2013a, Oliva *et al.* 2013). Moreover, NOD1-mediated release of pro-inflammatory cytokines from adipose tissue may directly contribute to the insulin resistance that is evident in pregnancies complicated by GDM (Catalano *et al.* 2002, Colomiere *et al.* 2010). In keeping with this, the data presented in this paper indicates that NOD1 activation induces attenuation of insulin signalling, which leads to suppression of insulin-induced glucose uptake.

Several lines of evidence support the importance of the COX–prostaglandin pathway in the pathophysiology of type 2 diabetes. *COX2* gene expression is induced in livers of diet-induced and genetic obesity mouse models (Henkel *et al.* 2012). Pharmacological inhibition of *COX2* has also been shown to improve insulin sensitivity in both rodents and humans (Gonzalez-Ortiz *et al.* 2001, Hsieh *et al.* 2008). Furthermore, PGE2, which is released from adipose tissue (Lappas *et al.* 2005a), can also attenuate insulin signalling (Henkel *et al.* 2009). Interestingly, genetic variation in the carbonyl reductase 3 gene, which encodes an enzyme for converting PGE2 to PGF2 α , is associated with type 2 diabetes (Chang *et al.* 2012). In this study, the NOD1 ligand induced *COX2* gene expression and this was associated with an increase in the secretion of PGE2 and PGF2 α from both subcutaneous and omental adipose tissues. Again, there was no effect of NOD2 activation on *COX2* expression and prostaglandin release. This is the first study, to my knowledge, to report that NOD1 activation upregulates the COX–prostaglandin pathway in adipose tissue; however NOD1 and NOD2 activation of *COX2* has previously been reported in placenta (Lappas 2013b) and macrophages (Tsai *et al.* 2011).

Development of excessive adipose tissue is associated with obesity and diabetes (Streuli 1999). Remodelling of the ECM is required for several aspects of adipose tissue differentiation, which is mainly mediated via the MMP systems. Proper ECM remodelling is critical for supporting adipose tissue malleability to accommodate changes in energy storage needs. In addition to their role in adipose tissue remodelling, MMPs play a role in inflammation by releasing, activating or degrading several growth factors and cytokines implicated in diabetes (Galis & Khatri 2002). MMP9, which is released from adipose tissue (Bouloumie *et al.* 2001), is a key enzyme involved in these processes. Higher plasma and tissue MMP9 levels are found in obese subjects (Laimer *et al.* 2005) and patients with the metabolic syndrome (Goncalves *et al.* 2009). In this study, the NOD1 ligand induced *MMP9* gene expression and secretion of pro MMP9 from both subcutaneous and omental adipose tissues. Similarly, NOD1 has been shown to induce MMP9 in human placenta (Lappas 2013b), and *in vivo* in the aortic root and pulmonary artery after oral administration of a NOD1 ligand (Nishio *et al.* 2011).

The adhesion molecules *ICAM1* and *VCAM1* play an important role in the development of endothelial dysfunction. Atherosclerosis is predominantly mediated by cellular adhesion molecules, which are expressed on the vascular endothelium and on circulating leukocytes in response to several inflammatory stimuli. Soluble forms of *ICAM1* and *VCAM1* have been found to enter the systemic circulation, as a result of proteolytic cleavage at the cell surface, releasing the extracellular domain. Indeed, sICAM1 and sVCAM1 levels are higher in insulin resistance, obesity and type 2 diabetes (Guler *et al.* 2002, Leinonen *et al.* 2003, Kent *et al.* 2004). There is also evidence indicating that women with GDM develop endothelial dysfunction during pregnancy (Knock *et al.* 1997), and despite returning to NGT, endothelial dysfunction is still evident in women 1-year post GDM pregnancy (Pleiner *et al.* 2007). Indeed, women with GDM have increased circulating sICAM1 and sVCAM1 levels (Mordwinkin *et al.* 2013). My recent studies have also shown that the expression and secretion of markers of endothelial cell dysfunction are increased in adipose tissue from women with GDM (Lappas 2014b). In this study, the NOD1 ligand iE-DAP induced adipose tissue expression and secretion of *ICAM1* and *VCAM1*. This is the first study, to my knowledge, to report that NOD1 activation upregulates the adhesion molecules in adipose tissue. However, ligand activation of NOD1 has been shown to increase *ICAM1* gene expression in human airway smooth

muscle cells (Kvarnhammar *et al.* 2013), bronchial epithelial cells (Wong *et al.* 2013) and human coronary artery endothelial cells (Nishio *et al.* 2011). *In vivo*, NOD1 ligands induce *ICAM1* and *VCAM1* gene expression in the aortic root, pulmonary artery, aorta and spleen (Nishio *et al.* 2011).

The NFκB signalling pathway has been shown to be a key link between inflammation and insulin resistance (Arkan *et al.* 2005). Similarly, my previous studies show that NFκB is a key regulator of inflammation-induced pro-inflammatory cytokine release from adipose tissue obtained from pregnant women (Lappas *et al.* 2005c, Lappas 2013a). There is increasing evidence that NODs elicit many of their actions via activation of NFκB (Inohara & Nunez 2003, Strober *et al.* 2006). This study also revealed that the mechanism underlying the pro-inflammatory gene expression and secretion downstream of NOD1 activation in subcutaneous and omental adipose tissues involves the NFκB signalling pathway. Specifically, the NFκB inhibitor BAY 11-7082 significantly attenuated NOD1-induced expression and secretion of *IL6* and *IL8* mRNAs, expression of the *COX2* gene and subsequent prostaglandin release and expression and secretion of *MMP9*, *ICAM1* and *VCAM1*. These data are in agreement with results from previous studies indicating an important role for NFκB in NOD1-induced adipocyte inflammation and differentiation (Zhou *et al.* 2012, Purohit *et al.* 2013).

A limitation of this study is that GDM mothers were treated with insulin to lower their blood sugar levels. This could influence the results as previous studies from my laboratory have shown differences in components of the insulin signalling pathway in adipose tissue between controls and insulin-treated GDM subjects but also between diet-controlled and insulin-managed GDM pregnancies (Colomiere *et al.* 2010). Nevertheless, this is, to my knowledge, the first study carried out in humans, which has identified changes in *NOD1* gene expression, induced by GDM, in both omental and subcutaneous adipose tissues. In addition, the present findings demonstrate a role for NOD1 in adipose tissue inflammation and in the development of insulin resistance in pregnancies complicated by GDM. In this study, I did not measure GLUT4 expression in the membrane fraction due to limitations in the amount of sample obtained. In addition, I was unable to detect phosphorylated AKT protein expression in these samples. Thus, from these studies, I am unable to conclude that the detected GLUT4 was translocated through the membrane. Nevertheless, insulin-stimulated glucose uptake was lower in the presence of the NOD1 ligand iE-DAP.

In conclusion, the data presented in this study provide evidence that omental and subcutaneous adipose tissues obtained from women with GDM are associated with increased NOD1 expression. NOD1 activation induced expression of a number of inflammatory markers including pro-inflammatory cytokines, COX2 and subsequent PGE2 and PGF2α production, the ECM remodelling enzyme MMP9 and the expression and secretion of the adhesion molecules *ICAM1* and *VCAM1*. These actions of NOD1 were mediated by NFκB. In addition, NOD1 activation impaired insulin signalling in adipose tissue. Together, these results indicate that NOD1 plays an important role in adipose tissue inflammation and insulin resistance that is evident in women with GDM.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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