SAGE Publishing

Page Proof Instructions and Queries

Thank you for choosing to publish with us. This is your final opportunity to ensure your article will be accurate at publication. Please review your proof carefully and respond to the queries using the circled tools in the image below, which are available **by clicking "Comment"** from the right-side menu in Adobe Reader DC.*

Please use *only* the tools circled in the image, as edits via other tools/methods can be lost during file conversion. For comments, questions, or formatting requests, please use [T]. Please do *not* use comment bubbles/sticky notes.

Comment ▾ ⬤ ✏ T T̄ T̄₌ T̄ᴬ T [T] ✏ ⌫ 🖊▾ 🔗▾ 📐▾

*If you do not see these tools, please ensure you have opened this file with **Adobe Reader DC**, available for free at get.adobe.com/reader or by going to Help > Check for Updates within other versions of Reader. For more detailed instructions, please see us.sagepub.com/ReaderXProofs.

No.	Query
	Please note, only ORCID iDs validated prior to acceptance will be authorized for publication; we are unable to add or amend ORCID iDs at this stage.
	Please confirm that all author information, including names, affiliations, sequence, and contact details, is correct.
	Please review the entire document for typographical errors, mathematical errors, and any other necessary corrections; check headings, tables, and figures.
	Please ensure that you have obtained and enclosed all necessary permissions for the reproduction of artworks (e.g. illustrations, photographs, charts, maps, other visual material, etc.) not owned by yourself. Please refer to your publishing agreement for further information.
	Please note that this proof represents your final opportunity to review your article prior to publication, so please do send all of your changes now.
	Please confirm that the Acknowledgement, Funding and Conflict of Interest statements are accurate.
1	Please check whether the inserted expansion for 'TP' is correct.
2	Please provide the manufacturer location details for 'Sigma' throughout the article.
3	Please provide the manufacturer location details for 'Santa Cruz' and 'Abcam'.
4	Please provide the manufacturer location details for 'LI-COR'.
5	Please check whether Table 1 is correct as set.
6	Please provide in-text citation for Figure 8.

Impaired vasocontractile responses to adenosine in chorionic vessels of human term placenta from pregnant women with pre-existing and gestational diabetes

Azlina A Razak[1,2], Lopa Leach[1] and Vera Ralevic[1] (iD)

Abstract

Background: There is clinical and experimental evidence for altered adenosine signalling in the fetoplacental circulation in pregnancies complicated by diabetes, leading to adenosine accumulation in the placenta. However, the consequence for fetoplacental vasocontractility is unclear. This study examined contractility to adenosine of chorionic vessels from type 1 diabetes mellitus, gestational diabetes mellitus and normal pregnancies.

Methods: Chorionic arteries and veins were isolated from human placenta from normal, gestational diabetes mellitus and type 1 diabetes mellitus pregnancies. Isometric tension recording measured responses to adenosine and the thromboxane A_2 analogue U46619 (thromboxane A_2 mediates adenosine fetoplacental vasoconstriction). Adenosine and thromboxane prostanoid receptor protein expression was determined by immunoblotting.

Results: Adenosine elicited contractions in chorionic arteries and veins which were impaired in both gestational diabetes mellitus and type 1 diabetes mellitus. Contractions to potassium chloride were unchanged. Adenosine A_{2A} and A_{2B} receptor protein levels were not different in gestational diabetes mellitus and normal pregnancies. Contractions to U46619 were unaltered in gestational diabetes mellitus arteries and increased in type 1 diabetes mellitus arteries. Overnight storage of vessels restored contractility to adenosine in gestational diabetes mellitus arteries and normalized contraction to U46619 in type 1 diabetes mellitus arteries.

Conclusion: These data are consistent with the concept of aberrant adenosine signalling in diabetes; they show for the first time that this involves impaired adenosine contractility of the fetoplacental vasculature.

Keywords

Adenosine, chorionic vessels, gestational diabetes, human placenta, vasoconstriction

Background

Diabetes mellitus (DM) during pregnancies, maternal diabetes, is recognized as a hostile environment for the development of the embryo and fetoplacental circulation. It can result in foetal morbidity and, later, an increased risk of cardiovascular diseases.[1,2] Maternal diabetes can be divided into pregestational and gestational diabetes mellitus (GDM). Pregestational DM refers to women previously diagnosed with either type 1 or type 2 diabetes mellitus (T1DM and T2DM, respectively) prior to the pregnancies. GDM, however, is defined as any degree of glucose intolerance with the onset of pregnancy. Optimal utero-placental and feto-placental blood flow, that is, efficient placental perfusion throughout gestation, is essential for oxygen and nutrient transport to the developing foetus. Human placenta at term from both pregestational type 1 and gestational diabetes have been shown to exhibit histopathological changes such as placental immaturity and abnormalities in both foetal and maternal placental vasculature.[3–8] While placentae from pregnancies complicated by T1DM exhibited increased angiogenesis and leaky blood vessels with loss of key junctional adhesion molecules, GDM placentae were also found to show less severe, but still statistically significant downregulation of endothelial cell–cell junctional molecules.[5,6]

[1]School of Life Sciences, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham, UK
[2]Faculty of Medicine & Health Sciences, UCSI University, Kuala Lumpur, Malaysia

Corresponding author:
Vera Ralevic, School of Life Sciences, University of Nottingham, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK.
Email: vera.ralevic@nottingham.ac.uk

The human fetoplacental unit (umbilical cord and foetal placental vessels) is known to be devoid of nerves; therefore, only non-nervous mechanisms can influence vascular tone and blood flow in this tissue.[9] Adenosine has been identified as one of the autacoids that is actively released in the foetal circulation and placental tissue of humans and other mammals,[10–14] and there is evidence that its signalling is altered in pregnancies complicated by diabetes. A higher adenosine concentration in venous umbilical blood, but not in arterial umbilical blood, was detected in pregnancies with GDM compared to normal pregnancies, suggesting an accumulation of extracellular adenosine from the placenta.[15] The extracellular concentration of adenosine in human umbilical vein endothelial cell (HUVEC) cultures from GDM was also higher than in HUVECs from normal pregnancies, and evidence was presented that this involved reduced human equilibrative nucleoside transporter 1 (hENT1)-mediated adenosine uptake.[16–19]

Very little is known about the effect of diabetes on the fetoplacental vasomotor response to adenosine. The only report to date used human umbilical vein, the largest fetoplacental vessel, and showed that endothelium-dependent relaxation to adenosine involving A_{2A} receptors was impaired in GDM.[19] Adenosine can also mediate contraction of the human placental vasculature (placental cotyledons and chorionic arteries and veins) via activation of A_{2B} receptors following synthesis of thromboxane A_2 or a related metabolite and subsequent activation of prostanoid thromboxane prostanoid (TP) receptors.[20,21] In this study, **[AQ: 1]** responses to adenosine of chorionic vessels (arteries and veins) from type 1 and gestational diabetes were compared to those of normal pregnancies. The selectivity of any changes was assessed by investigation of vasocontractile responses to a thromboxane A_2 analogue, U46619, and smooth muscle contractility of the placental vessels was assessed using a receptor-independent vasoconstrictor, potassium chloride (KCl). Adenosine and TP receptor protein expression levels were measured using immunoblotting. In light of the evidence for increased placental adenosine levels in maternal diabetes,[15] we also sought to investigate whether any changes in contractility to adenosine of the chorionic vessels could be recovered after an extended period in the absence of exposure to extracellular adenosine, namely, by overnight storage in physiological solution.

Methods

Human placenta samples

This study was based on pregnancies monitored at Queen's Medical Centre (QMC), Nottingham, UK, and had the approval of the QMC and University Hospital NHS Trust Ethics Committee (Ref: 0G010101; PI-LL). The study was carried out in accordance with the declaration of Helsinki.

Pregnant women with singleton pregnancy scheduled for elective Caesarean section were approached for written informed consent. The study groups included pregnant women who had pre-existing type 1 diabetes (T1DM), those diagnosed as having gestational diabetes (GDM) in the second trimester by either a fasting plasma glucose level of $\geqslant 5.6$ mmol/L or a 2-h plasma glucose level of $\geqslant 7.8$ mmol/L[22] and those who had normal, uncomplicated term pregnancies. Placentae were taken from elective sections only in order to avoid any parturition signals that may alter vascular reactivity. Pregnant women with asthma, smokers and pregnancies with other maternal or foetal conditions complicating the pregnancies (hypertension, preeclampsia and placenta praevia) were excluded.

Placentae taken were from full-term ($>$37 weeks) pregnancies. No significant difference was found in placental weight or birth weight between the three study groups. The GDM group was treated with metformin (3) or diet (4), while all T1DM were insulin treated. HbA1 were $<6.8\%$ in the diabetic groups.

Placentae (with cord clamped) were brought to the laboratory within 30 min of the delivery and transferred to a dissecting bath with physiological salt solution (PSS), which had been gassed with 5% CO_2 in air and adjusted to pH 7.4. The composition of the PSS was (mM): 127.76 NaCl, 25 $NaHCO_3$, 4.69 KCl, 2.4 $MgSO_4$, 1.18 KH_2PO_4, 6.05 glucose, 0.034 ethylenediaminetetraacetic acid (EDTA) and 1.6 $CaCl_2$. One superficial chorionic artery and one chorionic vein were carefully dissected from each placenta. Segments were derived between first- and third-order branches of chorionic vessels (diameter 1–2 mm). Two vessel ring segments (length ~0.5 cm) were obtained from each artery and vein from each placenta. The ring segments were used both as fresh tissues and after overnight-storage. The purpose of the overnight storage was to determine whether an extended washout could affect adenosine-mediated contractility. A total of 20 human term placenta from normal ($n=8$), GDM ($n=7$) and T1DM ($n=5$) pregnancies were prepared for the contractility studies. The fresh ring segments were quickly set up in organ baths for contractility studies, while overnight-stored ring segments were kept at 4°C in PSS containing 2% Ficoll (type 7000, Sigma).[23] **[AQ: 2]**

Contractility studies

Ring segments were suspended in a jacketed organ bath filled with PSS, gassed with 5% CO_2 in air and maintained at 37°C. The segments were suspended between two wires passed through the vessel lumen. The lower wire was fixed to a glass supporting rod, while the upper wire was connected via thread to a force transducer (Lectro-Med, Letchworth, UK) for isometric tension recording, linked to a PowerLab data acquisition unit (AD Instruments Ltd, Hastings, UK) via an amplifier. Segment rings were put
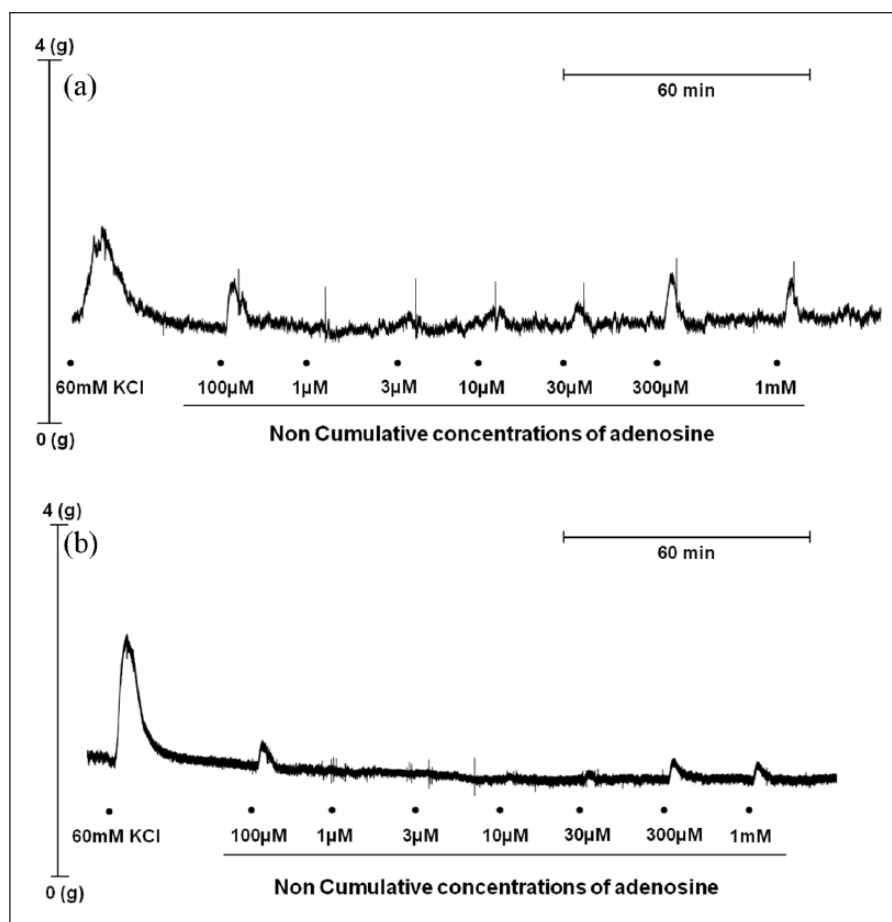
Figure 1. Representative traces showing contractions to adenosine of isolated chorionic arteries from placenta of (a) normal and (b) gestational diabetes (GDM) pregnancies. Adenosine was added as non-cumulative applications at concentrations of 1 μM–1 mM. Adenosine was washed out as soon as the contractile response had plateaued (4–5 min), and this was followed by an ~20-min recovery period before the next concentration was added.

under tension of 2.0 g for 45 min equilibration, with PSS washout every 15 min, and allowed to relax to a resting tension of around 1.0–1.5 g.[21,24]

Segment rings were initially contracted with two to three 60-mM KCl applications, with washout, at ~20-min intervals. The last KCl response was used for analysis. After the final KCl application was washed out, the tissue was rested for 15–30 min until it reached its resting tension of 1.0–1.5 g.[21,24] Preliminary studies showed that contractile responses to adenosine of the chorionic vessels rapidly desensitize, thus cumulative concentration-response curves could not be carried out. Thus, adenosine was initially applied to the vessel segments at a concentration of 100 μM as this consistently produced a robust and reproducible response which could be used to initially assess tissue responsiveness to adenosine. The adenosine was rapidly washed out as soon as a peak contraction was reached (about 3–5 min) and then left for 20 min before the next adenosine application. This was followed by the non-cumulative addition of adenosine (1 μM–1 mM) to elicit concentration-dependent contractions of chorionic vessels[21] (the order of

adenosine applications is shown in Figure 1). Lower concentrations of adenosine have been reported in blood from human umbilical artery and vein (~0.2–0.7 μM).[15] Contractions were evoked within 3–5 min of adenosine applications, and once the maximal contraction was reached, the tissues were rapidly washed. Applications were at 20-min intervals to minimize desensitization of the adenosine-induced contraction. At the end of the experiment, another 30 min was allowed before the cumulative addition of incremental doses (1 nM–30 μM) of U46619 at 6- to 10-min intervals.

Receptor quantification

Frozen chorionic vessel segments from GDM and normal groups were quantified for adenosine receptor subtypes (A_{2A}, A_{2B} and A_3), and TP receptors by immunoblotting using standard methods.[25] The antibodies used for the receptor quantifications were as follows: anti-A_{2A} antibody (rabbit polyclonal AA 373-391, Antibodies-online. com, 1:1000) tested positive against rat striatum, anti-A_{2B}

antibody (goat polyclonal sc-7505, Santa Cruz, 1:500) tested positive against pig bladder, anti-A_3 antibody (rabbit polyclonal A4229, Sigma, 1:1000) tested positive against A_3-transfected Chinese hamster ovary (CHO) cells and rabbit polyclonal anti-thromboxane (TBX)A_2 (rabbit monoclonal ab134959, Abcam, 1:500) antibody tested positive against pig heart. Full immunoblots and positive controls are shown in Supplementary Figures 1–3. A_{2B} receptor secondary antibody used was IRDye 800CW donkey anti-goat polyclonal (925-32214, LI-COR). A_{2A} and A_3 receptor secondary antibody was goat anti-rabbit 800CW (925-32211, LI-COR). Three different antibodies against the A_1 receptor, from Sigma, Abcam and Alomone, were used but due to their lack of specificity, the samples were not quantified for A_1 receptor expression. GAPDH was used (mouse monoclonal G8795, Sigma); secondary antibody used was IRDye 680CW goat anti-mouse (926-68070, LI-COR).

[AQ: 3]

[AQ: 4]

Data quantification and statistical analysis

Contractile responses were recorded in grammes of tension. Contractile responses to adenosine and U46619 were expressed as a percentage of the final KCl response and plotted as concentration-response curves. All data are presented as mean $\pm$ standard error of mean (s.e.mean) with number of placentae (*n*). Prism software version 6.04 (GraphPad Software, USA) was used to plot graphs and for statistical analysis. From concentration-response curves of each vessel segment, the agonist concentration producing 50% of the maximal response (EC_{50}) and maximal response (R_{max}) were determined. Concentration-response curves between study groups were tested using two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. EC_{50} and R_{max} were tested using one-way ANOVA followed by Fisher's LSD test. In a few vessel segments, response curves to adenosine and U46619 did not plateau so R_{max} and EC_{50} values could not be derived, and this accounts for the different *n* numbers in the figures and their corresponding tables. Immunoblotting data were analysed using Student's unpaired *t*-test. Significance was established by a *p*-value < 0.05.

Results

KCl contractions of chorionic vessels are similar in GDM and T1DM and after overnight storage

Contractile responses to KCl (60 mM) of arteries and veins were not significantly different between the control vessels, GDM and T1DM ($p > 0.05$, ANOVA) (Table 1). Given the evidence for an accumulation of adenosine from the placenta,[15] some vessels were stored in Krebs solution overnight, to determine whether this extended washout

Table 1. Contractile responses to KCl (60 mM) of chorionic arteries and veins from placentas of normal, gestational diabetes mellitus (GDM) and type 1 diabetes mellitus (T1DM) pregnancies. Data are expressed as mean $\pm$ s.e.mean.　[AQ: 5]

	Freshly collected	Overnight-stored
Chorionic arteries		
Normal	0.93 $\pm$ 0.08 g (*n* = 8)	0.78 $\pm$ 0.09 g (*n* = 6)
GDM	1.46 $\pm$ 0.22 g (*n* = 7)	0.99 $\pm$ 0.14 g (*n* = 7)
T1DM	1.00 $\pm$ 0.19 g (*n* = 5)	1.16 $\pm$ 0.21 g (*n* = 5)
Chorionic veins		
Normal	1.28 $\pm$ 0.19 g (*n* = 8)	1.40 $\pm$ 0.22 g (*n* = 6)
GDM	1.51 $\pm$ 0.20 g (*n* = 7)	1.33 $\pm$ 0.23 g (*n* = 7)
T1DM	1.84 $\pm$ 0.44 g (*n* = 5)	1.48 $\pm$ 0.38 g (*n* = 5)

period affected adenosine-mediated contractility. Overnight storage had no significant effect on contractile responses to KCl of the arteries and veins of normal, GDM and T1DM vessels compared to their corresponding fresh vessels ($p > 0.05$, ANOVA). Thus, the contractile machinery of the vascular smooth muscle of chorionic arteries and veins is fundamentally unimpaired by maternal diabetes.

Adenosine contractions are impaired in GDM arteries and veins

Adenosine (1 μM–1 mM) elicited concentration-dependent contractions in the chorionic arteries and veins [Figures 1, 2(a) and 3(a)] as reported by others.[21] Contractions to adenosine of fresh chorionic arteries from GDM were smaller than those of the normal arteries ($p < 0.0001$, ANOVA) [Figure 2(a)]. Contractile responses of chorionic veins were also smaller in freshly prepared GDM compared to normal veins ($p < 0.001$; ANOVA) [Figure 3(a)]. After overnight storage, there was an increase in responses to adenosine in GDM arteries, and the difference in contractions to adenosine between the GDM and normal arteries was abolished [Figure 2(b)]. After overnight storage contractions to adenosine remained smaller for chorionic veins from GDM compared to those of the normal vessels ($p < 0.01$; ANOVA) [Figure 3(b)]. Thus, adenosine contractility is impaired in chorionic arteries and veins in GDM, and overnight storage reverses the impaired contractility in chorionic arteries, but not veins.

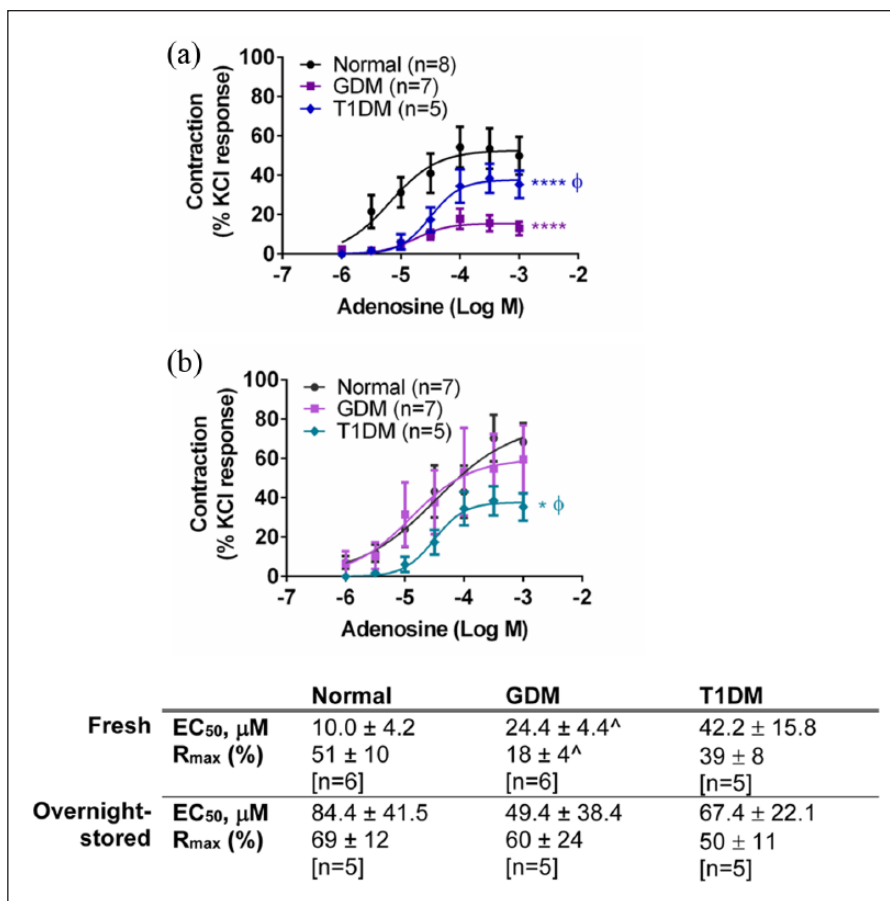### Adenosine contractions are impaired in T1DM arteries and veins

Contractions to adenosine in the chorionic arteries from T1DM were smaller than those of the arteries of normal pregnancies ($p < 0.0001$, ANOVA) [Figure 2(a)]. Contractile responses to adenosine of the chorionic veins were also smaller between the T1DM and normal groups ($p < 0.05$,

Figure 2. Response to adenosine in (a) fresh and (b) overnight-stored chorionic arteries from gestational diabetes mellitus (GDM), type 1 diabetes mellitus (T1DM) and normal pregnancies. Table indicates potencies (EC_{50}) and maximum response to adenosine (R_{max}). Values are mean $\pm$ s.e.mean with the number of placentae (n) shown in parentheses. % of contraction compared to potassium chloride (KCl, 60 mM).
*$p < 0.05$; ****$p < 0.0001$ compared to normal; $\phi p < 0.05$ compared to GDM, two-way ANOVA; ^$p < 0.01$ compared to normal, one-way ANOVA.

ANOVA) [Figure 3(a)]. After overnight storage, the significant difference in contractions to adenosine between the T1DM and normal arteries was still apparent ($p < 0.05$, ANOVA) [Figure 2(b)]. After overnight storage, the responses to adenosine remained smaller in T1DM compared to normal veins, although not significantly so ($p > 0.05$, ANOVA) [Figure 3(b)]. Thus, contractile responses to adenosine are impaired in both chorionic arteries and veins in T1DM, and this is not fundamentally altered by overnight storage.

Effect of GDM on vasocontractile responses to U46619

U46619 (1–300 nM) elicited concentration-dependent contractions in the chorionic arteries and veins (Figures 4 and 5). Contractile responses to U46619 were similar between GDM and normal groups in the arteries [Figure 4(a)] ($p > 0.05$, ANOVA) and were reduced in the GDM veins ($p < 0.0001$, ANOVA) [Figure 5(a)]. After overnight

storage, responses to U46619 in the chorionic arteries (normal and GDM) were increased overall [see Figure 4(a) and (b)]. However, the responses to U46619 in the arteries between GDM and normal groups remained similar [Figure 4(b)] ($p > 0.05$, ANOVA). After overnight storage, contractile responses to U46619 in chorionic veins also remained smaller compared to the normal veins ($p < 0.01$, ANOVA) [Figure 5(b)].

U46619 contractions are augmented in T1DM arteries and veins

Contractile responses to U46619 of the chorionic arteries were larger in the T1DM group compared to normal and GDM ($p < 0.0001$, ANOVA) with a significantly greater R_{max} in the T1DM group compared to the normal and GDM groups ($p < 0.05$, ANOVA) [Figure 4(a)]. Contractile responses to U46619 in chorionic veins were also greater in T1DM compared to the normal group ($p < 0.01$, ANOVA) [Figure 5(a)]. Overnight storage normalized the
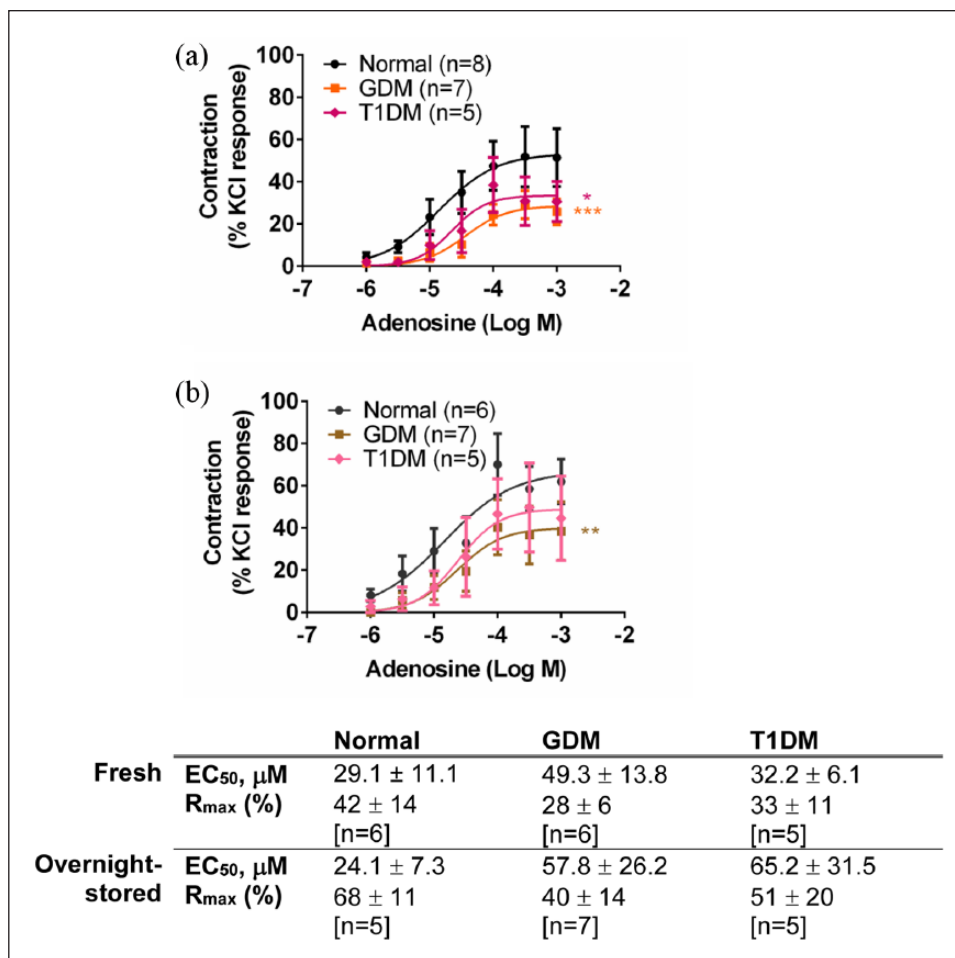
Figure 3. Response to adenosine in (a) fresh and (b) overnight-stored chorionic veins from gestational diabetes mellitus (GDM), type 1 diabetes mellitus (T1DM) and normal pregnancies. Table indicates potencies (EC_{50}) and maximum response to adenosine (R_{max}). Values are mean $\pm$ s.e.mean for number of placentae (*n*) shown in parentheses. % of contraction compared to potassium chloride (KCl, 60 mM).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to normal, two-way ANOVA.

differences in the responses between the normal and T1DM chorionic arteries, such that responses to U46619 were no longer significantly different between the two groups ($p > 0.05$, ANOVA) [Figure 4(b)]. After overnight storage, the responses to U46619 were slightly reduced in the veins in T1DM compared to the normal group ($p < 0.001$, ANOVA) [Figure 5(b)].

Immunoblotting for receptor quantification in normal and GDM vessels

Expression levels of A_{2A}, A_{2B} and A_3 receptor protein were investigated using immunoblotting in normal and GDM arteries and veins. A_1 receptor protein was not investigated as we could not demonstrate specificity of the antibodies. Bands were seen at ~45 kDa for the A_{2A} receptor protein and ~50 kDa for the A_{2B} receptor protein, which is similar to their predicted molecular weights of 45 kDa (Figure 6). The band size of the A_3 receptor protein was ~50 kDa in

both chorionic arteries and veins, which is greater than its predicted molecular weight of 36 kDa. There was no significant difference between normal and GDM arteries and veins with regard to expression of A_{2A} and A_{2B} receptor protein (Figure 6). A_3 receptor protein expression was similar in normal and GDM arteries, but was lower in veins of GDM compared to normal pregnancies ($p < 0.05$, Student's *t*-test) (Figure 6). Immunostaining for the TP receptor protein was observed at ~50 kDa, which is similar to its predicted molecular weight of 44 kDa. TP receptor protein expression was lower in GDM arteries compared to normal arteries ($p < 0.05$, Student's *t*-test) [Figure 7(a)], but expression levels were similar in normal and GDM veins [Figure 7(b)].

Discussion

This is the first study to investigate responses to adenosine in human chorionic vessels from pregnancies complicated
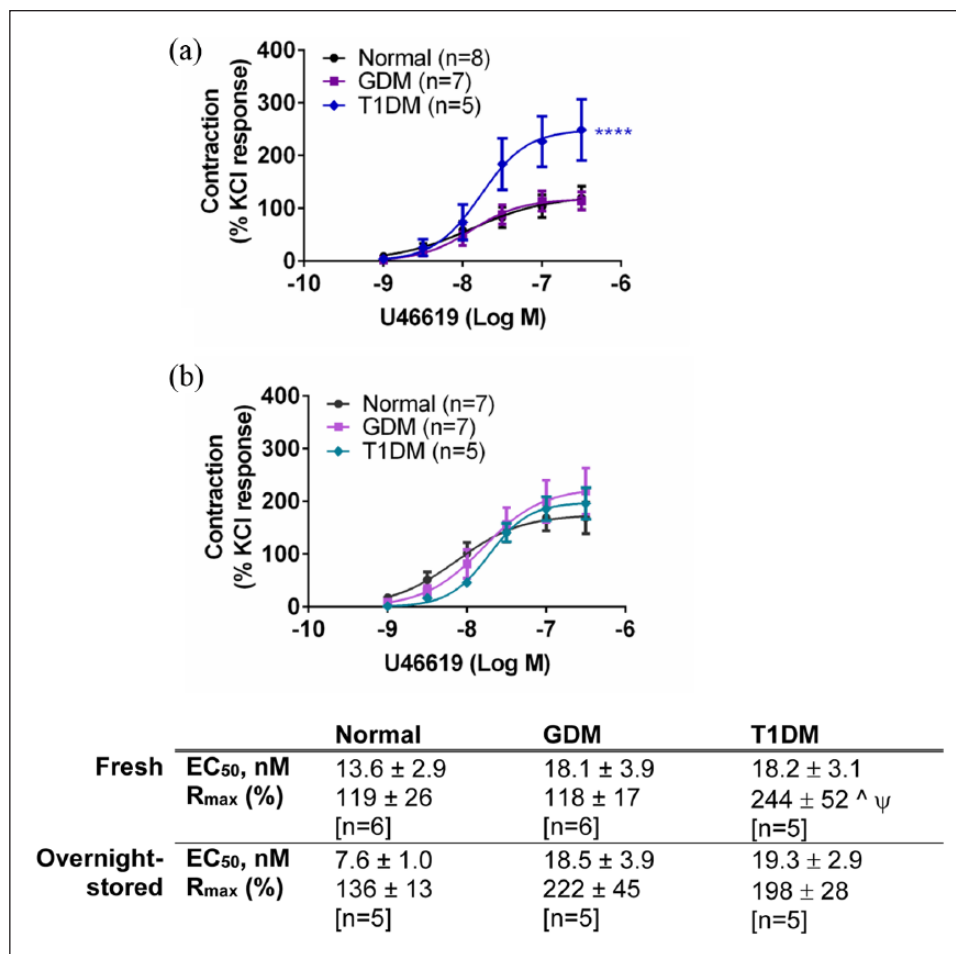
Figure 4. Response to U46619 in (a) fresh and (b) overnight-stored chorionic arteries from gestational diabetes mellitus (GDM), type 1 diabetes mellitus (T1DM) and normal pregnancies. Table indicates potencies (EC_{50}) and maximum response to U46619 (R_{max}). Values are mean $\pm$ s.e.mean for the number of placentae (*n*) shown in parentheses. % of contraction compared to potassium chloride (KCl, 60 mM).
****$p < 0.0001$ compared to normal, two-way ANOVA; ^$p < 0.05$ compared to normal; $^\psi p < 0.05$ compared to GDM, one-way ANOVA.

by two types of maternal diabetes, GDM and T1DM. The main finding in this study is that there is an impaired contractile response to adenosine in chorionic arteries and veins isolated from pregnancies with GDM and T1DM compared to vessels from normal pregnancies. Contraction to adenosine of human chorionic arteries and veins is mediated by A_{2B} receptors and a release of thromboxane A_2 or a related metabolite.[21] Here, we show that in chorionic arteries of both GDM and T1DM, the impaired adenosine contraction does not involve impaired signalling via TP receptors.

This study provides further evidence that adenosine induces vasoconstriction in human-isolated chorionic vessels and cotyledons.[20,21,26] Both the potency and maximal responses to adenosine in chorionic arteries and veins from normal pregnancies (artery EC_{50} 10 μM, R_{max} 51%; vein EC_{50} 29 μM, R_{max} 42%) are similar to those obtained by Donoso and colleagues (artery EC_{50} 38 μM, R_{max} 43%; vein EC_{50} 38 μM, R_{max} 41%).[21] Others have reported relaxation

of the human fetoplacental vasculature to adenosine, but either at substantially higher concentrations (EC_{50} ~1 mM adenosine) in isolated chorionic vessels[27] or as a slow response (developed over 20–30 min) in perfused cotyledons.[28] Biphasic responses of human isolated chorionic vessels to adenosine, with initial contraction (within 4 min) followed by relaxation (within 10–15 min), were also reported,[26] and similarly in the ovine placental vasculature.[29] The human placental vasculature expresses all four adenosine receptors, A_1, A_{2A}, A_{2B} and A_3.[21,30,31] The contractile adenosine receptor in human chorionic arteries and veins has been characterized as the A_{2B} subtype, located largely on the endothelium, which activates the cyclooxygenase pathway to mediate synthesis of thromboxane A_2 or a related metabolite, whereas the relaxation appears to involve smooth muscle A_2/A_3 receptors.[21,28] In vivo, the vascular responses are likely to depend on the adenosine levels, and endothelial integrity, such that an acute increase in adenosine will cause predominantly vasoconstriction,
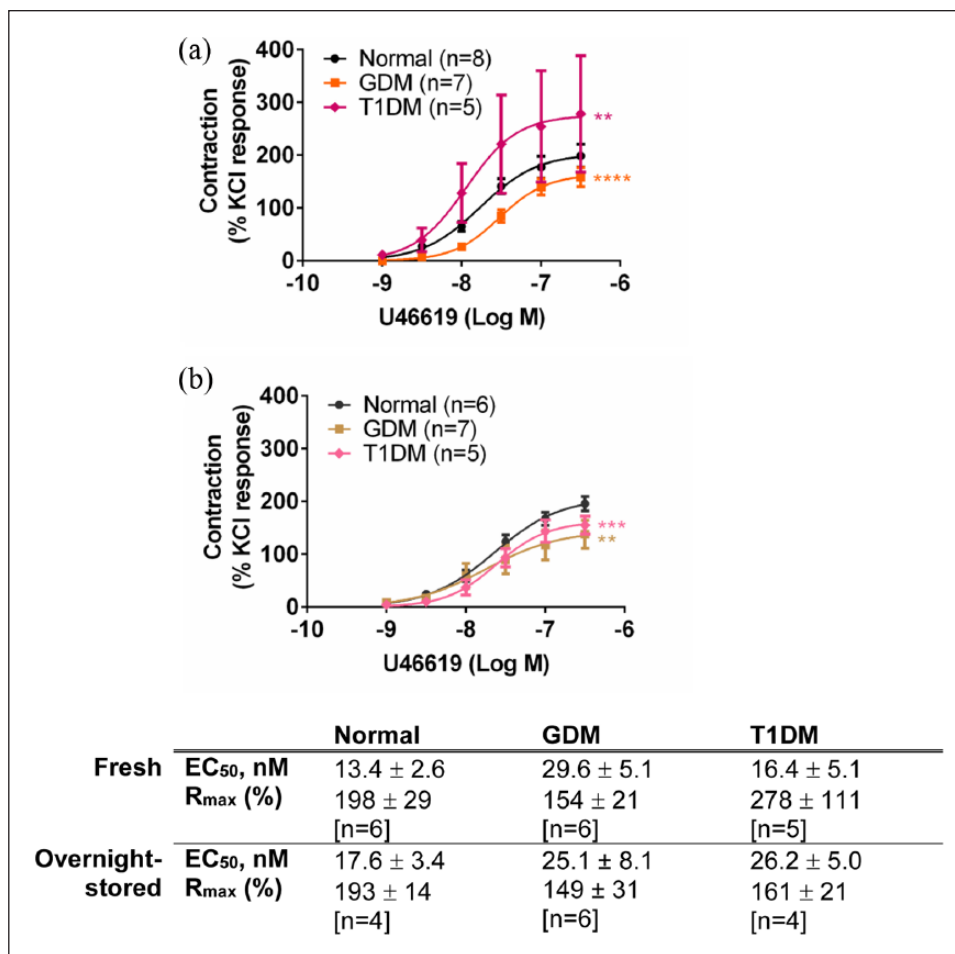
Figure 5. Response to U46619 in (a) fresh and (b) overnight-stored chorionic veins from gestational diabetes mellitus (GDM), type 1 diabetes mellitus (T1DM) and normal pregnancies. Table indicates potencies (EC_{50}) and maximum response to U46619 (R_{max}). Values are mean $\pm$ s.e.mean for the number of placentae (*n*) shown in parentheses. % of contraction compared to potassium chloride (KCl, 60 mM).
**p < 0.01; ***p < 0.001; ****p < 0.0001 compared to normal, two-way ANOVA.

whereas a sustained increase in adenosine will elicit additionally/predominantly relaxation, and this will help with the regional distribution of blood; an overall increase in the placental concentration of adenosine would, therefore, be expected to dampen adenosine-mediated blood flow regulation if accompanied by significant adenosine receptor desensitization.

Here, we report, for the first time, impaired contractions to adenosine of chorionic vessels (arteries and veins) from both GDM and T1DM, suggesting a systemic alteration of adenosine signalling in the fetoplacental circulation in maternal diabetes. This did not involve a generalized impairment of contractility of the vascular smooth muscle since responses to KCl, a receptor-independent vasoconstrictor, were similar between the normal and diabetic groups. Moreover, the impaired responsiveness to adenosine in chorionic arteries was selective with respect to responses to U46619, which were unaltered in GDM and increased in T1DM chorionic arteries. Selective impairment of the

response to adenosine was also observed in chorionic veins from T1DM, since U46619 mediated responses were augmented. In GDM, veins impaired responsiveness to U46619 accompanied the impaired contractile response to adenosine. Adenosine contractions involve the production and actions of thromboxane A_2,[21] but these data suggest that impaired adenosine contractions in artery and vein in T1DM, and in artery in GDM, do not involve defective signalling via TP receptors.

We used immunoblotting to investigate whether altered adenosine receptor expression could underlie the impaired contractile responses of the chorionic arteries and veins from GDM. There is a precedent for changes in placental adenosine receptor expression in complicated pregnancies since, in preeclampsia, the protein and messenger RNA (mRNA) of all four adenosine receptors were increased in placental biopsies and villous explants.[31] The band sizes of ~45 kDa for the A_{2A} receptor protein and ~50 kDa for the A_{2B} receptor protein are similar to their predicted molecular weights of 45
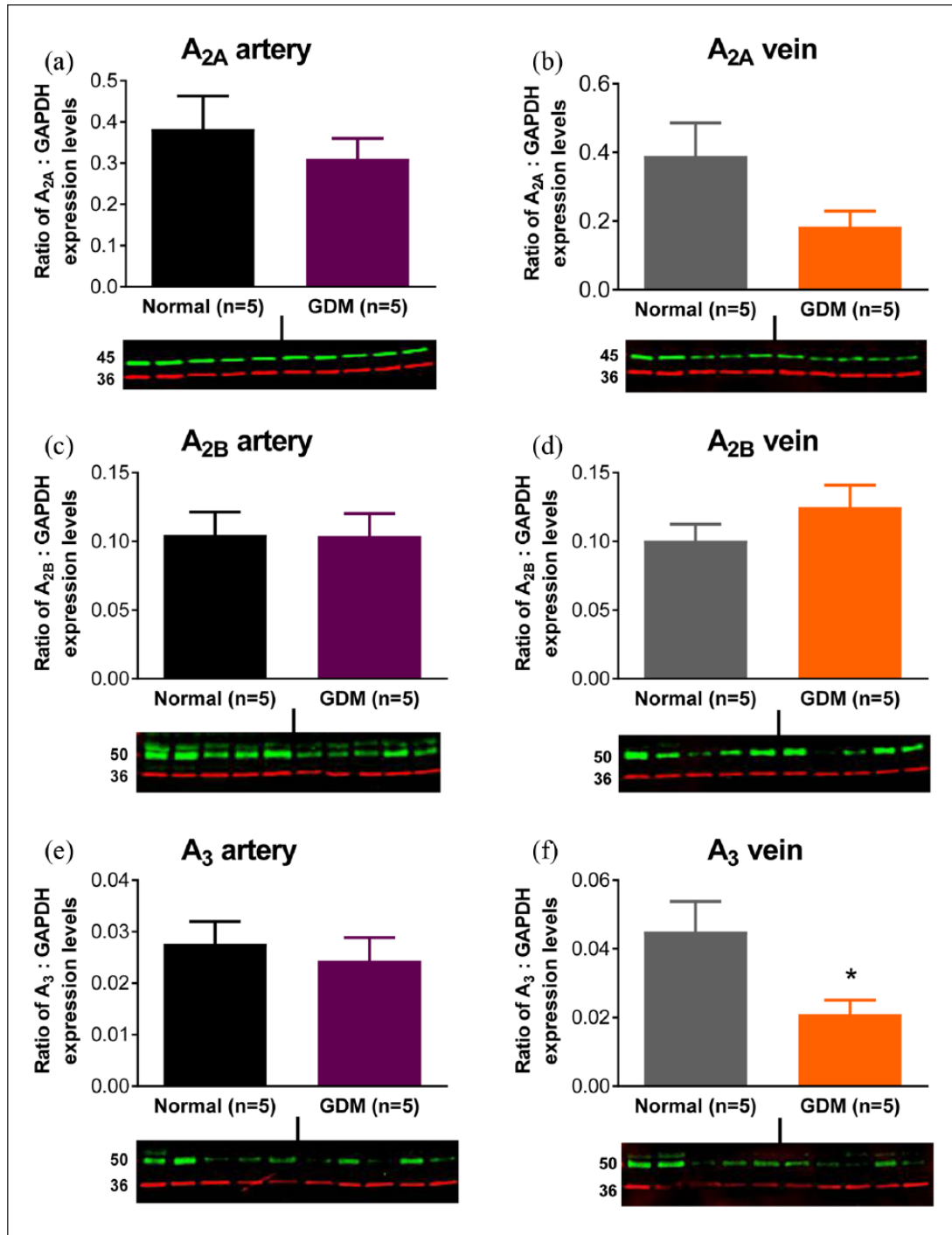
Figure 6. Expression levels of A_{2A} (~45 kDa) (a, b), A_{2B} (~50 kDa) (c, d) and A_3 (~50 kDa) (e, f) adenosine receptor proteins in chorionic arteries (a, c, e) and chorionic veins (b, d, f) from term placenta of normal and gestational diabetic mellitus (GDM) pregnancies. Blots show bands for adenosine receptor proteins (green) and GAPDH (~36 kDa) (red). Data are expressed as the ratio of A_{2A} to GAPDH band intensities and are the mean ± s.e.mean of five normal and five GDM samples. *$p < 0.05$, Student's *t*-test.

kDa, and to the bands observed in human placental biopsies (45 kDa, A_{2A}; 52 kDa, A_{2B}).[31] The band size of the A_3 receptor protein at ~50 kDa in both chorionic arteries and veins is greater than its predicted molecular weight of 36 kDa.

Immunostaining for the A_3 receptor protein in human placental biopsies[31] showed a band of ~36 kDa and an additional higher molecular weight band which could correspond to our band of ~50 kDa. We found no difference in A_{2B} (and A_{2A})
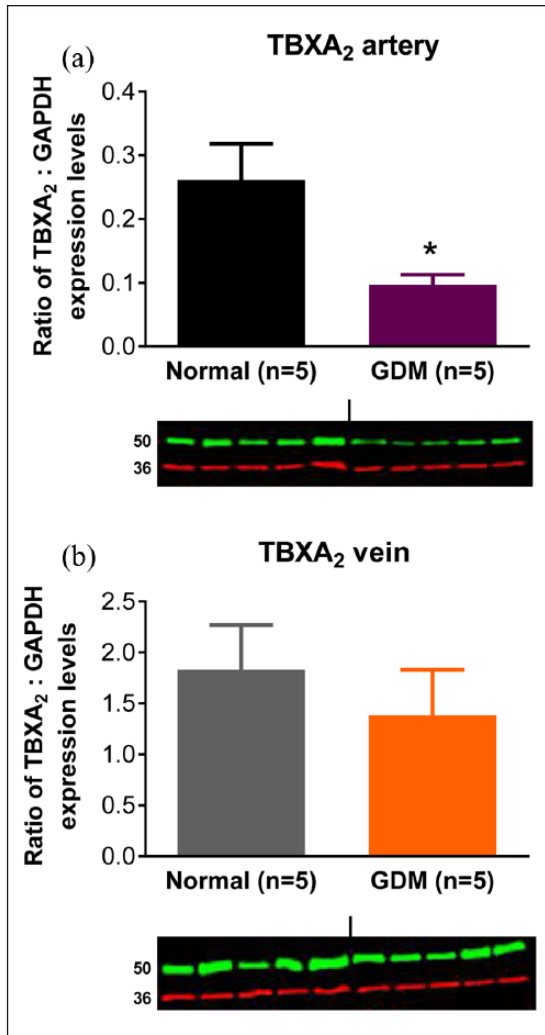
Figure 7. TP receptor protein (TBXA$_2$ ~50 kDa) and GAPDH (~36 kDa) expression levels in (a) chorionic arteries and (b) chorionic veins. Ratio of expression levels of TBXA$_2$ to GAPDH in chorionic arteries (a) and chorionic veins (b) from term placenta of normal and gestational diabetic mellitus (GDM) pregnancies based on the intensities of the bands. Data are expressed as the ratio of TBXA$_2$ protein to GAPDH band intensities and are the mean ± s.e.mean of five samples. *$p < 0.05$, Student's *t*-test.

adenosine receptor protein expression levels between GDM and normal chorionic arteries and veins. It is possible that the impaired contractility to adenosine in GDM (and possibly T1DM) involves A$_{2B}$ receptor desensitization/internalization,[32] perhaps as a consequence increased extracellular levels of adenosine in the GDM placenta.[15] Rapid fade of contractile responses to P2Y$_1$ receptor activation in human chorionic arteries was attributed to its exit from membrane lipid rafts and internalization.[33] In contrast, contractile responses of the human chorionic vasculature to U46619 and PGF$_2\alpha$ are sustained.[34–36] A limitation of our study is that we do not have data for receptor protein expression in chorionic

vessels from T1DM, but the data with GDM show that changes in agonist-induced contractile function can occur without a change in the level of receptor protein.

Whether the impaired contractility to adenosine in chorionic vessels from GDM and T1DM involves changes in plasma glucose concentrations is unclear. Maternal hyperglycaemia induces foetal hyperglycaemia and subsequent changes in the foetal circulation such as lower oxygen content and saturation.[37] However, hypoxia has been shown to increase mRNA expression of the trophoblast A$_{2B}$ receptor (which was shown to mediate proliferation, invasion and activation of cAMP response element binding protein).[38] A high glucose concentration impairs vascular endothelial function in vitro,[39] and since endothelium removal attenuates adenosine-mediated contractions in chorionic vessels,[21] this could contribute to the aberrant adenosine-mediated contractions in maternal diabetes. However, the blood glucose levels of the volunteers in our study are likely to have been well controlled, and others have reported no change in glucose in umbilical vein blood from normal and gestational diabetic pregnancies.[17] There is evidence that insulin receptors regulate placental vascular adenosine transport and nitric oxide generation,[15,19,40] and investigation of the impact of this in the study groups is an important avenue for future investigation.

Overnight storage in physiological solution reversed some of the changes, restoring contractility to adenosine in the GDM chorionic arteries. This recovery of function may involve resensitization/upregulation of contractile A$_{2B}$ receptors due to the removal of locally released or circulating adenosine. Extracellular levels of adenosine in the placental circulation may be elevated in maternal diabetes, an effect involving downregulation of placental hENT1,[15–17] and this may contribute to the reduced adenosine responses. An important limitation of this study is that adenosine levels were not measured in the tissue/blood so we do not have evidence that the differences that we have observed are a consequence of changes in adenosine levels. Changes in adenosine and adenosine triphosphate (ATP) release and metabolism could also be important in local elevations in placental vascular adenosine concentration.[41] KCl contractions were unaffected by overnight storage, suggesting that the recovery of function involved specific changes relevant to adenosine signalling. Reduced responses to KCl after overnight storage have reported by others,[42] but we did not see this. Overnight storage did not, however, reverse impaired contractility to adenosine in GDM chorionic veins, or in T1DM chorionic arteries and veins, suggesting more complex changes in contractility.

Contraction to U46619 in GDM arteries was unchanged, and in veins it was modestly reduced. This did not correlate with changes in TP receptor protein levels which were reduced in arteries and unchanged in veins. Interestingly, contractile responses to U46619 were much larger in normal veins than in arteries (R_{max} 198% veins; R_{max} 119%
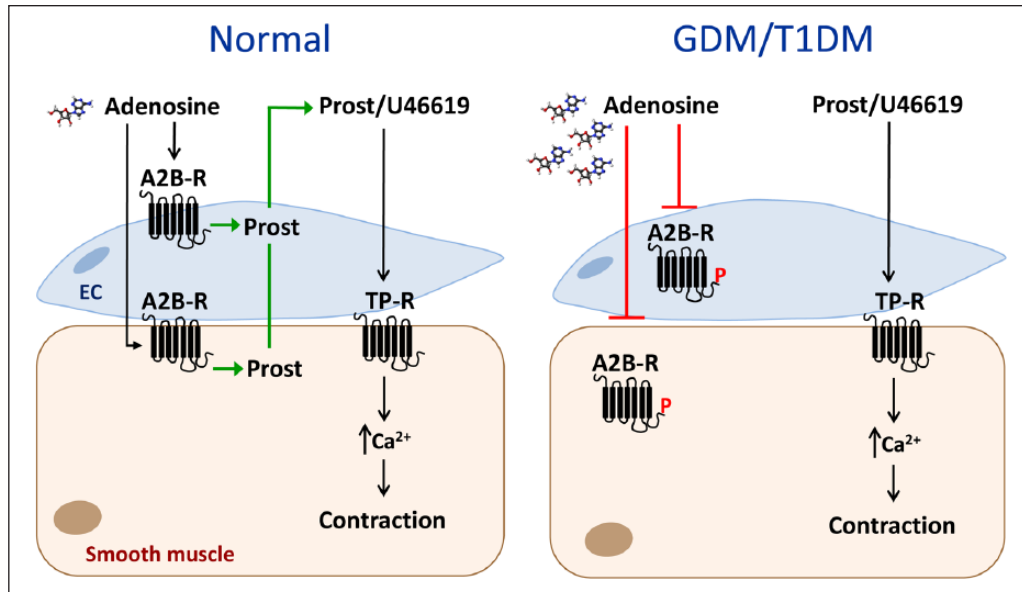
Figure 8. Summary diagram showing a putative pathway whereby adenosine contractility of the chorionic vasculature is impaired in maternal diabetes. Adenosine causes contraction of normal chorionic vessels through activation of A2B receptors (A2B-R) on the endothelium and smooth muscle, release of prostanoids (Prost) and activation of thromboxane receptors (TP-R). Thromboxane A2 is the principal endogenous agonist of TP-R and U46619 is a thromboxane A2 analogue. In gestational diabetes mellitus (GDM) and type 1 diabetes mellitus (T1DM), an accumulation of adenosine causes loss of the response to adenosine, possibly through receptor desensitization/downregulation, likely involving receptor phosphorylation (P). The contractile pathway involving prostanoid/U46619 activation of TP-R remains intact.
EC: endothelial cell.

arteries), which correlates with the greater (approximately 10-fold) TP receptor expression in the veins. Contraction to U46619 was enhanced in chorionic vessels from T1DM, in both arteries and veins, and this was reversed by overnight storage. In contrast, impaired vasoconstrictor responses to U46619 of perfused cotyledons from women with pregestational diabetes has been reported, attributed to a reduction in the affinity but not the number of placental TP receptors; placental thromboxane production was unchanged.[43] The reason for the increase in U46619 responses observed in this study in T1DM arteries and veins, but not in GDM vessels, is unclear. However, it is possible that the opposite changes in responses to adenosine and U46619 in T1DM vessels reflect a compensatory mechanism as placental vasoactive autacoids are known to work in concert to ensure optimum placental haemodynamic function in vivo.

Despite the evidence for a vasocontractile role of adenosine in the human placental vasculature,[20,21] there is an emphasis in the literature on adenosine-mediated placental vasorelaxation, possibly because adenosine is a vasodilator in most other vascular beds.[44] This study reinforces that overall control of fetoplacental haemodynamics by adenosine will involve the coordinated actions of vasocontractile as well as vasodilator adenosine receptors, likely differently expressed throughout the placental vascular tree as shown for the purine nucleotide receptors $P2Y_1$ and

$P2Y_2$.[45] The impaired adenosine-mediated contractility of chorionic vessels in maternal diabetes reported in this study might lead to a shift in balance towards adenosine-mediated placental vasorelaxation, although evidence for impaired adenosine relaxation in the umbilical vein in GDM,[19] suggests that adenosine-mediated blood flow regulation overall may be dampened.

Conclusion

This study reaffirms the presence of adenosine-induced vasocontraction in human chorionic vessels. The study has provided the first evidence of an altered contractile response to adenosine in chorionic vessels from pregnancies complicated by both pregestational and gestational diabetes. Impaired responses to adenosine in both chorionic arteries and veins suggest a systemic alteration of adenosine signalling in the fetoplacental circulation which may be contributed by both foetus and placenta in response to the maternal diabetes, and which may involve desensitization/downregulation of adenosine receptors. Our study contributes to a better understanding of differential function of the vasculature in diabetes.

technical assistance with the immunoblotting. Human tissue was obtained through the clinical connections and under the project licence of L.L. V.R. and A.A.R. designed the research study. A.A.R. performed the research and analysed the data. A.A.R. and V.R. wrote the initial draft. L.L. critically evaluated and commented on the results. All the authors read, modified and approved the final version of the manuscript.

Availability of data and material

All data generated or analysed during this study are included in this published article (and in its supplementary information file).

Consent for publication

Not applicable; no individual's data are reported.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Ethics approval and consent to participate

This study had the approval of the QMC and University Hospital NHS Trust Ethics Committee (Ref: 0G010101; PI-LL) and was carried out in accordance with the declaration of Helsinki. Pregnant women gave written informed consent.

Funding

ORCID iD

Vera Ralevic https://orcid.org/0000-0002-5252-280X

References

1. Nold JL and Georgieff MK. Infants of diabetic mothers. *Pediatr Clin N Am* 2004; 51: 619–637.
2. Leach L. Placental vascular dysfunction in diabetic pregnancies: intimations of fetal cardiovascular disease? *Microcirculation* 2011; 18: 263–269.
3. Jones CJP and Fox H. Placental changes in gestational diabetes: an ultrastructural study. *Obstet Gynecol* 1976; 48: 274–280.
4. Jones CJ and Fox H. An ultrastructural and ultrahistochemical study of the placenta of the diabetic woman. *J Pathol* 1976; 119: 91–99.
5. Babawale MO, Lovat S, Mayhew TM, et al. Effects of gestational diabetes on junctional adhesion molecules in human term placental vasculature. *Diabetologia* 2000; 43: 1185–1196.
6. Leach L, Gray C, Staton S, et al. Vascular endothelial cadherin and beta-catenin in human fetoplacental vessels of pregnancies complicated by Type 1 diabetes: associations with angiogenesis and perturbed barrier function. *Diabetologia* 2004; 47: 695–709.
7. Desoye G and Hauguel-de Mouzon S. The human placenta in gestational diabetes mellitus. The insulin and cytokine network. *Diabetes Care* 2007; 30: S120–S126.

8. Daskalakis G, Mariinopoulos S, Krielesi V, et al. Placental pathology in women with gestational diabetes. *Acta Obstet Gynecol Scand* 2008; 87: 403–407.
9. Boura ALA and Walters WAW. Autocoids and the control of vascular tone in the human umbilical placental circulation. *Placenta* 1991; 12: 453–477.
10. Slegel P, Kitagawa H and Maguire MH. Determination of adenosine in fetal perfusates of human placental cotyledons using fluorescence derivatization and reversed-phase high-performance liquid chromatography. *Ann Biochem* 1988; 171: 124–134.
11. Sawa R, Asakura H and Power GG. Changes in plasma adenosine during simulated birth of fetal sheep. *J Appl Physiol* 1991; 70: 1524–1528.
12. Blackburn MR, Xiang G, Airhart MJ, et al. Adenosine levels in the postimplantation mouse uterus: quantitation by HPLC-fluorometric detection and spatiotemporal regulation by 5'-nucleotidase and adenosine deaminase. *Dev Dyn* 1992; 194: 155–168.
13. Ball KT, Gunn TR, Power GG, et al. A potential role for adenosine in the inhibition of nonshivering thermogenesis in the fetal sheep. *Pediatr Res* 1995; 37: 303–309.
14. Yoneyama Y, Sawa R, Suzuki S, et al. The relationship between uterine artery Doppler velocimetry and umbilical venous adenosine levels in pregnancies complicated by preeclampsia. *Am J Obstet Gynecol* 1996; 174: 267–271.
15. Salomon C, Westermeier F, Puebla C, et al. Gestational diabetes reduces adenosine transport in human placental microvascular endothelium, an effect reversed by insulin. *PLoS ONE* 2012; 7: e40578.
16. Sobrevia L, Jarvis SM and Yudilevich DL. Adenosine transport in cultured human umbilical vein endothelial cells is reduced in diabetes. *Am J Physiol* 1994; 267: C39–C47.
17. Vasquez G, Sanhueza F, Vasquez R, et al. Role of adenosine transport in gestational diabetes-induced L-arginine transport and nitric oxide synthesis in human umbilical vein endothelium. *J Physiol* 2004; 560: 111–122.
18. San Martin R and Sobrevia L. Gestational diabetes and the adenosine/L-arginine/nitric oxide (ALANO) pathway in human umbilical vein endothelium. *Placenta* 2006; 27: 1–10.
19. Westermeier F, Salomon C, Gonzalez M, et al. Insulin restores gestational diabetes mellitus-reduced adenosine transport involving differential expression of insulin receptor isoforms in human umbilical vein endothelium. *Diabetes* 2011; 60: 1677–1687.
20. Maguire MH, Kitagawa H, Hosokawa T, et al. ATP and adenosine. Vasoconstrictors in human placenta. *Ann NY Acad Sci* 1990; 603: 443–444.
21. Donoso MV, Lopez RL, Miranda R, et al. A2B adenosine receptor mediates human chorionic vasoconstriction and signals through arachidonic acid cascade. *Am J Physiol Heart Circ Physiol* 2005; 288: H2439–H2449.
22. National Institute for Health Care Excellence (NICE). *Diabetes in pregnancy: management of diabetes and its complications from preconception to the postnatal period.* London: NICE, 2015.
23. Lot TY and Wilson VG. Overnight storage of the porcine isolated splenic artery enhances endothelium-dependent contractions to NG-nitro-L-arginine methyl ester without

impairing endothelium-dependent dilator function. *Naunyn Schmiedebergs Arch Pharmacol* 1994; 349: 95–100.

24. Valdecantos P, Briones R, Moya P, et al. Pharmacological identification of P2X1, P2X4 and P2X7 nucleotide receptors in the smooth muscles of human umbilical cord and chorionic blood vessels. *Placenta* 2003; 24: 17–26.

25. Rayment SJ, Ralevic V, Barrett DA, et al. A novel mechanism of vasoregulation: ADP-induced relaxation of the porcine isolated coronary artery is mediated via adenosine release. *FASEB J* 2007; 21: 577–585.

26. Krygicz J, Urban J and Malofiejew M. Influence of adenosine on contractility of isolated vessels of human placenta. *Am J Obstet Gynecol* 1978; 131: 587–589.

27. Reviriego J, Alonso MJ, Ibanez C, et al. Action of adenosine and characterization of adenosine receptors in human placental vasculature. *Gen Pharmacol* 1990; 21: 227–233.

28. Read MA, Boura ALA and Walters WAW. Vascular actions of purines in the fetal circulation of the human placenta. *Br J Pharmacol* 1993; 110: 454–460.

29. Reid DL, Davidson SR, Phernetton TM, et al. Adenosine causes a biphasic response in the ovine fetal placental vasculature. *J Dev Physiol* 1990; 13: 237–240.

30. Escudero C and Sobrevia L. A hypothesis for preeclampsia: adenosine and inducible nitric oxide synthase in human placental microvascular endothelium. *Placenta* 2008; 29: 469–483.

31. Von Versen-Höynck F, Rajakumar A, Bainbridge SA, et al. Human placental adenosine receptor expression is elevated in preeclampsia and hypoxia increases expression of the A2A receptor. *Placenta* 2009; 30: 434–442.

32. Mundell SJ, Mathary A-L, Nisar S, et al. Deletion of the distal COOH-terminus of the A2B adenosine receptor switches internalization to an arrestin- and clathrin-independent pathway and inhibits recycling. *Br J Pharmacol* 2010; 159: 518–533.

33. Norambuena A, Poblete MI, Donoso MV, et al. P2Y1 receptor activation elicits its partition out of membrane rafts and its rapid internalization from human blood vessels: implications for receptor signaling. *Mol Pharmacol* 2008; 74: 1666–1677.

34. Ralevic V, Burrell S, Kingdom J, et al. Characterization of P2 receptors for purine and pyrimidine nucleotides in human placental cotyledons. *Br J Pharmacol* 1997; 121: 1121–1126.

35. Wareing M, Crocker IP, Warren AY, et al. Characterization of small arteries isolated from the human placental chorionic plate. *Placenta* 2002; 23: 400–409.

36. Wareing M, Greenwood SL, Taggart MJ, et al. Vasoactive responses of veins isolated from the human placental chorionic plate. *Placenta* 2003; 24: 790–796.

37. Taricco E, Radaelli T, Rossi G, et al. Effects of gestational diabetes on fetal oxygen and glucose levels in vivo. *BJOG* 2009; 116: 1729–1735.

38. Darashchonak N, Koepsell B, Bogdanova N, et al. Adenosine A2B receptors induce proliferation, invasion and activation of cAMP response element binding protein (CREB) in trophoblast cells. *BMC Pregnancy Childbirth* 2014; 14: 2.

39. Dorigo P, Fraccarollo D, Santostasi G, et al. Impairment of endothelium-dependent but not of endothelium-independent dilatation in guinea-pig aorta rings incubated in the presence of elevated glucose. *Br J Pharmacol* 1997; 121: 972–976.

40. Aguayo C, Flores C, Parodi J, et al. Modulation of adenosine transport by insulin in human umbilical artery smooth muscle cells from normal or gestational diabetic pregnancies. *J Physiol* 2001; 534: 243–254.

41. Spaans F, de Vos P, Bakker WW, et al. Danger signals from ATP and adenosine in pregnancy and preeclampsia. *Hypertension* 2014; 63: 1154–1160.

42. Abad A, Estañ L, Morales-Olivas FJ, et al. Reactivity of isolated human chorionic vessels: analysis of some influencing variables. *Can J Physiol Pharmacol* 2003; 81: 1147–1151.

43. Wilkes BM, Mento PF and Hollander AM. Reduced thromboxane receptor affinity and vasoconstrictor responses in placentae from diabetic pregnancies. *Placenta* 1994; 15: 845–855.

44. Burnstock G and Ralevic V. Purinergic signaling and blood vessels in health and disease. *Pharmacol Rev* 2014; 66: 102–192.

45. Buvinic S, Poblete MI, Donoso MV, et al. P2Y1 and P2Y2 receptor distribution varies along the human placental vascular tree: role of nucleotides in vascular tone regulation. *J Physiol* 2006; 573: 427–443.