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Myo-inositol alters ^{13}C -labeled fatty acid metabolism in human placental explants

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Abstract

We postulate that myo-inositol, a proposed intervention for gestational diabetes, affects transplacental lipid supply to the fetus. We investigated the effect of myo-inositol on fatty acid processing in human placental explants from uncomplicated pregnancies. Explants were incubated with ^{13}C -labeled palmitic acid, ^{13}C -oleic acid and ^{13}C -docosahexaenoic acid across a range of myo-inositol concentrations for 24 h and 48 h. The incorporation of labeled fatty acids into individual lipids was quantified by liquid chromatography mass spectrometry. At 24 h, myo-inositol increased the amount of ^{13}C -palmitic acid and ^{13}C -oleic-acid labeled lipids (median fold change relative to control = 1). Significant effects were seen with 30 μM myo-inositol (physiological) for ^{13}C -palmitic acid-lysophosphatidylcholines (1.26) and ^{13}C -palmitic acid-phosphatidylethanolamines (1.17). At 48 h, myo-inositol addition increased ^{13}C -oleic-acid-lipids but decreased ^{13}C -palmitic acid and ^{13}C -docosahexaenoic-acid lipids. Significant effects were seen with 30 μM myo-inositol for ^{13}C -oleic-acid-phosphatidylcholines (1.25), ^{13}C -oleic-acid-phosphatidylethanolamines (1.37) and ^{13}C -oleic-acid-triacylglycerols (1.32) and with 100 μM myo-inositol for ^{13}C -docosahexaenoic-acid-triacylglycerols (0.78). Lipids labeled with the same ^{13}C -fatty acid showed similar responses when tested at the same time point, suggesting myo-inositol alters upstream processes such as fatty acid uptake or activation. Myo-inositol supplementation may alter placental lipid physiology with unknown clinical consequences.

Key Words

- ▶ placenta
- ▶ pregnancy
- ▶ fatty acids
- ▶ phospholipids
- ▶ metabolism

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Introduction

The naturally occurring carbohydrate myo-inositol is the most abundant form of inositol and is present in all living cells (Noventa *et al.* 2016). It is endogenously synthesized by the kidney and is ingested in food (Noventa *et al.* 2016). Perturbations in myo-inositol synthesis, metabolism and

excretion have been associated with the insulin-resistant conditions of polycystic ovary syndrome (PCOS), diabetes mellitus and metabolic syndrome (Croze & Soulage 2013), and with pregnancy disorders such as gestational diabetes (GDM) (Crawford *et al.* 2015, D'Anna & Santamaria 2018),

pre-eclampsia (D'Oria *et al.* 2017), intrauterine growth restriction (Dessi & Fanos 2013) and fetal neural tube defects (Copp & Greene 2010). However, the extent and the focus of the myo-inositol-related pathophysiological events in these conditions are unclear (Zhang *et al.* 2019).

Inositols are the building blocks for phosphatidylinositol lipids, inositol phosphate derivatives and inositol-phospho-glycans (IPG). These compounds act as signaling molecules and participate in regulating membrane fluidity, trafficking and transport, organelle function, intracellular compartmentalization and enzyme activity (Balla 2013). Many inositol derivatives act as insulin mimetics or interact with insulin second messenger pathways and therefore modulate glucose and lipid metabolism (Larner *et al.* 2010, Hansen 2015). Any disruption in the bioavailability of inositols and inositol-derived signaling compounds could therefore have far reaching and pathological consequences.

In non-pregnant animal studies, myo-inositol deficiency increases lipid mobilization from adipose tissue and increases hepatic lipid accumulation (Hayashi *et al.* 1978). Myo-inositol treatment during pregnancy reduced intra-abdominal adiposity in women (Croze & Soulage 2013) and reduced gonadal and perirenal adiposity in mice (Plows *et al.* 2017).

The placenta plays a central role in the generation of GDM pathology, in part through the release of pro-diabetic biological signals (Jayabalan *et al.* 2017). Furthermore, the placenta regulates transplacental lipid transfer from the mother to the fetus, and placental lipid uptake and metabolism are known to be disrupted in GDM (Uhl *et al.* 2015, Gallo *et al.* 2017). This has been postulated to contribute to dysregulated fetal growth and development (Herrera & Ortega-Senovilla 2018).

Myo-inositol supplementation for GDM prevention is being trialed (Godfrey *et al.* 2017, Zhang *et al.* 2019) and, if demonstrated to be effective, widespread supplementation would invariably involve exposure of uncomplicated pregnancies to additional exogenous myo-inositol. Thus, its impact needs to be understood, not only in the diseased state, but also in normal pregnancies. Given the critical role of the placenta in regulating the physiology of both the mother and fetus, there is a need to understand the effects of myo-inositol on placental lipid biology and this, to our knowledge, has never been explored.

Our overarching hypothesis is that myo-inositol plays a physiological role in the regulation of placental FA uptake and lipid metabolism and that both deficient and excessive myo-inositol states could impact placental lipid metabolism with consequences for fetal development.

In this study we sought to address the specific hypothesis that myo-inositol treatment alters the processing of fatty acids in normal human term placenta.

We previously reported that human term placental explants incubated with stable-isotope-labeled palmitic acid (PA: saturated fatty acid), oleic acid (OA: monounsaturated fatty acid) or docosahexaenoic acid (DHA: long-chain polyunsaturated fatty acid (LC-PUFA)) incorporated the labeled fatty acids into phosphatidylcholines (PC), triacylglycerols (TAG), phosphatidylethanolamines (PE) and lysophosphatidylcholines (LPC) in a fatty acid-specific manner (Watkins *et al.* 2019). Here, we aimed to use this placental explant model to investigate changes in the amounts of stable-isotope-labeled lipids with exposure to three myo-inositol concentrations consistent with deficient, physiologically normal or supraphysiological states. For hypothesis generation, we also explored the potential persistent influence of maternal first trimester BMI and antenatal glucose regulation on the myo-inositol responsiveness of placental explants, as well as the potential association between myo-inositol responsiveness and fetal size.

Materials and methods

Ethical approval

Women were recruited from the National University Hospital, Singapore with informed written consent. Ethical approval was obtained from the National Healthcare Group Domain Specific Review Board (2016/00183).

Placental tissue collection

Seven placentas from uncomplicated singleton pregnancies, delivered by elective cesarean section at term (mean $\pm$ s.d.: 38 weeks and 2 days' gestation $\pm$ 5 days) were obtained. All mothers (aged 34 ± 6 years; maternal first trimester BMI 27 ± 6 kg/m²) were non-smokers with normal glucose tolerance at mid-gestation as assessed by a three time point 75g oral glucose tolerance test (OGTT) using WHO 2013 criteria (mean fasting plasma glucose: 4.3 ± 0.3 mmol/L, 1 h post-load plasma glucose: 7.3 ± 1.3 mmol/L, 2 h post-load plasma glucose: 6.2 ± 1.0 mmol/L). There were four male and three female babies who were all appropriately grown for gestational age based on customized birth weight centiles (for maternal age, ethnicity, BMI, parity and fetal sex) (Gardosi *et al.* 2018), with a group mean of 55% ($\pm$ 35).

Placental tissue culture

Placental explant culture was completed as previously described (Watkins *et al.* 2019) but with the addition of 0, 30 or 100 μM myo-inositol (Sigma, >99% pure) representing a myo-inositol-deficient, physiological or supra-physiological environments (Brusati *et al.* 2005). Myo-inositol was added to basal serum-free CMRL media (GIBCO 1066-L Glutamine) containing 12 μM of myo-inositol and 5 mM glucose. Fresh placental explants (approximately $3 \times 3 \times 3$ mm of villous placental tissue) were cultured for 24 h in CMRL media containing 1.5% BSA (HI Clone fraction V, Culture grade, pH 7.00 lyophilized powder, GE Life Sciences), with the addition of 0, 30 or 100 μM myo-inositol. The normal physiological circulating concentration of maternal myo-inositol is 30–50 μM (Brusati *et al.* 2005). After 24 h the media was replaced with fresh media containing the same myo-inositol concentration and either no FA or $^{13}\text{C}_{16}$ -palmitic acid (^{13}C -PA; 99 atom % ^{13}C , 99% CP; Sigma-Aldrich, 300 μM), $^{13}\text{C}_{18}$ -oleic acid (^{13}C -OA; 99 atom % ^{13}C , 99% CP, Sigma-Aldrich, 300 μM) or $^{13}\text{C}_{22}$ -docosahexaenoic acid (^{13}C -DHA; 99 atom % ^{13}C , 99% CP, Cambridge Isotope Laboratories, 100 μM). Explants cultured in 12-well plates were incubated in 2 mL of media at 37°C in a humidified atmosphere of 5% CO_2/air .

Explants and the corresponding conditioned media were harvested at 24 h and 48 h after FA addition. DHA was only tested at 48 h. Harvested explants were washed with PBS and particulate matter removed from the supernatant by centrifugation before being stored at -80°C . Experiments were performed in triplicate for each placenta. To confirm the specificity of myo-inositol effects, explant culture with additional 30 μM myo-inositol was also performed ($n=3$) in the presence of 1 mM phlorizin (Cayman Chemical Company), a competitive inhibitor of myo-inositol-binding sites in membrane transporters and enzymes (Van Steenberg *et al.* 2017). We found that lactate dehydrogenase (LDH) and human chorionic gonadotropin (HCG) levels were similar with 0, 30 and 100 μM myo-inositol at each time point suggesting that myo-inositol did not affect explant viability (Fig. 1). Our previous work showed that explant viability was also not significantly affected by the addition of stable-isotope-labeled FA (Watkins *et al.* 2019).

Lipid quantification

Lipid extraction and analysis was completed as previously described (Watkins *et al.* 2019). Briefly, placental explants

were freeze dried and weighed, and then lysed using an Omni bead Rupter homogenizer followed by lipid extraction using a modified Bligh and Dyer method (Bligh & Dyer 1959). Lysate (100 μL) or conditioned media (50 μL) were extracted with chloroform and methanol (1:2, 450 μL , containing internal standards; Watkins *et al.* 2019). Water (300 μL) was added and the samples vortexed

Figure 1

Lactate dehydrogenase (LDH; A) and human chorionic gonadotropin (HCG; B) levels in conditioned media were not changed by the addition of 30 or 100 μM myo-inositol. Levels of LDH and HCG in media were measured after initial 24 h of placental explant culture and at 24 and 48 h after media change. Boxes show median and the interquartile range, whiskers show 5th–95th percentile. LDH was measured using a Roche Cytotoxicity detection kit^{PLUS} (04744934001) and HCG using a DRG HCG ELISA kit (EIA 1469; Marburg, Germany) following the manufacturers' protocol ($n=6$ placenta). Media from well-triplicates were combined and measured in duplicate. Media + 1.5% BSA was used to quantify background. Data were normalized such that each assay well contained the equivalent of 30 mg dry placental explant weight and 2 mL of media. Data were normally distributed (Shapiro–Wilk normality test), so data were analyzed by repeated-measures two-way ANOVA with multiple comparisons tests using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (GraphPad Prism 7). No significant differences were caused by the addition of myo-inositol.

then centrifuged. The chloroform layer was collected and the aqueous layer re-extracted with chloroform (500 μ L). Chloroform layers were combined, dried, dissolved in 200 μ L 90% IPA, 5% chloroform, 5% methanol then stored at -80°C until use. Triplicates were extracted as separate samples.

Analysis of lipids incorporating one or more $^{13}\text{C}_{16}$ -PA, $^{13}\text{C}_{18}$ -OA or $^{13}\text{C}_{22}$ -DHA was performed on the Agilent 6490 triple quadrupole liquid chromatography-mass spectrometry (LC-MS) instrument using reverse phase chromatographic separation and mass spectrometry with a targeted dynamic multiple reaction monitoring method (Watkins *et al.* 2019).

Data processing and statistical analyses

Data were processed using Mass Hunter quantitative analysis and Excel. Metabolites were quantified by integration, followed by normalization against internal standards (Watkins *et al.* 2019). Lipid amount is expressed as a function of dried placental weight ($\mu\text{mol/g}$). These data were used to calculate the equivalent lipid amount that would be expected in a normalized standard well system containing 30 mg dry weight explant and 2 mL media. Molar amount ^{13}C -lipid by class is the sum of the molar amounts for all quantified individual ^{13}C -lipids in class. Triplicate data were averaged to give a mean result for each placenta, which was then used for subsequent analysis. The effects of myo-inositol were expressed relative to controls (with no additional myo-inositol) whose mean lipid amount was assigned an arbitrary value of 1 for each lipid from each placenta. Myo-inositol response (i.e. fold change) was calculated as amount of labeled lipid in explants exposed to additional myo-inositol divided by amount of labeled lipid in control explants.

Non-parametric Wilcoxon signed-rank tests were used to test whether (i) the addition of myo-inositol (30 or 100 μM) changed the amount of labeled lipid in placental explants at either 24 or 48 h, for any lipid class; that is whether the myo-inositol response was significantly different to 1 and (ii) the myo-inositol response at 24 h was significantly different to the myo-inositol at 48 h, for any lipid class, with 30 or 100 μM myo-inositol. Multiple comparisons were accounted for using false discovery rate (FDR)-adjusted *P* values (MetaboAnalyst 3.0; Xia *et al.* 2015). The non-parametric Friedman test followed by the Dunn's *post hoc* test (GraphPad Prism 7.00) were used to determine whether any lipid class showed a significantly different MI response to any other lipid class at each time and for each myo-inositol concentration.

Correlations were measured using the non-parametric Spearman method (GraphPad Prism 7.00). Statistical significance was taken as $P < 0.05$.

Results

Placental explants incubated with myo-inositol and stable-isotope-labeled ^{13}C -PA or ^{13}C -OA demonstrated incorporation of labeled FAs into LPCs, PCs, PEs and TAGs at 24 h and 48 h. ^{13}C -DHA incorporation was only reliably quantifiable in TAGs and was only tested at 48 h. Labeled LPCs were the only class of labeled lipids detectable by our LC-MS method in the conditioned media after incubation with placental explants (Watkins *et al.* 2019).

Myo-inositol responses in placental explants and conditioned media

Figures 2 and 3 show the effects of 30 μM and 100 μM additional myo-inositol on the net incorporation of labeled FA into placental LPCs, PCs, PEs and TAGs in placental explants and conditioned media. This myo-inositol response is expressed as the relative amounts of labeled lipids in the treated experiment compared with controls (i.e. molar amount of ^{13}C -lipid with 30 or 100 μM myo-inositol/molar amount of ^{13}C -lipid with 0 μM myo-inositol).

Individual lipid species in the same class demonstrated similar myo-inositol responses (Fig. 2), so the amounts for all quantified individual ^{13}C -lipids in a given lipid class were summed in subsequent analysis (Fig. 3). Lipid class data showed that the direction and magnitude of myo-inositol response was also similar across lipid classes (i.e. between TAGs, PCs, PEs and LPCs) for each labeled FA when tested at the same time point (Fig. 3A and C).

In placental explants at 24 h, myo-inositol addition increased the amounts of labeled lipids compared to controls, with a similar effect seen for ^{13}C -PA and ^{13}C -OA labeled lipids. Myo-inositol response (median (interquartile range 25th–75th percentile)) in explants treated with 30 μM myo-inositol was significantly different ($P < 0.05$) to the 0 μM myo-inositol control (assigned value of 1) for ^{13}C -PA-LPC (1.26 (1.07–1.38)) and ^{13}C -PA-PE (1.17 (1.03–1.62)) (Fig. 3A). DHA was not tested at 24 h.

At 48 h, myo-inositol addition increased the amount of ^{13}C -OA-labeled lipids compared to controls. Myo-inositol response in explants treated with 30 μM myo-inositol was significantly different to the 0 μM myo-inositol control (assigned value of 1) for ^{13}C -OA-PC

Figure 2

Heat map (median fold change) showing the effect of myo-inositol on net fatty acid incorporation into individual placental lipids, 24 h and 48 h after the addition of stable-isotope labeled fatty acids. Effects were assessed by quantifying the amount of stable-isotope labeled lipids in placental explants with myo-inositol treatment (30 μM , $n = 7$; 100 μM , $n = 6$) relative to the respective controls (no added myo-inositol). Increased lipids are shown in red, while decreased lipids are shown in blue. Myo-inositol response (fold change) = amount of ^{13}C -lipid with additional myo-inositol/amount of ^{13}C -lipid with 0 μM additional myo-inositol. DHA, docosahexaenoic acid; MI, myo-inositol; LPC, lyso-phosphatidylcholine; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

(1.25 (1.02–1.75)), ^{13}C -OA-PE (1.37 (1.16–1.52)) and ^{13}C -OA-TAG (1.32 (1.21–1.75)) (Fig. 3A). Treatment with a higher dose of 100 μM myo-inositol showed a similar effect as treatment with 30 μM myo-inositol, but the effect was not significantly different compared to the control (Fig. 3C).

At 48 h, myo-inositol addition resulted in an overall decrease in the amount of ^{13}C -PA-labeled lipids compared with controls, but the effect was not significant. However, the difference between the myo-inositol response for ^{13}C -PA compared with that for ^{13}C -OA were statistically

significant for every lipid class (Fig. 3A). At 48 h, 100 μM myo-inositol also significantly decreased the amount of ^{13}C -DHA-TAG (0.78 (0.72–0.86)) compared with 0 μM myo-inositol control (Fig. 3C). The ^{13}C -DHA response was also significantly different to that for ^{13}C -OA ($P < 0.05$). It is to be noted that these decreases represent decreases with respect to the control with no added MI and not changes in absolute amounts over the period in culture.

When comparing the difference in myo-inositol response between 24 h and 48 h, only ^{13}C -PA showed divergent effects of an increase at 24 h but a decrease at 48 h for all four lipid classes. Differences in amount were significant for ^{13}C -PA-PE (24 h: 1.17 (1.03–1.62) vs 48 h: 0.75 (0.57–0.99)) and ^{13}C -PA-TAG (24 h: 1.05 (0.84–1.80) vs 48 h: 0.73 (0.58–1.14)) with the addition of 30 μM myo-inositol (Fig. 3A). For ^{13}C -OA there was no further significant increase in labeled lipids from 24 h to 48 h compared to controls.

Labeled LPC levels were highly variable in conditioned media and no statistically significant effects in labeled LPC levels in conditioned media were observed between myo-inositol treatment groups or FA type (Fig. 3B and D).

To demonstrate that the changes seen in the amount of labeled lipid were a specific effect of myo-inositol, placental explants treated with the physiological dose of myo-inositol (additional 30 μM) were cultured in the presence and absence of the myo-inositol competitive inhibitor, phlorizin. The presence of phlorizin suppressed the 48-h myo-inositol-induced increases in ^{13}C -OA-labeled lipid classes relative to control (Fig. 4).

Maternal and fetal characteristics associated with the amount of labeled lipid and MI responsivity

For hypothesis generation purposes, we investigated if the preceding antenatal characteristics of first trimester maternal BMI and mid-gestation maternal glucose tolerance, reflected by the fasting glucose concentration and post-load glucose concentrations at 1 h and 2 h of an OGTT, could leave a placental 'imprint' that persists into the time of *in vitro* culture post-delivery to influence placental lipids. We also explored whether differences in placental MI responses could be associated with fetal size, by examining the relationship between placental labeled lipids and customized birthweight percentile.

We first examined the correlations between these characteristics and absolute molar amount of labeled lipid at the physiological myo-inositol concentration (additional 30 μM). In general, across the BMI and glycemic ranges of our study population, LPC, PC and PEs

Figure 3

Effect of myo-inositol on the net fatty acid incorporation into placental explant lipids by class, 24 h and 48 h after addition of stable-isotope-labeled fatty acids. Effects were assessed by quantifying the amount of stable-isotope-labeled lipids in placental explants (A and C) with myo-inositol treatment (A, B: 30 μ M, $n = 7$ or C, D: 100 μ M, $n = 6$) and conditioned media (i.e. media incubated with placental explants, B, D), expressed relative to the respective controls (no added MI). Myo-inositol response (median fold change with 25th and 75th percentiles) = amount of $^{13}\text{C-lipid}$ in each lipid class with additional myo-inositol/amount of $^{13}\text{C-lipid}$ in each lipid class with 0 μ M additional myo-inositol. Statistical significance * by Wilcoxon signed-rank test if the FDR-adjusted $P < 0.05$ compared with control (dashed line = 1) or between different time points (indicated by * above brackets). Letters indicate statistically significant differences in myo-inositol response between lipid classes at 48 h by Friedman test followed by Dunns *post hoc* tests with $P < 0.05$. Significantly different with 30 μ M myo-inositol: a vs b. Significantly different with 100 μ M myo-inositol: c vs d or e vs f; significantly different with 100 μ M myo-inositol. DHA, docosahexaenoic acid; LPC, lyso-phosphatidylcholine; MI, myo-inositol; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

labeled with $^{13}\text{C-PA}$ and $^{13}\text{C-OA}$ positively correlated with maternal first trimester BMI, but negatively correlated with antenatal fasting glucose concentrations as assessed at mid-gestation.

Meanwhile TAGs labeled with $^{13}\text{C-PA}$ and $^{13}\text{C-OA}$ positively correlated with antenatal 2-h glucose values and birthweight percentiles (Fig. 5). Next we investigated the correlation between these factors and lipid class myo-inositol responsivity (ratio of amount with added myo-inositol/amount with no added myo-inositol). Correlations were derived only for conditions in which a significant myo-inositol response was observed (24 h, 30 μ M myo-inositol for $^{13}\text{C-PA}$ lipids and 48 h, 30 μ M myo-inositol for $^{13}\text{C-OA}$ lipids). Myo-inositol response for $^{13}\text{C-PA-LPC}$ increased with fasting glucose (Rho 0.9, $P < 0.01$, Fig. 6A) in contrast to the negative correlation with the absolute amount of placental $^{13}\text{C-PA-LPC}$ (Rho -0.9 , $P < 0.01$, Fig. 6A). In comparison, the higher the maternal BMI, the lower the myo-inositol response for

$^{13}\text{C-PA-PC}$ (Rho -0.89 , $P = 0.01$, Fig. 6B) even though the amount of $^{13}\text{C-PA-PC}$ was positively associated with BMI. For $^{13}\text{C-OA}$ at 48 h, the higher the post-load glycemia at 1 h and 2 h, the greater the 48-h myo-inositol response for $^{13}\text{C-OA-PE}$ synthesis (Rho 0.77, $P = 0.05$ and Rho 0.86, $P = 0.02$ respectively, Fig. 6D) and the higher the absolute amounts of placental $^{13}\text{C-OA-TAG}$ (Rho 0.77, $P = 0.05$ and Rho 0.93, $P = 0.007$ respectively, Fig. 6C). Birthweight percentile was positively correlated with both the absolute amount of $^{13}\text{C-PA-TAG}$ with 30 μ M myo-inositol at 24 h (Rho 0.79, $P = 0.048$, Fig. 6E) and myo-inositol response for $^{13}\text{C-OA-TAG}$ (Rho 0.79, $P = 0.048$, Fig. 6F). These findings suggest that placental TAG metabolism could be associated with fetal growth. No significant correlations were observed for $^{13}\text{C-DHA}$ -labeled lipids with any of the maternal or fetal characteristics. Our study was underpowered to conclusively determine the effect of sex and ethnicity, and larger studies will be needed to investigate this variable.

Figure 4

Influence of the myo-inositol inhibitor, phlorizin (1 mM), on myo-inositol response of ^{13}C -OA lipids with 30 μM myo-inositol at 48 h. Effects assessed by quantifying the amount of stable-isotope labeled lipids in placental explants ($n = 3$) with myo-inositol treatment (crosses) and myo-inositol with phlorizin (dots) relative to the respective controls (no added myo-inositol). Lines show the decrease with myo-inositol inhibition for each placenta. Amount relative to control = amount of ^{13}C -lipid in each lipid class with additional MI/amount of ^{13}C -lipid in each lipid class with 0 μM additional myo-inositol. LPC, lyso-phosphatidylcholine; MI, myo-inositol; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLz, phlorizin; TAG, triacylglycerol.

Discussion

This study has demonstrated that myo-inositol affects the metabolism of ^{13}C -labeled PA, OA and DHA, in human term placental explants from uncomplicated pregnancies. At each time point, all measured lipids incorporating the same labeled FA showed a similar response to myo-inositol treatment, with myo-inositol initially increasing then decreasing the incorporation of the saturated PA into placental lipids over 48h, but continued increasing incorporation of the monounsaturated OA and decreasing incorporation of the long-chained polyunsaturated docosahexaenoic acid compared to controls. This indicates that myo-inositol does not specifically act on biological pathways affecting the synthesis of any one lipid or lipid class. It instead suggests that myo-inositol affects common upstream processes such as FA transport into the placenta or the activation of FAs that initiates the incorporation of FA into lipids.

The effect of myo-inositol did differ between the type of FA and over time. This suggests that several different FA-selective biological processes are affected by myo-inositol. The rate-limiting step for each time and for each FA would depend on the placental capacity and speed of each process and whether these processes were close

Spearman Rho of ^{13}C -lipid amount at 30 μM myo-inositol **Figure 5**

Heatmap showing correlations (Spearman Rho) between maternal glycemia, maternal BMI and birthweight percentile, with the amount of ^{13}C -PA or ^{13}C -OA labeled placental lipids when incubated with 30 μM MI ($n = 7$). Rho values are presented to show general trends. Positive correlations are shown in red while negative correlations are shown in blue. The sample size is too small to determine significance for all factors and lipids. Specific statistically significant correlations for lipids by class are highlighted in Fig. 6. LPC, lyso-phosphatidylcholine; MI, myo-inositol; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

to equilibrium. We speculate that FA transport processes facilitating cellular entry should exert the greatest effect soon after FA addition to culture media and have less influence later. Synthetic and catabolic processes, in contrast, would exert a greater effect later. A balance of all these different processes would be reflected by the net incorporation represented by the absolute amount of labeled lipids quantified.

Myo-inositol most likely impacts lipid transport and metabolism though the effects of MI-derived signaling molecules such as phosphoinositides, phosphatidylinositol phosphates or inositol phosphoglycans, which are known to regulate lipid metabolism in various tissues (Larner *et al.* 2010, Hansen 2015). Myo-inositol may affect enzymes that catalyze the initial steps of lipid synthesis such as the

Figure 6

Illustrated correlations between ^{13}C lipid amount, myo-inositol (MI) response and fetal and maternal characteristics with 30 μM MI ($n = 7$). Relative MI response (fold change, dots) = amount of ^{13}C -lipid in each lipid class with additional myo-inositol/ amount of ^{13}C -lipid in each lipid class with 0 μM additional MI. Absolute molar amount of ^{13}C -fatty acid-lipid (crosses) expressed as the amount (nmol) in an average well system containing placental explants (30 mg dry) and media (2 mL). Overlapping points are shown by overlapped X and +. Correlations were measured using the non-parametric Spearman's method. LPC, lyso-phosphatidylcholine; MI, Myo-inositol; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

acyl-CoA long-chain synthetases (ACSL, ACSL_V), which are important for facilitating placental FA uptake (Tobin *et al.* 2009, Araújo *et al.* 2013) or the glycerol phosphate acyl transferases (GPAT) (Wendel *et al.* 2009). Alternatively myo-inositol could affect enzymes that regulate whether FA acyl-CoAs get directed into lipid synthesis or FA catabolism such as carnitine palmitoyltransferase-1 (CPT, increases catabolism) (Wolf 1992, Keung *et al.* 2013) and acetyl-CoA carboxylase (ACC, decreases catabolism) (Wendel *et al.* 2009). Whether alterations in the activities of these enzymes occur in our explant model with myo-inositol treatment remains to be determined, but there is some literature evidence consistent with our hypothesis that these enzymes are likely candidates (Hayashi *et al.* 1976, Kunjara *et al.* 1999, Hammond *et al.* 2002, Araújo *et al.* 2013).

The activities of GPAT and ACC are influenced by inositol-derived inositol phosphoglycans (Huang *et al.* 1993, Farese *et al.* 1994, Kunjara *et al.* 1999). Furthermore, myo-inositol deficiency in yeasts increased ACC activity resulting in an accumulation of lipids containing unsaturated FAs (Hayashi *et al.* 1976), an effect reversed by myo-inositol treatment. Intriguingly, phospholipids from Gpat1^{-/-} mice contain less PA and DHA, and more OA, than lipids from control mice (Hammond *et al.* 2002); a pattern that matches our data at 48 h. Furthermore different isoforms of these enzymes are known to display different FA selectivity and could be differentially regulated by myo-inositol (Hammond *et al.* 2002, Watkins 2008, Wendel *et al.* 2009, Yan *et al.* 2015).

ACSL in human trophoblasts and CPT in placental explants are decreased by GDM (Araújo *et al.* 2013,

Visiedo *et al.* 2013) and when exposed to high glucose *in vitro* (Visiedo *et al.* 2013). Furthermore, ACC (Abu-Elheiga *et al.* 2003, Kreuz *et al.* 2009, Xu *et al.* 2014, Harriman *et al.* 2016), CPT (Wolf 1992, Keung *et al.* 2013) and GPAT (Bates & Saggerson 1977, Farese *et al.* 1994) are affected by glucose, insulin resistance and diabetes in other organs. This could explain why placental myo-inositol response was associated with maternal glycemia.

Correlations of myo-inositol response with maternal and fetal characteristics

Maternal obesity and hyperglycemia are associated with dysregulated placental lipid metabolism (Uhl *et al.* 2015, Gallo *et al.* 2017, Delhaes *et al.* 2018). In our present study, the participants had a range of BMI but none were morbidly obese (BMI >40 kg/m²) and none had gestational diabetes. Even within the relatively narrow range of maternal glycemia and non-morbid BMI range, and the small sample size, correlations with placental explant incorporation of labeled FA and myo-inositol responsiveness were already evident. It is intriguing that maternal BMI and glycemia is associated with divergent effects on labeled lipid incorporation in placenta with physiological myo-inositol exposure. This strongly suggests that these maternal characteristics antenatally leave a lasting influence on the placenta that persists into *in vitro* culture, consistent with previous reports (Araújo *et al.* 2013, Visiedo *et al.* 2015) and that this contributes to biological variability in myo-inositol response.

At 24 h higher maternal fasting glycemia was associated with a lower absolute amount of freshly synthesized placental ¹³C-PA-lipid; this contrasted with the increase in ¹³C-PA-lipid amount following placental myo-inositol treatment *in vitro* (reflected by an increasing myo-inositol response). Furthermore, the magnitude of increase in ¹³C-PA incorporation induced by myo-inositol treatment was larger for placenta from pregnancies of higher fasting maternal glycemia. This suggests that increasing placental myo-inositol exposure could potentially promote compensatory mechanisms to combat the glycemia-induced disruptions in placental lipid processing. However, myo-inositol decreased ¹³C-PA lipid incorporation at 48 h suggesting that potential compensatory mechanisms may be transient, contrasting and complicated, involving multiple biological pathways.

With respect to ¹³C-OA incorporation at 48 h, myo-inositol response was also higher in those with higher levels of maternal glycemia, but only with higher 1-h

and 2-h post-load glycemia rather than fasting glycemia. This further emphasizes the potential differences in the regulation of PA and OA metabolism in human placenta.

Interestingly, the maternal glycemia-associated changes in the placental ¹³C-PA-lipid amount and myo-inositol response are opposite to those associated with maternal BMI, suggesting that maternal glycemia and BMI impact on placental lipid pathways differently, even though both characteristics are associated with increasing fetal adiposity and size. This may explain the differences between clinical trials on the effects of myo-inositol supplementation on birthweight. Three small Italian trials had previously shown that myo-inositol supplementation reduced the GDM rate in at-risk women; while a reduction in birthweight and fetal abdominal circumferences was found in the two studies that recruited women with clear dysglycemic risk (a borderline fasting glucose at booking or family history of diabetes) (D'Anna *et al.* 2013, Matarrelli *et al.* 2013), no birthweight reduction was found in the one conducted in women with a raised BMI (D'anna *et al.* 2012).

The link between FA incorporation and myo-inositol response with maternal glycemia and fetal size is intriguing, because placental lipid metabolism is thought to regulate fetal size (Haggarty 2002, Lager & Powell 2012). We previously suggested that placental TAGs act as an accessible FA reserve for transfer to the fetus (Watkins *et al.* 2019) to promote growth and hypothesized that fetal size may be partly regulated by how much FA is found in accessible placental lipid reserves such as TAGs compared with other less accessible reserves such as phospholipids. Our data of increasing absolute amounts of PA- and OA-labeled TAG with rising glycemia (2 h) and birthweight percentile is consistent with this idea and consistent with other studies which have reported that placental TAGs are increased by hyperglycemia and associated with fetal macrosomia (Visiedo *et al.* 2013, 2015).

Conversely, sequestration of FA into inaccessible phospholipids could mean less FA available for transfer to the fetus, thus suppressing fetal growth. If high placental myo-inositol *in vivo* could increase FA incorporation into phospholipids, as we observed *in vitro* at 24 h of culture, this would be consistent with the observation of intrauterine growth restriction in a sheep model of high placental inositol (Regnault *et al.* 2010). However, in our small study, absolute amounts of labeled phospholipids were not significantly correlated with birthweight percentile, and myo-inositol's effect on phospholipid synthesis was contrasting, depending on FA type and

time in culture. Further research is therefore needed to explore if myo-inositol could sufficiently influence the regulation of FA sequestration into phospholipids to have appreciable impact on birthweight. Nonetheless, our data overall suggest that myo-inositol has the potential to alter placental lipid synthesis, and this could affect fetal growth.

DHA is important for fetal development, particularly the brain (Yessoufou *et al.* 2015). The reduction in DHA incorporation into TAGs induced by myo-inositol could result from decreased DHA uptake or from altered DHA metabolism. This could be problematic if myo-inositol is to be used in GDM management, since this could potentially exacerbate the already reduced placental DHA uptake in this condition (Gallo *et al.* 2017). On the other hand, it would be less concerning if our results reflect a myo-inositol-induced reduction in DHA incorporation into TAGs, implying that there could be more non-esterified DHA available for fetal transfer, rather than a reduction in overall DHA uptake. Unfortunately, non-esterified DHA was not quantified by our method to enable conclusions to be drawn.

This study is limited by its relatively small sample size ($n=7$) and only involved placentas from uncomplicated pregnancies without GDM. Thus, it is difficult to predict whether myo-inositol would be useful in preventing the macrosomia associated with GDM. Larger studies will be needed to confirm the effects of MI on the placenta and fetal growth with consideration of multiple maternal and fetal characteristics and confounders. Even though our placental explant treatment with myo-inositol *in vitro* attempted to mimic the effects of maternal myo-inositol supplementation, this may not entirely recapitulate the *in vivo* situation, where the placenta synthesizes its own myo-inositol, while also receiving exogenous supply from both mother and fetus (Quirk & Bleasdale 1983). Since the relative contribution of each myo-inositol source is not yet known, it remains uncertain whether maternal myo-inositol supplementation will substantially increase placental myo-inositol and how placental lipid metabolism would be affected. Moreover, our placental explant method limits the interpretation of our findings since it cannot examine transplacental FA transfers between the maternal and fetal compartments. Placental explants also comprise many different cell types and can only indicate the composite response of the entire tissue. Other complementary methods such as placental perfusion or *in vivo* studies will therefore be necessary to further investigate the effects of myo-inositol.

Conclusion

This study shows that myo-inositol induces FA-specific effects on upstream placental lipid processes such as FA uptake or activation. Myo-inositol supplementation may have the potential to alter placental lipid physiology across a wide range of lipid classes even in uncomplicated pregnancies, with as yet unknown clinical consequences. The magnitude of the myo-inositol effect appears to be associated with maternal glycaemia, BMI and fetal birthweight indicating that the placental lipid-processing effects of myo-inositol supplementation in pregnancy will likely depend on the population, consistent with the notion that placental metabolism is regulated by the maternal environment.

Declaration of interest

S Y Chan, N Karnani and K M Godfrey are part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone for work unrelated to this manuscript. K M Godfrey and S Y Chan are co-inventors on a pending patent, which covers the use of a novel inositol composition in human health applications. This invention is unrelated to the submitted work. The other authors have nothing to disclose.

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