Activation of AMPK improves inflammation and insulin resistance in adipose tissue and skeletal muscle from pregnant women

Running title: AMPK and gestational diabetes

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ABSTRACT

Gestational diabetes mellitus (GDM) is characterised by maternal peripheral insulin resistance and inflammation. Sterile inflammation and bacterial infection are key mediators of this enhanced inflammatory response. Adenosine monophosphate (AMP)-activated kinase (AMPK), which is decreased in insulin resistant states, possesses potent pro-inflammatory actions. There are, however, no studies on the role of AMPK in pregnancies complicated by GDM. Thus, the aims of this study were (i) to compare the expression of AMPK in adipose tissue and skeletal muscle from women with GDM and normal glucose tolerant (NGT) pregnant women; and (ii) to investigate the effect of AMPK activation on inflammation and insulin resistance induced by the bacterial endotoxin lipopolysaccharide (LPS) and the pro-inflammatory cytokine IL-1 β . When compared to NGT pregnant women, AMPK α activity was significantly lower in women with GDM as evidenced by a decrease in threonine phosphorylation of AMPK α . Activation of AMPK, using two pharmacologically distinct compounds, AICAR or phenformin, significantly suppressed LPS- or IL-1 β induced gene expression and secretion of pro-inflammatory cytokine IL-6, the chemokines IL-8 and MCP-1, and COX-2 and subsequent prostaglandin release from adipose tissue and skeletal muscle. In addition, activators of AMPK decreased skeletal muscle insulin resistance induced by LPS or IL-1 β as evidenced by increased insulin-stimulated phosphorylation of IRS-1, GLUT-4 expression and glucose uptake. These findings suggest that AMPK may play an important role in inflammation and insulin resistance.

Keywords: adipose tissue; skeletal muscle; GDM; AMPK; inflammation; infection; insulin resistance

INTRODUCTION

Gestational diabetes mellitus (GDM), defined as glucose intolerance of varying severity with onset or first recognition during pregnancy, affects up to 18% of all pregnant women. GDM is associated with increases in pregnancy complications and potential long-term consequences for both mother and child [5]. Pregnancy complications include higher rates of macrosomia, shoulder dystocia, birth trauma and Caesarean birth. Neonatal complications include hypoglycaemia, jaundice and respiratory distress. Long-term risks for both the mother and her child include increased rates of type 2 diabetes and cardiovascular disease later in life [5]. The health care and fiscal costs are enormous [12].

Inflammation is a central feature of pregnancies complicated by GDM [26]. Pro-inflammatory cytokines and bacterial infections are recognised as key mediators of this inflammation [19]. A myriad of pro- and anti-inflammatory cytokines, secreted from pregnant adipose tissue [18, 19] and skeletal muscle [18] are thought to play a central role in a pathogenesis and/or progression of GDM. Pregnancies complicated by GDM are also associated with maternal skeletal muscle insulin resistance [4]. The resultant increase in nutrient availability increases the risk of fetal fat accretion which predisposes the offspring of women with GDM to childhood obesity and later adult disease [5]. Little, however, is known about the cellular mechanisms responsible for this altered skeletal muscle insulin resistance and/or enhanced inflammation.

In non-pregnant tissues, adenosine monophosphate (AMP)-activated protein kinase (AMPK), has been implicated in modulating glucose metabolism and acute inflammatory reactions. AMPK is a serine/threonine protein kinase that consists of three heterogenic subunits, a catalytic (α) subunit and two regulatory subunits (β and γ); all of which exist in at least 2 isoforms. There is much evidence to demonstrate that AMPK regulates skeletal muscle glucose metabolism [9]. However,

recent data indicate that activation of AMPK might also be a useful strategy for beneficial regulation of the inflammatory responses. For example, *in vitro*, activation of AMPK is associated with inhibition of cytokine production in LPS-stimulated murine neutrophils [34], LPS-stimulated primary astrocytes, microglia, and peritoneal macrophages [8], and IL-1 β stimulated adipose tissue and skeletal muscles [21]. *In vivo*, activation of AMPK has been shown to inhibit the production of pro-inflammatory mediators in serum and their expression in central nervous system of rats injected with a sublethal dose of LPS [8]. Enhanced activation of AMPK also resulted in diminished severity of LPS-induced acute lung injury in mice [34].

Diminished AMPK activity or impairment of its activation has been observed in skeletal muscle of individuals with obesity and diabetes [28]. Similarly, in adipose tissue, AMPK activity is decreased in morbidly obese individuals who are insulin resistant than in comparably obese people who are insulin sensitive [31]. Recently, we have published that AMPK activity is lower in skeletal muscle obtained from women with GDM [1]. However, a limitation of these studies was that all women were obese and treated with insulin for optimal glucose control. In addition, there no studies that have determined the effect of GDM on the expression of AMPK in human pregnant adipose tissue. Thus, the aim of this study was to determine the AMPK protein expression in omental adipose tissue and skeletal muscle from non-obese women with diet-controlled GDM and compare them to BMI-matched normal glucose tolerant (NGT) women. In addition, the effect of AMPK activators on adipose tissue and skeletal muscle inflammation and skeletal muscle insulin resistance induced by endotoxemia (i.e. LPS) or the pro-inflammatory cytokine IL-1 β were also determined from NGT women. In this study, two well-described AMPK activators were used: AICAR (5-amino-1- β -D-ribofuranosylimidazole-4-carboxamide), an analogue of adenosine that enters cells and stimulates AMPK [22]; and an antidiabetic drug from the biguanide class, phenformin which activates AMPK by increasing cytosolic AMP concentration [33].

MATERIALS AND METHODS

78

Tissue collection and preparation

80 Human omental adipose tissue and skeletal muscle (pyramidalis) described in this study was
obtained (with the Research Ethics Committee of Mercy Health approval) from consenting women
82 who delivered healthy, singleton infants at term (>37 weeks gestation). Indications for Caesarean
section were breech presentation and/or previous Caesarean section. Tissues were obtained and
84 processed within 15 min of delivery.

86 Omental adipose tissue (n=12 patients) and skeletal muscle (n=12 patients) was obtained from non-
obese normal glucose tolerant (NGT) women and BMI-matched women with GDM. The clinical
88 details of the patients used in this study are detailed elsewhere [20]. Women with any underlying
medical conditions such as pre-existing diabetes, asthma, polycystic ovarian syndrome,
90 preeclampsia and macrovascular complications were excluded. Women with GDM were diagnosed
according to the criteria of the Australasian Diabetes in Pregnancy Society (ADIPS) by either a
92 fasting venous plasma glucose concentrations of ≥ 5.5 mmol/l glucose, and/or ≥ 8.0 mmol/l glucose 2
h after a 75 g oral glucose load at approximately 28 weeks gestation. All women with GDM were
94 managed by diet alone. All pregnant women were screened for GDM, and women participating in
the normal group had a negative screen. Tissues were thoroughly washed in ice-cold PBS to remove
96 any blood. Dissected fragments were stored at -80°C until assayed as detailed below.

Tissue explant culture

Tissue explants were performed to determine the effect of AMPK activators on inflammation in
100 pregnant adipose tissue and skeletal muscle. For these studies, adipose tissue and skeletal muscle
was obtained from non-obese NGT pregnant women, and tissue explants were performed as
102 previously described [18, 19]. Briefly, adipose tissue and skeletal muscle was finely diced and

placed in DMEM at 37°C in a humidified atmosphere of 21% O₂ and 5% CO₂ for 1 h. Tissues were
104 blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg for adipose
tissue and 50 mg for skeletal muscle). The explants were incubated in 1 ml DMEM containing 100
106 U/ml penicillin G and 100 µg/ml streptomycin. Tissues were incubated in the absence or presence
of 2 mM AICAR (AdooQ BioScience; Sapphire Bioscience, NSW, Australia) or 1 mM phenformin
108 (Cayman Chemical Co; Sapphire Bioscience, NSW, Australia) for 60 min before the addition of 10
µg/ml LPS (derived from Escherichia coli 026:B6; Sigma-Aldrich; St. Louis, MO, USA) or 1 ng/ml
110 IL-1β (PeproTech; Rocky Hill, NJ, USA) for 20 h. After final incubation, tissue and media were
collected separately and stored at -80°C for further analysis as detailed below. The concentrations of
112 AICAR and phenformin were based on previous studies in non-gestational tissues [8, 34]. Each
treatment was performed on tissues obtained from at least five patients.

114

To assess the effect of AMPK activators on the insulin signalling pathway, skeletal muscle explants
116 were performed as detailed above, however, after 20 h incubation, tissues were incubated with 0.1
µM insulin for 30 minutes. After final incubation, tissue was collected and assessment of insulin
118 signalling proteins performed by qRT-PCR or Western blotting as detailed below. Each treatment
was performed on tissues obtained from four patients.

120

Glucose uptake

122 Skeletal muscle explants were performed as detailed above and glucose uptake was performed as
previously described [19]. Briefly, after final incubation with treatment, tissues were pre-incubated
124 in the absence or presence of 20 µM cytochalasin B in Krebs buffer for 5 mins. 2-Deoxy-D-glucose
(2DG) uptake was measured by adding 3 µCi/ml [¹⁴C]-2DG (Perkin Elmer) and 1 mM 2DG to
126 Krebs buffer containing 0.5% BSA (fatty acid free) and 0.1 µM insulin for 20 min. Tissues were
then collected and washed three times in PBS, blotted dry on filter paper and the solubilised for 4 h
128 in 0.5 ml 1 M NaOH at 60°C. Tissues were neutralised with 0.5 ml 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
130 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
132 in the presence of 20 μM cytochalasin B. Fold change was calculated relative to LPS or IL-1 β ,
which was set at 1. Glucose uptake was performed on tissues obtained from four patients.

134

Western blotting

136 Tissue lysates and Western blotting were prepared as previously described [20]. Twenty
micrograms of protein was separated on polyacrylamide gels (Bio-Rad Laboratories; Gladesville,
138 NSW, Australia) and transferred to PVDF. Protein expression was identified by comparison with
the mobility of protein standard. Blots were incubated with 1 $\mu\text{g}/\text{ml}$ rabbit polyclonal
140 phosphorylated Thr172 AMPK α (sc-33524; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1
 $\mu\text{g}/\text{ml}$ mouse monoclonal AMPK α 1/2 (sc-74461; Santa Cruz Biotechnology, Santa Cruz, CA,
142 USA), 1 $\mu\text{g}/\text{ml}$ rabbit polyclonal phosphorylated (Tyr1229) IRS-1 (sc-17202; Santa Cruz
Biotechnology; Santa Cruz, CA, USA), 1 $\mu\text{g}/\text{ml}$ rabbit polyclonal IRS-1 (sc-560; Santa Cruz
144 Biotechnology; Santa Cruz, CA, USA), 2 $\mu\text{g}/\text{ml}$ rabbit polyclonal GLUT-4 (sc-7938; Santa Cruz
Biotechnology; Santa Cruz, CA, USA) diluted in blocking buffer (5% BSA in TBS with 0.05%
146 Tween-20) for 16 h at 4°C. Membranes were viewed and analysed using the XRS ChemiDoc
system (Bio-Rad Laboratories; Gladesville, NSW, Australia). Semi-quantitative analysis of the
148 relative density of the bands in Western blots was performed using Image Lab v3 analysis software
(Bio-Rad Laboratories; Gladesville, NSW, Australia). For Figures 1 and 2, the levels of
150 phosphorylated AMPK α were normalised to the levels of total AMPK α 1/2 and fold change was
calculated relative to the NGT group. For the explant studies, the levels of phosphorylated IRS-1
152 were normalised to the levels of total IRS-1 while the levels of GLUT-4 were normalised to the
levels of β -actin (1:20,000; A5316; Sigma-Aldrich; St. Louis, MO, USA). Fold change was
154 calculated relative to LPS or IL-1 β , which was set at 1.

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

Total RNA was extracted from tissues using TRIsure™ according to manufacturer's instructions
158 (Bioline, Alexandria, NSW, Australia). RNA concentration and purity were measured using a
NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific; Scoresby, Vic, Australia). RNA
160 was converted to cDNA the Tetro cDNA synthesis kit (Bioline, Alexandria, NSW, Australia)
according to the manufacturer's instructions. The cDNA was diluted fifty-fold, and 4 µl of this was
162 used to perform RT-PCR using SensiFAST™ SYBR (Bioline, Alexandria, NSW, Australia) and
100 nM of pre-designed and validated QuantiTect primers (Qiagen, Chadstone Centre, Vic,
164 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
166 average 18S Ct values of the same cDNA sample. Fold differences were determined using the
comparative Ct method. For the explant studies, there was a large variability in the response to LPS
168 or IL-1β which is normal for tissues derived from different patients. Thus, fold change was
calculated relative to LPS or IL-1β, which was set at 1.

170

Cytokine and prostaglandin immunoassays

172 The release of MCP-1, IL-6 and IL-8 was performed by sandwich ELISA according to the
manufacturer's instructions (Life Technologies; Mulgrave, Vic, Australia). The release of PGE₂ into
174 the incubation medium was assayed using a commercially available competitive enzyme
immunoassay kit according to the manufacturer's specifications (Kookaburra Kits from Sapphire
176 Bioscience; Waterloo, NSW, Australia). The limit of detection of the PGE₂ assay was 16 pg/ml. All
data were corrected for total protein and expressed as either pg or ng per mg protein. The protein
178 content of tissue homogenates was determined using BCA protein assay (Thermo Fisher Scientific;
Scoresby, Vic, Australia), using BSA as a reference standard, as previously described [18]. The
180 calculated interassay and intraassay coefficients of variation (CV) were all less than 10%.

182 **Statistical analysis**

Statistics was performed on the normalised data unless otherwise specified. All statistical analyses
184 were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For two sample
comparisons, an unpaired Student's t-test was used to assess statistical significance between
186 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
188 assessed by the Bartlett test, and when significant, the data were logarithmically transformed before
further analysis using a one-way ANOVA (using LSD correction to discriminate among the means).
190 Statistical significance was ascribed to *P* value <0.05. Data were expressed as mean $\pm$ standard
error of the mean (SEM).

192

RESULTS

AMPK α activity is decreased in adipose tissue and skeletal muscle from women with GDM

Adipose tissue and skeletal muscle was obtained from non-obese women with NGT or diet-controlled GDM. Demographic data of all participants involved in this study are detailed elsewhere [20]. There was no difference in maternal pre-pregnancy BMI, maternal BMI at delivery, maternal age, gestational weight gain, fetal birthweight, or gestation age at delivery. Fasting, one hour and two hour plasma glucose during the antenatal OGTT were significantly higher in women with GDM when compared to NGT pregnant women.

Western blotting was used to determine the expression of total AMPK α and AMPK α phosphorylated at Thr172 (p-AMPK α). The protein expression of p-AMPK α was significantly lower adipose tissue (Figure 1A) and skeletal muscle (Figure 1B) from women with GDM when compared to BMI-matched NGT women.

Activators of AMPK decrease LPS-induced pro-inflammatory cytokines and chemokines in adipose tissue and skeletal muscle

The above studies show that AMPK activity is decreased in adipose tissue and skeletal muscle of women with GDM. Thus, the next aim was to determine the effect of the AMPK activators on TLR4-induced expression and secretion of pro-inflammatory cytokines, chemokines and adhesion molecules. Of note, in all subsequent experiments in this study, adipose tissue and skeletal muscle were obtained from NGT pregnant women and stimulated with either LPS or IL-1 β as a model of GDM. The data for adipose tissue are presented in Figure 2 while the data for skeletal muscle are presented in Figure 3. In both tissues, treatment with LPS induced a significant increase in the mRNA expression and release of IL-6, IL-8 and MCP-1. Treatment with the AMPK activators AICAR or phenformin significantly suppressed LPS-induced cytokine and chemokine expression

and secretion. Of note, there was no effect of AMPK activators on cytokine release under basal
conditions (data not shown).

Activators of AMPK decrease IL-1 β -induced pro-inflammatory cytokines and chemokines in adipose tissue and skeletal muscle

The data for adipose tissue are presented in Figure 4 while the data for skeletal muscle are presented in Figure 5. As expected, IL-1 β significantly increased IL-6, IL-8 and MCP-1 gene expression and release from both adipose tissue and skeletal muscle. Co-incubation with AICAR or phenformin significantly attenuated IL-1 β -stimulated cytokine and chemokine gene expression and secretion.

Activators of AMPK decrease LPS- and IL-1 β -induced COX-2 expression and PGE₂ production in adipose tissue

Our previous studies have shown that LPS and IL-1 β can induce COX-2 expression and subsequent prostaglandin release from adipose tissue [20]. COX-2 and the prostaglandin PGE₂ have been shown to contribute to the pathophysiology of diabetes [24]. Therefore, the next aim was to determine the effect of AMPK on the COX-2-prostaglandin pathway in adipose tissue is presented in Figure 6. As expected, LPS and IL-1 β significantly induced COX-2 expression (Figures 6A,C) and PGE₂ secretion (Figures 6B,D). Co-incubation with AICAR or phenformin significantly suppressed both LPS and IL-1 β -induced activation of the COX-2-prostaglandin pathway. Of note, there was no effect of LPS or IL-1 β with or without AMPK activators on COX-1 expression (data not shown).

Activators of AMPK improve LPS- and IL-1 β induced defects in the insulin pathway

Skeletal muscle insulin resistance is a key feature of pregnancies complicated by GDM [4]. Thus, the final aim of this study was to determine the effect of AMPK activation on insulin signalling in the presence of LPS or IL-1 β . Both LPS (Figure 7) and IL-1 β (Figure 8) significantly attenuated

phosphorylated IRS-1 protein expression (Figures 7A,8A), GLUT-4 protein expression (Figures
246 7B,8B) and 2DG uptake (Figures 7C,8C). In contrast, co-incubation with AICAR and phenformin
in skeletal muscle and adipose tissues treated with either LPS or IL-1 β , restored phosphorylated
248 IRS-1 (Figures 7A,8A) and GLUT-4 protein expression (Figures 7B,8B) and 2DG uptake (Figures
7C,8C) to basal levels.

250

DISCUSSION

A novel finding of this study is that AMPK α activity is decreased in omental adipose tissue and skeletal muscle obtained from women with diet-controlled GDM when compared to BMI-matched NGT women. Moreover, activation of AMPK α could efficiently prevent the expression and release of pro-inflammatory mediators in omental adipose tissue and skeletal muscle that were stimulated with TLR4 ligand and bacterial product LPS and the pro-inflammatory cytokine IL-1 β . AMPK also improved glucose metabolism in skeletal muscle stimulated with LPS and IL-1 β . Collectively, these results show the importance of AMPK activation in improving inflammation and insulin resistance in pregnant adipose tissue and skeletal muscle.

In this study, threonine phosphorylation of AMPK α was lower in adipose tissue and skeletal muscle obtained from women with GDM who were managed by diet when compared to BMI-matched NGT pregnant women; thus, suggesting decreased AMPK α activity in pregnant GDM tissues. Of note, and in agreement of these findings, we have also recently reported lower threonine phosphorylation of AMPK α in skeletal muscle of obese GDM who were managed by insulin during pregnancy [1]. These findings are also in line with studies in non-pregnant individuals and in experimental animal models of diabetes. For example, studies in skeletal muscle [28] or adipose tissue [31] of obese, insulin-resistant humans show decreased AMPK activity or impaired activation than in comparably obese people who are insulin sensitive. Moreover, reduced AMPK activity has been observed in skeletal muscle of rodents with the metabolic syndrome, including Zucker diabetic fatty rats [32] and *ob/ob* mice [32]. Collectively, these findings show that insulin-resistant individuals, regardless of BMI, have decreased AMPK activity. The upstream mediators involved in the decrease in AMPK α in GDM adipose tissue and skeletal muscle are not known. However, a number of diabetes-related stimuli including high glucose, oxidative stress, inflammation, and fatty acids have been shown to diminish AMPK activity in rodents *in vivo* [27]. Nevertheless, the

findings of this study suggest that AMPK may thus play a role in the pathophysiology of insulin
278 resistance that is independent of obesity.

280 It should be noted that a strong point of this study is the well-defined study population that was
used for assessment of AMPK expression. Patients were excluded for maternal or clinical factors
282 which may influence the maternal inflammatory profile such as asthma, preeclampsia, pre-existing
diabetes, hypertension and smoking. In addition, tissues were obtained from women at the time of
284 term Caesarean section in the absence of labour which is an inflammatory state [16]. Importantly,
and in addition to including both lean and obese patients, patients were matched for gestational
286 weight gain. This is important as fasting and exercise have been reported to activate AMPK [2]. A
further strength of this study was the inclusion of tissues from GDM women controlled by diet
288 therapy only ensuring that the changes in AMPK expression were not due to insulin therapy.

290 In non-pregnant tissues, activation of AMPK has been shown to reduce inflammation in response to
a number of stimuli including LPS and IL-1 β [8, 34]. GDM is associated with enhanced
292 inflammation [19]. Both sterile inflammation and bacterial infection are thought to play a role in
mediating this inflammatory response. TNF- α induces the secretion of pro-inflammatory cytokine
294 expression in human placenta, and adipose tissue and skeletal muscle from pregnant women [18].
Similarly, bacterial products such as LPS, iE-DAP (a dipeptide present in the peptidoglycan of all
296 Gram-negative and certain Gram-positive bacteria) and muramyl dipeptide (MDP, the minimal
bioactive peptidoglycan motif common to all bacteria) can induce the gene expression and secretion
298 of a pro-inflammatory cytokines and chemokines from human placenta, and/or pregnant adipose
tissue and skeletal muscle [18]. Given that AMPK was decreased in adipose tissue and skeletal
300 muscle from women with GDM, it was of interest to determine if AMPK also regulates the
inflammatory response in these tissues. Inflammation was induced by the TLR4 ligand bacterial
302 endotoxin LPS and the pro-inflammatory cytokine IL-1 β . In this study, the AMPK activators

AICAR or phenformin decreased the expression and secretion of the pro-inflammatory cytokines
304 IL-6, IL-8 and MCP-1 in adipose tissue and skeletal muscle stimulated with LPS or IL-1 β . To our
knowledge, this is the first study to report AMPK regulates inflammation in skeletal muscle and
306 adipose tissue.

308 Pro-inflammatory cytokines play a myriad of roles in pregnancy and GDM. They may induce
alterations in placental transport that are observed in GDM pregnancies. In keeping with this, IL-6
310 stimulates trophoblast fatty acid accumulation [17] and increases trophoblast System A amino acid
transporter activity and expression [14]. Pro-inflammatory cytokines may also induce the peripheral
312 insulin resistance observed in women with GDM [4]. Indeed, previous studies have shown that IL-
1 β , which is increased in women with GDM [19], interferes with the insulin signalling pathway in
314 pregnant adipose tissue [19]. Collectively, increased peripheral insulin resistance and/or increased
placental nutrient transport may contribute to the fetal overgrowth and or increased fat deposition
316 observed in infants of women with GDM [6].

318 In addition to its role in regulating inflammation, AMPK may affect key metabolic processes,
including skeletal muscle glucose metabolism [9]. For example, in skeletal muscle AMPK
320 activation leads to increase GLUT4 transcription [35]. In insulin resistant rats, activation of AMPK
improved insulin sensitivity [13]. GLUT4 is an important transporter channel for glucose uptake by
322 cells. Skeletal muscle insulin resistance is a central feature of pregnancies complicated by GDM [4].
Thus, in order to further understand the role of AMPK in the pathophysiology of GDM, the effect
324 of the AMPK activators AICAR and phenformin on the insulin signalling pathway in skeletal
muscle was determined. Both IL-1 β and LPS attenuated phosphorylated IRS-1 and GLUT-4 protein
326 expression and 2DG uptake in skeletal muscle. Activation of AMPK with AICAR and phenformin
subsequently rescued these defects in the insulin pathway induced by IL-1 β and LPS. Thus, it is

328 possible that impaired insulin signalling and glucose uptake in pregnant skeletal muscle may be
associated with reduced or defective AMPK activation.

330

COX-2 and prostaglandins may also play a role in the pathophysiology of diabetes [24]. For
332 example, *in vivo* studies have demonstrated inhibition of COX-2 prevents the onset of diabetes in
mice [29]. Other studies have also shown COX-2 and prostaglandins to promote inflammation, and
334 more specifically, to induce IL-6 production [11]. It has been demonstrated in hepatocytes that
prostaglandins, in particular PGE₂, blunts the insulin signalling pathway via ERK1/2-dependent
336 serine phosphorylation of insulin receptor substrates [10]. Thus, it was also of interest to determine
the role of AMPK in regulating the expression of COX-2 and the secretion of the prostaglandin
338 PGE₂. In keeping with studies in non-pregnant tissues [7, 25], both LPS and IL-1 β increased COX-2
mRNA expression and PGF_{2 α} secretion in adipose tissue. This increase was significantly decrease
340 by pre-treatment with the AMPK activators AICAR or phenformin. In concordance with this study,
other studies have also shown AMPK activation is associated with decreased production of COX-2
342 and prostaglandins [3]. To our knowledge, however, this is the first study to report that AMPK
regulates COX-2-prostaglandin pathway in adipose tissue.

344

Recent studies suggest a link between AMPK and IL-1 β production via the inflammasome [30].
346 Specifically, this study found that activation of the inflammasome, using palmitate-BSA plus LPS,
decreased phosphorylation of the AMPK α subunit while increasing caspase-1 mediated IL-1 β
348 production. This decrease in AMPK α and increase in IL-1 β was significantly attenuated using the
AMPK activator AICAR. It is interesting to note that we have recently described increased
350 activation of the inflammasome in adipose tissue of women with GDM [19]. In these studies, we
also showed that activation of the inflammasome was required to activate caspase-1 mediated IL-1 β
352 production in pregnant adipose tissue. Furthermore, treatment of pregnant adipose tissue with IL-1 β
resulted in a decrease in insulin stimulated glucose uptake, suggesting that IL-1 β may contribute to

354 the pathophysiology of GDM. It is thus possible that AMPK may also regulate caspase-1 activation
of IL-1 β in adipose tissue.

356

Limitations of this study includes the effect of the AMPK activation on attenuating pro-
358 inflammatory cytokine and insulin resistance was not assessed in adipose tissue and skeletal muscle
obtained from women with GDM. In this study, LPS and IL-1 β were used to model a GDM
360 environment. Thus, the findings of this study should be interpreted with some caution. Furthermore,
the pathway(s) involved in AMPK-induced suppression of inflammation and insulin sensitivity
362 remains yet to be elucidated. Recent studies show AICAR and phenformin inhibits LPS-induced
NF- κ B signalling and also the transcription of NF- κ B-dependent genes [15]. NF- κ B is a
364 transcription factor that regulates the expression of pro-inflammatory mediators and has been
described to play an important role in the pathogenesis of diabetes and its associated complications
366 [23]. Thus, further work is required to determine how AMPK and its activators regulate NF- κ B.

368 In conclusion, the data presented in this study show that AMPK α activity is decreased in omental
adipose tissue and skeletal muscle obtained from women with GDM. Activation of AMPK, using
370 two pharmacologically distinct compounds, significantly suppressed LPS- or IL-1 β induced gene
expression and secretion of pro-inflammatory cytokines, chemokines, and prostaglandins from
372 adipose tissue and skeletal muscle. AMPK activation also suppressed LPS- or IL-1 β -induced insulin
resistance and improved glucose uptake by skeletal muscle. Inflammation, by further inciting
374 inflammation and/or inducing insulin resistance, plays a central in the pathophysiology of GDM.
Thus, it would be of interest to determine the effect of AMPK activation on inflammation and
376 glucose metabolism in animal models of GDM. Further studies on the role of AMPK in GDM are
warranted.

378

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392

DISCLOSURE SUMMARY

394 The author has nothing to declare.

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FIGURE LEGENDS

Figure 1. AMPK α activity is lower in adipose tissue and skeletal muscle from GDM women

(A) Omental adipose tissue and (B) skeletal muscle was obtained from non-obese women with NGT (n=6 patients per group) and diet-controlled GDM (n=6 patients per group) at the time of term Caesarean section. Phosphorylation of AMPK α at Thr172 (p-AMPK α) was analysed by immunoblotting and normalised to total AMPK α 1/2 protein expression. The fold change was calculated relative to NGT and data is displayed as mean $\pm$ SEM. * P <0.05 vs. NGT (Student's t-test). Representative Western blot from 2 NGT and 2 GDM patients is also shown.

Figure 2. Effect of AMPK activators on LPS-induced pro-inflammatory cytokines and chemokines in adipose tissue

Human omental adipose tissue from NGT pregnant women was incubated with 10 μ g/ml LPS in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h (n=6 patients). (A-C) Gene expression for IL-6, IL-8 and MCP-1 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to LPS. Data displayed as mean $\pm$ SEM. * P <0.05 vs. LPS (one-way ANOVA). (D-F) The incubation medium was assayed for concentration of IL-6, IL-8 and MCP-1 by ELISA. Data displayed as mean $\pm$ SEM. * P <0.05 vs. LPS (one-way ANOVA).

Figure 3. Effect of AMPK activators on LPS-induced pro-inflammatory cytokines and chemokines in skeletal muscle

Human skeletal muscle from NGT pregnant women was incubated with 10 μ g/ml LPS in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h (n=6 patients). (A-C) Gene expression for IL-6, IL-8 and MCP-1 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to LPS. Data

displayed as mean $\pm$ SEM. * P <0.05 vs. LPS (one-way ANOVA). **(D-F)** The incubation medium was assayed for concentration of IL-6, IL-8 and MCP-1 by ELISA. Data displayed as mean $\pm$ SEM. * P <0.05 vs. LPS (one-way ANOVA).

Figure 4. Effect of AMPK activators on IL-1 β -induced pro-inflammatory cytokines and chemokines in adipose tissue

Human omental adipose tissue from NGT pregnant women was incubated with 1 ng/ml IL-1 β in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h (n=5 patients). **(A-C)** Gene expression for IL-6, IL-8 and MCP-1 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to IL-1 β . Data displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β (one-way ANOVA). **(D-F)** The incubation medium was assayed for concentration of IL-6, IL-8 and MCP-1 by ELISA. Data displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β (one-way ANOVA).

Figure 5. Effect of AMPK activators on IL-1 β -induced pro-inflammatory cytokines and chemokines in skeletal muscle

Human skeletal muscle from NGT pregnant women was incubated with 1 ng/ml IL-1 β in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h (n=5 patients). **(A-C)** Gene expression for IL-6, IL-8 and MCP-1 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to IL-1 β . Data displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β (one-way ANOVA). **(D-F)** The incubation medium was assayed for concentration of IL-6, IL-8 and MCP-1 by ELISA. Data displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β (one-way ANOVA).

Figure 6. Effect of AMPK activators on LPS- and IL-1 β -induced COX-2 expression and prostaglandin release in adipose tissue

Human adipose tissue from NGT pregnant women was incubated with (A,B) 10 µg/ml LPS or (C,D) 1 ng/ml IL-1β in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h (n=6 patients). (A,C) Gene expression for COX-2 was analysed by qRT-PCR. Gene expression was normalised to 18S mRNA expression and the fold change was calculated relative to LPS or IL-1β. Data displayed as mean ± SEM. **P*<0.05 vs. LPS (one-way ANOVA); ***P*<0.05 vs IL-1β (one-way ANOVA). (B,D) The incubation medium was assayed for concentration of PGE₂ by EIA. Data displayed as mean ± SEM. **P*<0.05 vs. LPS (one-way ANOVA); ***P*<0.05 vs IL-1β (one-way ANOVA).

Figure 7. Effect of AMPK activators on LPS-induced insulin resistance in skeletal muscle

Human skeletal muscle from NGT pregnant women was incubated with 10 µg/ml LPS in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h followed by a 30 min incubation with 0.1 µM insulin (n=4 patients). (A) Phosphorylated IRS-1 (pIRS-1) protein expression was normalised to total IRS-1, and the fold change was calculated relative to LPS. Data is presented as mean ± SEM. **P*<0.05 vs. LPS (one-way ANOVA). A representative Western blot from 1 patient is also shown. (B) GLUT-4 protein expression was normalised to β-actin, and the fold change was calculated relative to LPS. Data is presented as mean ± SEM. **P*<0.05 vs. LPS (one-way ANOVA). A representative Western blot from 1 patient is also shown. (C) 2DG uptake data is displayed as mean ± SEM. **P*<0.05 vs. LPS (one-way ANOVA).

Figure 8. Effect of AMPK activators on IL-1β-induced insulin resistance in skeletal muscle

Human skeletal muscle from NGT pregnant women was incubated with 1 ng/ml IL-1β in the absence or presence of 2 mM AICAR or 1 mM phenformin (Phen) for 20 h followed by a 30 min incubation with 0.1 µM insulin (n=4 patients). (A) Phosphorylated IRS-1 (pIRS-1) protein expression was normalised to total IRS-1, and the fold change was calculated relative to IL-1β. Data is presented as mean ± SEM. **P*<0.05 vs. IL-1β (one-way ANOVA). A representative Western blot

from 1 patient is also shown. **(B)** GLUT-4 protein expression was normalised to β -actin, and the fold change was calculated relative to IL-1 β . Data is presented as mean $\pm$ SEM. * P <0.05 vs. IL-1 β (one-way ANOVA). A representative Western blot from 1 patient is also shown. **(C)** 2DG uptake data is displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β (one-way ANOVA).

Figure 1

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