Human myometrial artery function and endothelial cell calcium signalling are reduced by obesity: can this contribute to poor labour outcomes?

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Abstract

Aims: Determining how obesity affects function in human myometrial arteries, to help understand why childbirth has poor outcomes in obese women.

Methods: Myometrial arteries were studied from 84 biopsies. Contraction (vasopressin and U-46619) and relaxation (carbachol, bradykinin, SNAP) was assessed using wire myography. eNOS activity was assessed using L-NAME. Cholesterol was reduced using methyl-β-cyclodextrin to determine whether it altered responses. Differences in endothelial cell intracellular Ca^{2+} signalling were assessed using confocal microscopy.

Results: The effects of BMI on relaxation were agonist specific and very marked; all vessels, irrespective of BMI, relaxed to bradykinin but 0% of vessels (0/13) from obese women relaxed to carbachol, compared to 59% (10/17) from normal weight women. Cholesterol-lowering drugs did not restore carbachol responses (n=6). All vessels, irrespective of BMI, relaxed when NO was directly released by SNAP (n=19). Inhibition of eNOS with L-NAME had a significant effect in normal but not overweight/obese vessels. Compared to bradykinin, a lower proportion of endothelial cells responded to carbachol and the amplitude of the calcium response was significantly less, in all vessels. Furthermore, a significantly lower proportion of endothelial cells responded to carbachol in the overweight/obese group compared to control. In contrast to relaxation, the effect of contractile agonists was unchanged with increasing BMI.

Conclusions: The ability of human myometrial arteries to relax is significantly impaired with obesity, and our data suggest this is due to a deficit in endothelial calcium signalling. This inability to recover following compression during contractions, might contribute to poor labours in obese women.

Key words: myometrial arteries, BMI, obesity, endothelium, Ca^{2+} signalling

Introduction

According to the World Health Organisation, worldwide obesity rates have more than doubled since 1980, showing 39% of adults aged 18 years and over to be overweight and 13% obese.[1] Similarly raised levels of obesity are observed in pregnant women registering for antenatal care,[2-4] with Iverson and co-workers[5] showing a 16.4% increase in obesity in pregnant women from 2004 to 2012. In 2006, the incidence of obesity in the pregnant population of Liverpool was 17.7%.[3] Similar values were observed in the Irish pregnant population[6] and recent data from the USA[7, 8] found the incidence of obesity in the pregnant population to be even higher (20.6% and 27.8% respectively). Maternal obesity (BMI≥30) is a well-recognised risk factor for difficult pregnancies and adverse outcomes, such as hypertension, pre-eclampsia, post-dates, still birth, congenital abnormalities, aberrant fetal growth, gestational diabetes and intervention in labour (C-section, induction).[3, 4, 9, 10]

A healthy pregnancy depends on the maternal cardiovascular system and uterine vasculature undergoing complex physiological adaptations.[11] During labour, contraction of the myometrium exerts external pressure on myometrial blood vessels, causing partial or total occlusion.[12-15] This leads to repeated transient bouts of myometrial hypoxia. Larcombe-McDouall and co-workers[16] showed that in a rat *in vivo* model, uterine contraction led to reduced blood flow, resulting in altered metabolite levels (decreased ATP and phosphocreatine, increased inorganic phosphate) and a decreased intracellular pH (pH_i). Other *in vitro* assays have shown uterine pH_i to fall during hypoxia or metabolic inhibition and if prolonged, to lead to reduced myometrial contractility.[17-19] Recently however it has been shown that shorter, repeated periods of hypoxia, can actually trigger increases in force in the uterus, a mechanism known as HIFI, hypoxia-induced force increase.[20] Thus uterine

tissue in labour is critically dependent upon its environment, which in turn, depends upon blood flow and the myometrial blood vessels. These vessels however have been little studied.

The removal of the metabolites of oxidative stress and intracellular acidification from one contraction to the next requires the occluded vessels to dilate and reperfusion of the tissue to occur. This process of perfusion, and vessel dilation occurs hundreds of time over the course of labours. If myometrial vessels in obese women are compromised or less resilient, then perfusion will decrease and recovery from occlusions will be incomplete. This will change the environment surrounding the myofibrils. It is known that lactate will increase and the associated decrease in pH can reduce myometrial contractions.[21] The HIFI mechanism may also be impaired. For these reasons, the increase in adverse pregnancy outcomes experienced by obese women may be related to myometrial blood vessel function.

Obesity is associated with alterations in vascular function. In particular endothelial dysfunction, frequently caused by deficits in the nitric oxide (NO) system, and hypertension are common findings in the obese population worldwide.[22] In addition, vascular reactivity, that is dilation and constriction responses, are compromised. As noted above, labour with its repetitive episodes of vascular occlusions, will result in stress on the myometrial vasculature's reactivity. Endothelial dysfunction is associated with pregnancy complications, most notably, pre-eclampsia.[23, 24] Myometrial vessels from women with pre-eclampsia have been shown to lose endothelial-dependent relaxation.[25] Work by Myers *et al*.[26] found that even in women without pregnancy complications, high BMI attenuated endothelium-dependent relaxation. Furthermore, a recent study has indicated that signalling in vessels from obese women differs from that in vessels from non-obese women.[27]

We have studied human myometrial vessels to better understand their physiology and pathophysiology and how obesity contributes to complications in labour. Our focus has been on endothelial function and the effects and mechanisms whereby obesity can alter this. Our aims therefore were to examine whether maternal BMI affects the ability of the human myometrial arteries to contract and to relax in vitro. We have investigated the underlying causes of alterations in function in several ways: i) We have used the cholesterol sequestering agent, methyl-β-cyclodextran (MCD) to determine whether dyslipidaemia (elevated cholesterol) was contributing to any dysfunction. ii) We have used L-NAME to inhibit eNOS, in order to determine if there is a deficit in the endothelial NO system as BMI increases and iii) we have examined smooth muscle function by using SNAP to directly generate NO and bypass the endothelial cell NO production. iv) A rise in intracellular Ca^{2+} is required in endothelial cells in order to activate eNOS and generate NO. Therefore, we have also used confocal microscopy to examine whether the calcium signalling of myometrial artery endothelial cells is altered with increasing maternal BMI.

Results

Baseline characteristics.

Data were obtained on 84 singleton pregnancies with biopsies obtained during elective C-sections after otherwise uncomplicated term pregnancies. The reason for C-section (CS) and mean data for maternal age, body mass index (BMI), parity, gestational age and weight of the baby is shown in Table 1. As expected, there is a significant difference in BMI between all groups. Median gestational dates were longer (by one day) in the overweight group.

	Normal weight (n=35)	Overweight (n=27)	Obese (n=22)	P value
Maternal age (years)	32 (29-37)	34 (29-37)	30.5 (27.75-35.5)	0.31
Maternal BMI (kg/m^2)	23 (22.2-23.6)	27.3 (26.0-27.8)	34.4 (31.1-37.5)	<0.0001
Parity	1 (0 – 5)	1 (0 - 3)	1 (0 - 3)	0.19
Gestational age (days)	273 (267.8-275)	274.5 (273-276)	273 (269.5-274.5)	0.003
Birth weight (grams)	3325 (3058-3563)	3590 (3300-3890)	3460 (2980-3770)	0.04*
Reason for CS				
Previous CS, n (%)	20 (57.1%)	15 (55.6%)	13 (59.1%)	
Previous difficult birth, n (%)	5 (14.3%)	5 (18.5%)	2 (9.1%)	
Breech, n (%)	3 (8.6%)	3 (11.1%)	1 (4.5%)	
Other, n (%)	7 (20%)	4 (14.8%)	6 (27.3%)	

Table 1. Maternal demographic and obstetric data for the women participating in this study; data expressed as median (IQR) or number n (%). Parity is expressed as median (range). Maternal BMI was measured at the booking appointment. * Kruskal-Wallis test p=0.04 but Dunn's multiple comparison post-hoc test did not find a difference between groups.

Contractility assays

Mean vessel diameter was 354.7±9.9µm, with a range from 160 to 680µm. There were no statistical differences in vessel size for any experimental group described below. Data is divided into 3 BMI groups; normal weight (BMI<25), overweight (BMI 25-29.9) and obese (BMI≥30).

Vessels from an additional 7 myometrial biopsies (not included in Table 1) were unresponsive to K^+ and were thus discarded (3x BMI<25, 4x BMI≥25, these are not included in Table 1). In the remaining vessels from 67 biopsies, the magnitude of the response to K^+ was not significantly different between groups (BMI<25; 0.85 (0.11-2.7) mN/mm, n=28, overweight: 0.78 (0.33-2.32) mN/mm, n=21, obese: 0.79 (0.23-1.92) mN/mm, n=18; Mann Whitney test p=0.75).

Effect of maternal BMI on contraction

Two well characterised contractile agonists were studied: the thromboxane A2 analogue, U-46619 and arginine vasopressin (AVP).

Contraction with U-46619

Cumulative concentration-response curves to U-46619 (1nM - 3µM) were obtained in 33 human myometrial biopsies. U-46619 produced a sustained contraction of the myometrial arteries (Figure 1A & B). When samples were separated by BMI, there was no significant

difference in EC_{50} between the BMI groups (see Figure 1C: BMI<25 pEC_{50}=7.02±0.19, n=12; overweight pEC_{50}=6.64±0.20, n=12; obese pEC_{50}=6.84±0.19, n=7, Kruskal-Wallis test p=0.053). The maximum amplitude of the response (normalised to a high K^+ response) was also unaltered (BMI<25 Max=713.4±122.4%, n=12; overweight Max=675.0±139.5%, n=12; obese Max=473.0±96.2%, n=7, Kruskal-Wallis test p=0.54). There was also no significant difference in vessel size between groups (BMI<25 380.1 (276.5-484.1) µm; overweight 299.9 (227.4-359.5) µm; obese 249.7 (227.1-369.2) µm, Kruskal-Wallis test p=0.059). Based on these data, a concentration of 1µM U-46619 was chosen to contract vessels for the subsequent study of relaxation.

Contraction with arginine vasopressin

AVP also produced a sustained contraction of the myometrial arteries (Figure 1D & E). Cumulative concentration-response curves (0.1nM - 10nM) were obtained in 25 human biopsies. There was no significant difference in vessel size between groups (BMI<25; 420.6±27.8µm; overweight 405.1±56.4µm; obese 347.0±73.1µm, ANOVA p=0.60).When samples were separated by BMI, there was no significant difference in EC_{50} between the BMI groups (see Figure 1F: BMI<25 pEC_{50}=8.88±0.20, n=15; overweight pEC_{50}=8.73±0.26, n=6; obese pEC_{50}=9.01±0.24, n=3; ANOVA p=0.60). The maximum response between the two groups was not significantly different BMI<25 Max=500.0±87.0%, n=15; overweight Max=411.7±115.2%, n=6; obese Max=453.4±105.6%, n=3; ANOVA p=0.89).
Based on these data, a concentration of 10nM AVP was chosen to contract vessels for the subsequent study of relaxation.

Effect of maternal BMI on relaxation

Endothelium-dependent relaxation: Bradykinin (BK)

Vessels were pre-contracted with 10nM AVP. Once the contraction had stabilised, a concentration-response curve to BK was obtained (1nM – 1µM). All vessels from normal weight, overweight and obese women relaxed equally well to BK (BMI<25 pEC_{50}=7.63±0.23, n=10; overweight pEC_{50}=7.91±0.07, n=7; obese pEC_{50}=7.60±0.08, n=6; ANOVA p=0.31, Figure 2A).

Concentration-response curves to bradykinin were also obtained in vessels pre-contracted with 1µM U-46619. Again all vessels relaxed equally well to BK (BMI<25 pEC_{50}=7.88±0.18, n=15; overweight pEC_{50}=7.46±0.17, n=10; obese pEC_{50}=7.27±0.75, n=5; ANOVA p=0.94,Figure 2B). There was no significant difference in the maximum relaxation achieved in each BMI group (ANOVA, p=16). The nature of the pre-contractile agent did not alter the potency of the BK response, however the amplitude response in the overweight and obese groups was significantly smaller when the tissue was precontracted with U-46619 than with AVP (Student's t-test: overweight p=0.046, obese p=0.024).

Endothelium-dependent relaxation: Carbachol (CCh)

Vessels were pre-contracted with 10nM AVP. Once the contraction had stabilised, the response to CCh was obtained. In contrast to BK, not all vessels relaxed when challenged with CCh (Figure 3A & B). When samples were separated by BMI, vessels from 10 of 17 biopsies (59%) from normal weight women responded to CCh (mean inhibition of 58.6±0.06%), whereas vessels from the remaining 7 biopsies (41%) failed to relax in response to CCh (mean inhibition of 6.3±0.02%). There was no significant difference in BMI between responders and non-responders (Responders 22.34±0.60; Non-responders 22.9±0.71,

unpaired t-test p=0.56) and no significant difference in vessel size (Responders 390.8±36.3 µm; Non-responders 384.3±30.9 µm, unpaired t-test p=0.91).

In biopsies from overweight women, vessels from only 4 of 13 biopsies (31%) responded to CCh (mean inhibition of 73.0±8.8 %), whereas 9 of 13 (69%) did not relax (mean inhibition of 3.0±1.2 %) when stimulated with CCh. Again, there was no significant difference in BMI between responders and non-responders (Responders 26.9±0.38; Non-responders 26.2±0.23, unpaired t-test p=0.13) and no significant difference in vessel size (Responders 333.9±51.6 µm; Non-responders 333.5±25.8 µm, unpaired t-test p=0.99). In biopsies from obese women, no response to CCh was observed in any of 13 vessels (mean inhibition of 10.0±1.4 %, mean vessel size 331.0±26.5). It should be noted however that all of these vessels responded normally to BK (Figure 3B).

If we separate the data by response to CCh, then the mean BMI of women whose vessels responded to CCh was 23.64±0.72 (n=14), which was significantly lower than those that did not respond to CCh 29.33±1.15 (n=29, unpaired t-test p=0.002, Figure 3C). There was no significant difference in vessel size (Responders 373.9±28.93 µm; Non-responders 342.7±16.33 µm, unpaired t-test p=0.32). Concentration-response curves for CCh were obtained in responding vessels (Figure 3E), where pEC_{50}=7.27±0.33 (n=6 (of which 5 normal weight, 1 overweight)).

In vessels pre-contracted with 1µM U-46619, 10µM CCh produced relaxation in vessels from 3 of 9 biopsies with BMI<25 and in 0/7 biopsies from women with BMI≥25 (overweight n=6, obese n=1).

Endothelium-independent relaxation: SNAP

In order to determine if the impaired relaxation was due to signalling changes or impairment at the level of the contractile machinery, the NO donor SNAP (20μM) was used to directly activate the myometrial artery smooth muscle cells. Application of SNAP produced a rapid and complete relaxation of AVP pre-contracted myometrial vessels from all women (BMI<25 % inhibition=96.8 (95.6-101) %, n=13: overweight % inhibition=97.5 (95.7-101) %, n=10 biopsies; obese % inhibition=90.9 (90.3-95.6) %, n=9; Dunn's multiple comparison test shows no difference between these groups, Figure 4A & B). When vessels were pre-contracted with U-46619, again there was no difference in degree of relaxation observed in the 3 BMI groups (BMI<25 % inhibition=90.3 (86.3-92.7) %, n=17: overweight % inhibition=91.4 (86.7-94.1) %, n=11 biopsies; obese % inhibition=72.0 (62.3-91.5) %, n=4; Kruskal-Wallis test p=0.23).

Effect of lowering cholesterol levels with methyl-β-cyclodextrin (MCD)

Previous data has shown that the dyslipidemic environment often associated with high BMI can impair vascular function. [28, 29] We have therefore investigated whether lowering cholesterol, by sequestration with MCD, would alter the responsiveness of the human myometrial arteries to CCh. This method has previously been shown to reduce the cholesterol content of myometrial strips by 50% over a 20min incubation period.[30] Vessels from women with BMI≥30 consistently failed to respond to CCh. Pre-treatment of vessels with 2% MCD to modulate cholesterol (and thus caveolae) did not restore a response to CCh. No response to CCh was observed in these vessels from obese women either before or after MCD treatment (Control n=6 versus MCD n=6). Additionally, after MCD treatment, the amplitude of the pre-contraction to AVP (10nM, not shown), the response to SNAP (20μM, not shown) and the EC_{50} and amplitude of the BK concentration-response curve (Figure 4C) were not significantly altered.

Effect of L-NAME inhibition

In order to determine whether nitric oxide synthase (NOS) activity is altered in the endothelial cells of vessels from women of normal weight and overweight/obese women, the response to BK was assessed in the absence and presence of 100μM L-NAME. Since our previous experiments demonstrated no significant difference in the response to BK in overweight and obese vessels, we have compared BMI<25 vessels with BMI≥25 vessels in this experiment. Vessels were pre-contracted with 10nM AVP. The 30min application of L-NAME caused a significant increase in the amplitude of the contraction in normal weight (% increase; Control 2.02±4.9%, n=6 versus L-NAME 26.08±4.6%, n=6; p=0.005) and BMI≥25 vessels (% increase; Control 4.73±3.7%, n=5 versus L-NAME 27.77±5.3%, n=5; p=0.007, see Figure5A & B).

In vessels from normal weight women, L-NAME caused a significant rightward shift of the BK concentration-response curve (BK 7.78±0.28, n=5; BK with L-NAME 7.06±0.20, n=6; p=0.023, see Figure 5C) and the maximum relaxation achieved with BK was significantly smaller (BK 0.28±0.12; BK with L-NAME 0.57±0.06; p=0.04). In vessels from overweight/obese women (BMI≥25), L-NAME did not have a significant effect on the BK concentration-response curve (BK 7.88±0.24, n=5; BK with L-NAME 7.93±0.49, n=4; Mann-Whitney test p=0.73, see Figure 5D).

Confocal microscopy

Using confocal microscopy, we examined the endothelial cell layer in myometrial arteries that were between 50μm and 1000μm in size, from 22 myometrial biopsies. The endothelium was confirmed to be intact following tissue loading and preparation (Figure 6A) and normal weight and overweight/obese samples were indistinguishable by eye. In all tissues, BK

produced a rise in intracellular Ca^{2+} in endothelial cells (expressed as a ratio F/F_0). This rise was of a similar amplitude in vessels in both the normal weight (F/F_0 2.49 (2.41-3.03), n=8) and BMI≥25 groups (F/F_0 3.08 (2.53-3.94), n=9, Mann Whitney test, p=0.24, Figure 6 and 7A-B). The response was characterised by a large, rapid upstroke of intracellular Ca^{2+}; sometimes this response was transient, sometimes it was followed by a sustained plateau phase, as seen in Figures 6C and D, respectively. These patterns were exhibited irrespective of BMI.

In vessels from normal weight women, a response to 10µM CCh was observed. However the amplitude of the Ca^{2+} response was significantly smaller than that to BK (F/F_0: CCh 1.79±0.20 versus BK2.55±0.16, n=6, paired t-test p=0.013, Figure 7A) and a lower proportion of cells responded (CCh 12.3±5.8 % versus BK 78.8±5.4 %, n=9, paired t-test p=0.0001, Figure 7C, Video 1).

In most vessels from women with BMI≥25, only one or two endothelial cells in the field of view (or frequently no cells), responded to stimulation with CCh (Figures 6C-D & 7D). The amplitude of the CCh response, when it was present, was similar to that seen in vessels from normal weight women (unpaired t-test p=0.33, Figures 7A & B), meaning that again the CCh response was significantly smaller than the BK response (F/F_0: CCh 2.11±0.21 versus BK 2.97±0.40, n=4, paired t-test p=0.049, Figure 7B). Only 0.4 (0 - 2.2) % of cells responded to CCh compared to 91.4 (72.9 – 98.9) % to BK (Wilcoxon signed rank test p<0.004, n=9, Figure 7D, Video 2). A significantly lower percentage of endothelial cells responded to CCh in the BMI≥25 group than in the normal weight group (Mann Whitney test p=0.037), whereas a similar number of cells responded to BK irrespective of BMI (Mann Whitney test p=0.76).

We examined whether SNAP (20µM) generated a Ca^{2+} response in myometrial artery endothelial cells and observed no response in endothelial cells from all BMI groups (n=8, see Video 3).

We also examined the timecourse of the BK endothelial cell Ca^{2+} response, by comparing the time differential between the first and last cells to respond with a rise in intracellular Ca^{2+} to agonist application. No significant difference was observed in the timecourse of the Ca^{2+} response to BK in vessels from normal weight women (21.85±5.8 sec from 1st to last response) and overweight/obese women (BMI≥25, 22.04±4.0 sec). The same analysis could not be carried out for CCh, as so few cells responded to CCh in the overweight/obese preparations.

These confocal experiments to measure endothelial cell responses were carried out under resting conditions, unlike the functional myograph assays, where endothelial responses were assessed in the presence of the contractile agonists AVP or U46610. In order to demonstrate that the presence of the contractile agonist had no effect on the endothelial cell Ca^{2+} response, we compared the amplitude of the Ca^{2+} response to BK in the absence and presence of 10nM AVP in vessels from normal weight women. No differences were observed (Resting conditions F/F_0 2.49 (2.41-3.03), n=8; with AVP F/F_0 2.78 (2.38-3.22), n=4; Mann Whitney test p=0.68) and a similar % of cells responded (Resting conditions 78.8±5.4 %, n=9; with AVP 86.6±7.2 %, n=4, p=0.42).

Discussion

Obesity has been consistently associated with vascular dysfunction.[31, 32] Obese and overweight women have an increased risk of problems in pregnancy and delivery.[4, 10] Healthy pregnancies and deliveries depend on the maternal cardiovascular system and uterine

vasculature undergoing complex physiological adaptations[11] and impairment of these processes will have significant consequences. Obesity is a significant risk factor for maternal and fetal complications, and intervention in labour, especially Caesarean-sections.[4, 10] Despite this, the vessels within the myometrium have received little attention. Given the clear importance of the vasculature to a healthy pregnancy, it is necessary to understand if obesity is associated with impairment of the myometrial vessels. As both vascular contraction and relaxation are needed for proper functioning, we investigated the effects of obesity on both.

We found a clear relationship between impaired vasorelaxation and increasing BMI, and this was an agonist-specific, not a global, effect. All vessels from normal weight, overweight and obese women relaxed to bradykinin, but the response to another relaxatory agonist, carbachol, decreased with increasing BMI. It is worth emphasising that no vessels from obese women responded to carbachol and only a third of vessels from overweight women responded. The contractile responses of these vessels did not alter with increasing maternal BMI.

Hayward et al. (2014)[27] are the only other group to have studied the effect of maternal BMI on myometrial artery function. They reported deficits in specific regulatory pathways in vessels from obese women: impaired contraction with U-46619 but not AVP (with no concomitant change in TBXA2 receptor expression), impaired relaxation to BK when the vessel was pre-contracted with AVP but not U-46619 and similarly, impaired relaxation to the NO donor SNP when the vessel was pre-contracted with AVP but not U-46619. The magnitude of the deficits were small. In our study, despite similar subjects and experimental procedures, we see no effect of BMI on contractile responses and BK-mediated relaxation is maintained in overweight and obese groups (no matter which pre-contractant is used).

Hayward et al. did not study carbachol. Our data showed a significant deficit in the response to carbachol.

In our hands, direct donation of NO to the vascular smooth muscle results in unimpaired relaxation, suggesting that the deficit is in the endothelial production of NO, rather than the ability of the vessel to respond to it. In pregnant human myometrial arteries, BK can generate relaxation via activation of the endothelial derived hyperpolarising factor (EDHF) pathway, as well as via the NO pathway and in fact both pathways need to be blocked in order to inhibit the BK response.[33] The results of our L-NAME experiment suggest a deficit in the NO system in obese women, since the BK response was sensitive to eNOS inhibition in normal weight but not overweight/obese vessels. The BK response however remains intact in these vessels presumably because the EDHF pathway can compensate for the loss of NO. Carbachol may not be able to activate the EDHF pathway to the same extent, making the muscarinic response more susceptible to impairment with obesity.

Obesity, pregnancy and vasculature function

Obesity is associated with endothelial dysfunction in a range of animal models of diet-induced obesity,[34, 35] as well as obese humans.[32, 36, 37] Oxidative stress coupled with reductions in NO bioavailability appear to contribute to the endothelial dysfunction in obesity.[38-40] Although less well studied, it has been found that myocyte function *per se* is also altered in vessels from obese individuals.[41, 42] It is likely that other pathways may compensate, at least initially, for changes in vascular function with obesity, suggesting that some receptors and pathways are more vulnerable to the obese environment; for example in coronary arterioles of obese rats, an increased activity of soluble guanylate cyclase leads to enhanced sensitivity to NO, which contribute to the maintenance of NO-mediated dilations and coronary perfusion.[43]

Obesity in pregnancy, as in non-pregnant individuals, leads to dyslipidemia, impaired vascular function and a perturbed metabolic and inflammatory state.[26, 44-46] For example, Sarno et al. (2015) found that obese women have increased umbilical artery resistance.[45] Similarly, our data in myometrial vessels showed an agonist specific impairment of vasorelaxation, impaired NO production by the endothelium, and alterations of endothelial calcium signalling, all associated with obesity.

Work conducted to elucidate how obesity affects vascular function, indicates that agonist-stimulated endothelium-dependent vasodilation is multiply impaired in obesity (see e.g. Van Guilder *et al*.[47]). Muscarinic dysfunction i.e. responses to ACh, carbachol or methacholine were the first receptor signalling pathway to be shown to be affected by obesity. Subsequent *in vivo* work confirmed these findings. It has been reported that forearm vasodilation responses *in vivo* to ACh in pregnant women is impaired, and that this response develops throughout gestation.[46] We found impaired vasodilation to carbachol but not bradykinin. In an earlier study by Myers *et al*.[48] they failed to demonstrate carbachol relaxation in human myometrial arteries, so used bradykinin instead to investigate vasodilation, but no details of BMI were given. This suggests the response to carbachol in myometrial arteries is less robust than that to bradykinin, which is consistent with our calcium signalling data. There is some evidence that oxidative stress, which is a feature of obesity, can cause a reduction in expression of M3 receptors.[49, 50] More specifically, Lin et al.[51] recently showed that a high fat diet led to decreased muscarinic M3 receptor expression in rat bladder. A reduction in the expression of M3 receptors could account for the decreased relaxation and endothelial cell calcium signalling we found in vessels from obese women but further studies would be required to confirm this. We can find no evidence for bradykinin B2 receptor expression

changes with obesity, and previous functional studies are contradictory, with BK-mediated relaxation either impaired with obesity[52, 53] or unchanged.[54]

Confocal microscopy and Calcium signalling.

We have performed the first confocal analysis of calcium signals in pregnant human myometrial artery endothelial cells. We could distinguish endothelial cell Ca^{2+} signals from myocyte signals and could observe intact sheets of endothelial cells and thus be confident that the endothelium had not been damaged.[29, 55] We show that even in vessels from normal weight women, which relax to both carbachol and bradykinin, the amplitude of the intracellular Ca^{2+} response was significantly smaller to carbachol compared with bradykinin and a significantly lower proportion of endothelial cells responded. These inhomogeneous Ca^{2+} responses were apparent in vessels from overweight/obese women too and thus are independent of BMI. Similar inhomogeneous distribution of endothelial cell responses to agonist stimulation has been shown in mouse aorta.[56] When we compared responses in vessels from overweight and obese women, a significant reduction in the number of endothelial cells that respond to carbachol was found. The response to bradykinin in endothelial cells from the vessels of overweight and obese women was similar to that in the normal weight group. These data, taken alongside our findings on signal amplitude, suggest that carbachol Ca^{2+} signalling in human myometrial arteries, is significantly less robust to the effects of an environment changed by obesity, than is bradykinin signalling. In turn, these direct measurements of Ca^{2+} responses also support the functional responses we found, i.e. carbachol relaxation is impaired in obesity and bradykinin responses are not.

Vasoconstriction

Although there is evidence that vasoconstriction is elevated in obese individuals,[57, 58] our data in myometrial arterial vessels do not suggest increased tone associated with obesity in pregnant women. Using AVP, Hayward et al.[27] also reported no difference in vasoconstrictor responses with BMI. They also found no difference with U46619 in overweight, compared to normal weight women, in agreement with our data. They did however find a small but significant difference in the obese group. While we did not find significance, examination of our Figure 1C, shows all the data points in the obese women lying beneath those from normal weight women. Our data were normalised to K^+-depolarization, whereas Hayward et al. report pressure values; it may be that this led to the different findings.

Caveolae and cholesterol manipulation

Our work with ApoE knockout mice, which have elevated cholesterol and LDLs, has shown a selective, reduced relaxation to carbachol in aortic preparations compared to wildtype mice.[28] In myometrial myocytes, contractions were increased when cholesterol was lowered.[30] It has been postulated that because of the dyslipidaemia usually associated with obesity, membrane domains and cholesterol-rich membrane caveolae may be altered.[59, 60] Of particular note, NO-synthase (NOS) is inactive in caveolae. It is only as agonists stimulate a rise in Ca^{2+}, which binds with calmodulin and this complex interacts with NOS, that it can move to a non-caveolar location and activate. Dreja *et al*.[61] found that MCD reduced the vasopressin response by 50% in rat tail arteries, suggesting V2 receptors are also affected by cholesterol and caveaolae. Our data with MCD, to lower cholesterol and reduce caveolae, however, showed no improvement in relaxation to carbachol in the human myometrial arteries.

Study limitation and implication

Our data come from biopsies from 84 women. While this represents a considerable accomplishment in obtaining human samples, nevertheless there will always be concerns how representative the group is. We used biopsies from elective sections from women not in labour, to reduce variability between groups e.g. length and progress of labour. Thus, any effects of labour on the myometrial vessels could not be studied. Our data was also collected from women having uncomplicated pregnancies despite being obese; excluding complications may mean we are under representing the size of the effects found.

The vessels were dissected from myometrial biopsies and placed on wire myographs. This methodology meant it was impossible to study vessels below approx. 100µm, which may be more affected by obesity than the larger arteries. Responses to SNAP confirmed that the vessels had not been damaged by dissection and mounting for study. There were also no significant differences in the size of vessels with BMI, removing this as a factor influencing the results. While we demonstrated a deficiency in the NO system in endothelial cells from obese women, we did not measure muscarinic M3 receptor expression and therefore cannot discount that changes in receptor number or function could also contribute to the dysfunctional relaxation observed. Although MCD has been documented to effectively reduce cholesterol in a wide variety of tissues and cells,[62] we did not directly determine cholesterol levels in this study.

Relation to labour outcome

As noted earlier, during labour there is repetitive compression of the uterine blood vessels and hypoxia and alterations in metabolites.[16, 63, 64] Repetitive transient hypoxia can increase myometrial contractions, HIFI, and may be a mechanism to counter the metabolic changes.[20] Much less well documented however is how the vascular myocytes themselves are affected by the compressions, which will increase as labour progresses. There will be a fall in pH_i in

the vascular myocytes due to stimulation of glycolysis. This will reduce their ability to produce force and relaxation will also be slowed.[65] In the myometrium, the removal of the metabolites of oxidative stress and intracellular acidification from one contraction to the next, requires the occluded vessels to dilate fully.

Our data show that although the ability to constrict is unaffected by BMI, impaired relaxation in the myometrial vessels occurs in obese women; they are compromised and less resilient, and thus recovery from occlusions will be incomplete. We suggest this produces a gradual change in the environment surrounding the myofibrils, e.g. build-up of lactate that will reduce contractions.[21] Such a lactate acidosis is associated with dysfunctional labours[66] and would directly relate to the increase in instrumental delivery experienced by obese women.

Methods

Non-labouring human myometrial biopsies were collected from 84 women with full term, uncomplicated singleton pregnancies undergoing elective caesarian section (median gestation 39 weeks (range 37^{+1} to 40^{+4} weeks)), at Liverpool Women's Hospital, UK. All women gave written informed consent to participate. The study was approved by the North West (Liverpool East) Research Ethics committee (Ref: 10/H1002/49) and by the Research and Development director at Liverpool Women's Hospital NHS Foundation Trust, Liverpool, UK. Indications for caesarian section were previous caesarian section (48), previous traumatic vaginal delivery (12), breech presentation (7) and other reasons (17), such as placenta previa, tocophobia, fetal abnormalities and maternal spina bifida or cerebral palsy. The average BMI of the women was 32.52 (range 19 – 47) and average weight of the babies was 3441g (range 2400 – 4550g). Biopsies were taken from the upper edge of the lower uterine segment incision immediately after delivery of the baby.[67] All biopsies were placed in

Hanks balanced salt solution at 4°C and experiments were performed within 24 hours of biopsy excision.

Contractility

Using a stereomicroscope, small myometrial arteries (mean (range): 354.7µm (160-680µm) were dissected, cut into 2mm sections and mounted in a DMT dual wire myograph. Vessels were maintained in a physiological salt solution (composition (mM): NaCl 154, KCl 5.6, $MgSO_4.7H_2O$ 1.2, HEPES 10.9, glucose 8, and $CaCl_2$ 2 (adjusted to pH7.4)) at 35ºC and gently bubbled with oxygen. The vessels were set to their normalized diameter according to the method of Mulvany and Halpern.[68] The aim of normalization is to determine the internal circumference the vessel would have if relaxed and under a transmural pressure of 100mmHg (L_{100}), with the vessel being set to $L_1 = 0.9x\ L_{100}$, where active force production has been shown to be maximal. Briefly, vessels were stretched in small increments and passive tension was recorded. The vessel was stretched until the passive tension had reached the limit of 13.3 kPa (100 mmHg). Vessels were then normalized to an internal circumference calculated as 90% of this limit for the remainder of the experiment ($0.9 \times IC_{100}$). Normalized lumen diameter is taken as L_1/π (as described in 'Procedures for investigation of small vessels using small vessel myograph', by Mulvaney).[69]

Vessels were divided into 3 groups, those from women with a BMI<25 (i.e. normal weight) and those with BMI25-29.9 (overweight) and those with BMI≥30 (obese). All tissues were challenged with 60mM K^+ to test viability.

Vessel contractility was assessed by obtaining cumulative concentration-response curves to the agonists arginine vasopressin (AVP, 0.1 – 10nM) and the thromboxane analogue, U-46619 (1nM – 3μM). The response to each concentration was allowed to reach a plateau before the next concentration was applied. To measure relaxation, vessels were pre-contracted with either 10nM AVP or 1μM U-46619. Once the contractile response had stabilised, vessels were challenged with bradykinin (BK, 1nM - 3μM), carbachol (CCh, 1nM - 10μM or single concentration of 10μM) or the NO donor, S-nitroso-N-actetyl-DL-penicillamine (SNAP, 20μM). Again each agonist was applied for as long as required to attain a steady state response, or for 10 min if no response was observed. In parallel experiments, membrane cholesterol was extracted from vessels (from women with BMI>30) using methyl-β-cyclodextrin (MCD), a cyclic oligosaccharide which sequesters cholesterol as previously described. [28, 30, 62] Vessels were pre-treated with 2% MCD for 20 min, washed x2 and equilibrated for 30 min prior to assessing contractility and relaxation.[28] L-NAME was used to assess the effect of inhibiting nitric oxide synthase (NOS). Vessels were precontracted with 10nM AVP. Once the response had stabilised, 100μM L-NAME was applied for 30min, after which BK concentration-response curves were obtained.

Confocal microscopy

Isolated myometrial arteries and strips of myometrium with vessels running through them were incubated with 15μM Cal-520 for 2.5h at 34ºC, in the presence of 0.25% of the non-ionic detergent Pluronic F-127. The tissue was then placed in physiological salt solution to allow de-esterification of the dye. Vessels and strips were mounted under a small amount of isometric tension between two fixed aluminium foil clips at the bottom of the chamber, on the stage of an Olympus inverted microscope, as previously described. [28, 29, 55] The chamber was perfused with physiological salt solution at a constant flow rate (1ml/min) and maintained at

34°C. Experiments were performed using an Ultraview LCI spinning (Nipkow) disc, wide-field confocal microscope (Perkin Elmer, Cambridge, UK), equipped with an Orca ER cooled CCD camera (Hamamatsu Photonics, UK) and a 20x objective (N.A. 0.7; see [70]). Mean fluorescence intensity was measured on-line from regions of interest drawn over individual cells using UltraView software. Well-loaded cells were chosen for analysis, with up to 10 individual cells analysed per tissue strip. Movement artefacts were occasionally a problem, however if substantial movement occurred, measurements were not made. The numerical data obtained were saved to an ASCII file for further analysis using Origin 7.0 software. The amplitude of the $[Ca^{2+}]_i$ signal was expressed as a normalised pseudo ratio of Cal-520 fluorescence (F/F_0). Once a stable baseline signal was established, the agonist carbachol was applied at 10μM and bradykinin at 1μM. The resulting Ca^{2+} signals were measured in terms of the peak amplitude of the response and the percentage of cells responding to CCh and bradykinin. Similar experiments were carried out in the presence of 10nM AVP. The timecourse of BK-mediated calcium responses was assessed by measuring the time difference between the first and last cells to respond to the agonist.

Data Analysis

Contractility data were recorded using LabChart Data Acquisition software (ADInstruments). Contractile amplitude of KCl was expressed as mN/mm and other agonist data (AVP, U46619) were normalised to the KCl response obtained in that tissue (expressed as % of the KCl response). Relaxation is expressed as % reduction of the initial pre-contraction. If multiple vessels were obtained from one biopsy, then the data were averaged (n=number of biopsies). Concentration-response curves were fitted to the logistic equation using non-linear regression (PRISM, version 5.0; Graph Pad Software Inc., San Diego, CA, USA). Data that is

normally distributed is expressed as mean ± s.e.m. and non-parametric data is expressed as median (interquartile range). Statistical differences were tested with the Student's *t* test, Mann Whitney test, ANOVA or Wilcoxon signed rank test as appropriate, using PRISM. $p < 0.05$ was considered statistically significant.

Drugs and solutions

Unless otherwise specified, chemicals were obtained from Sigma (UK). Pluronic-F127 was obtained from Invitrogen, Cal-520 from Stratech Scientific Ltd (UK) and U46619 from Cambridge Bioscience (UK). Cal-520 was made up to a stock concentration of 15mM in DMSO, and diluted to 15µM in physiological salt solution as required. A SNAP stock solution (100mM) was prepared in DMSO and bradykinin (10mM) was prepared in 0.1M acetic acid, however the final concentration of DMSO and acetic acid in the myograph bath was negligible and no vehicle control was required (0.02% DMSO or 0.00006% acetic acid). All other drugs were made up in aqueous solution.

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Conflict of Interest

There are no Conflicts of Interest to declare.

Figure legends

Figure 1: Effect of BMI on vessel contractility. Experimental traces showing the effect of U-46619 in a myometrial artery from **(A)** a normal weight woman and **(B)** a woman with BMI≥25 (BMI 29). **(C)** Mean concentration-response curves comparing the effect of U-46619 in the normal weight (n=13), overweight (n=12) and obese (n=7) groups. Experimental traces showing the effect of AVP in a myometrial artery from **(D)** a normal weight woman and **(E)** a woman with BMI≥25 (BMI 36.3). **(F)** Mean concentration-response curves comparing the effect of AVP in the normal weight (n=15), overweight (n=8) and obese (n=3) groups.

Figure 2: Effect of BMI on bradykinin-mediated relaxation. Mean concentration-response curves comparing the relaxatory effect of BK in vessels pre-contracted with **(A)** 10nM AVP (normal weight (n=10), overweight (n=7) and obese (n=6)), and **(B)** pre-contracted with 1μM U-46619 (normal weight (n=15), overweight (n=10) and obese (n=5)).

Figure 3: **Effect of BMI on carbachol-mediated relaxation. (A)** Experimental trace from the myometrial artery of a normal weight woman (BMI<25) showing a relaxatory concentration-response curve to CCh (after pre-contraction with AVP). **(B)** Experimental trace from the myometrial artery of an overweight woman (BMI 26.3), showing no response to CCh, but normal relaxation to BK (after pre-contraction with AVP). **(C)** Bar chart demonstrating that the mean BMI of vessels that respond to CCh is significantly lower than the BMI of vessels that did not respond to CCh. **(D)** Graph demonstrating that the % of

vessels responding to CCh is related to maternal BMI. **(E)** Concentration-response curve for CCh in vessels from normal weight women.

Figure 4: Effect of SNAP and MCD. (A) Experimental trace showing the relaxatory effect of SNAP (20μM) in myometrial arteries pre-contracted with AVP (BMI 26.3). **(B)** Bar chart demonstrating that SNAP produced a similar degree of relaxation in vessels from normal weight, overweight and obese women. **(C)** Concentration-response curves for BK in vessels from obese women (BMI>30) in the absence or presence of MCD.

Figure 5: The role of eNOS in bradykinin-mediated relaxation. Effect of 100μM L-NAME on AVP pre-contraction in vessels from (**A**) normal weight (n=6) and (**B**) BMI≥25 women (n=5). Effect of L-NAME on the BK concentration-response curve in vessels from (**C**) normal weight (n=5-6) and (**D**) BMI≥25 (n=4-5) women.

Figure 6: Confocal imaging of endothelial cell calcium responses. (A) Confocal image of myometrial artery endothelial cells (loaded with Cal-520) from a normal weight woman (BMI<25). **(B)** Experimental trace showing the effect of 10μM CCh and 1μM BK in one of the endothelial cells shown (BMI<25). **(C & D)** Experimental traces showing the effect of 10μM CCh and 1μM BK in myometrial artery endothelial cells from women with BMI≥25. The Ca^{2+} response to BK can be transient **(C)** or sustained **(D)** in nature.

Figure 7: Comparison of carbachol and bradykinin-mediated calcium responses. Amplitude of the Ca^{2+} response to BK in myometrial artery endothelial cells was significantly larger than the response to CCh in both normal weight **(A)** and BMI≥25 women **(B)**. **(C)** A significantly larger proportion of myometrial artery endothelial cells from normal

weight women responded to BK with a rise in intracellular Ca^{2+} compared to CCh. **(D)** In myometrial arteries from women with BMI≥25, almost no endothelial cells responded to CCh, whereas most cells still responded to BK with a rise in intracellular Ca^{2+}.

Video legends

Video file 1: Endothelial cell response to CCh and BK in a myometrial artery from a normal weight woman (BMI 22), loaded with the calcium-sensitive indicator Cal-520.The initial response seen is due to application of 10µM CCh and the subsequent larger calcium response it due to application of 1µM BK.

Video file 2: Endothelial cell response to CCh and BK in a myometrial artery from a woman with BMI 29.9, loaded with the calcium-sensitive indicator Cal-520. 10µM CCh is applied at the beginning but no response is seen. When 1µM BK is applied (45s), a large endothelial cell response is observed.

Video file 3: Endothelial cell response to SNAP in a myometrial artery from a normal weight woman, loaded with the calcium-sensitive indicator Cal-520. 20µM SNAP is applied from 5-65s but no response is observed.

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Figure 1

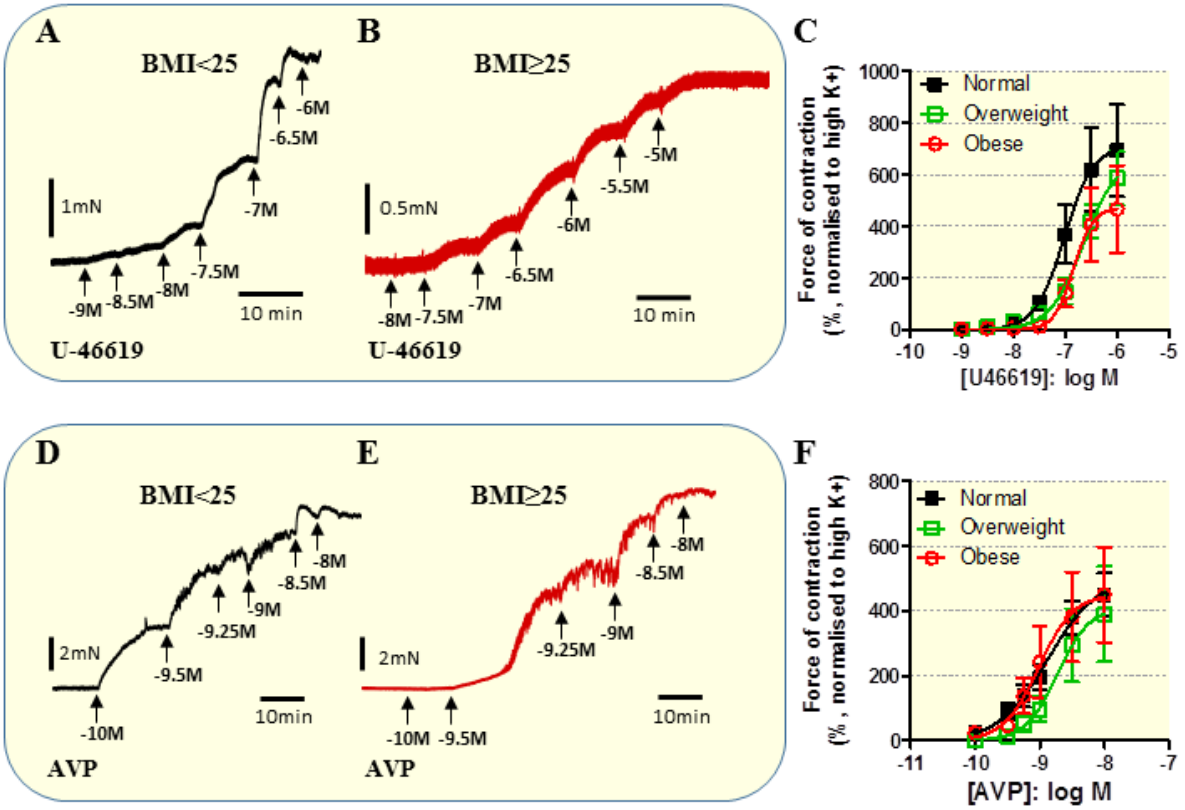

A
BMI<25
1mN
-6M
-6.5M
-7M
-7.5M
-8M
-8.5M
-9M
10 min
U-46619
B
BMI≥25
0.5mN
-5M
-5.5M
-6M
-6.5M
-7M
-7.5M
-8M
10 min
U-46619
C
Force of contraction (%, normalised to high K+)
Normal
Overweight
Obese
[U46619]: log M
D
BMI<25
2mN
-8M
-8.5M
-9M
-9.25M
-9.5M
-10M
10min
AVP
E
BMI≥25
2mN
-8M
-8.5M
-9M
-9.25M
-9.5M
-10M
10min
AVP
F
Force of contraction (%, normalised to high K+)
Normal
Overweight
Obese
[AVP]: log M

Figure 2

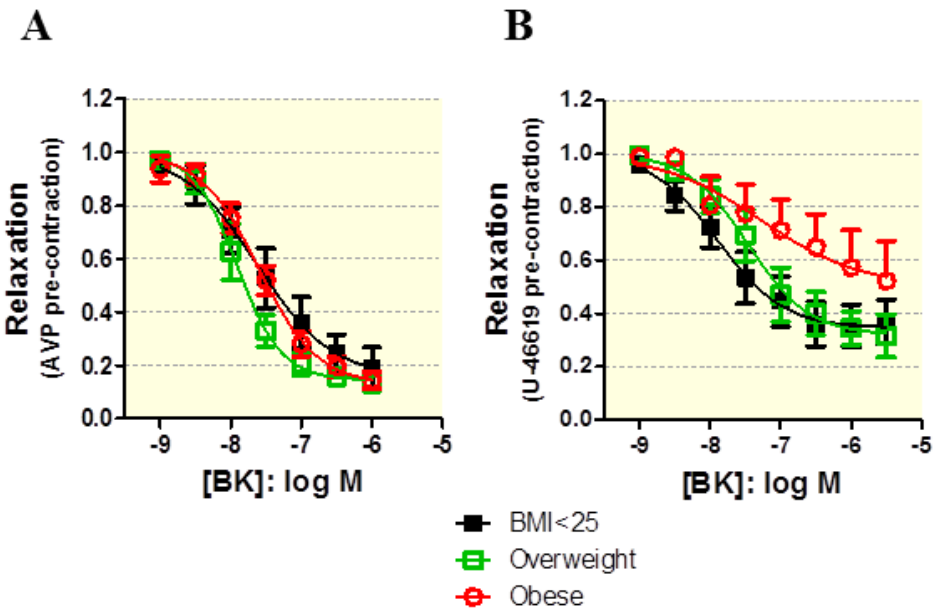

A
B
Relaxation
(AVP pre-contraction)
Relaxation
(U-46619 pre-contraction)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9
-8
-7
-6
-5
[BK]: log M
[BK]: log M
BMI<25
Overweight
Obese

Figure 3

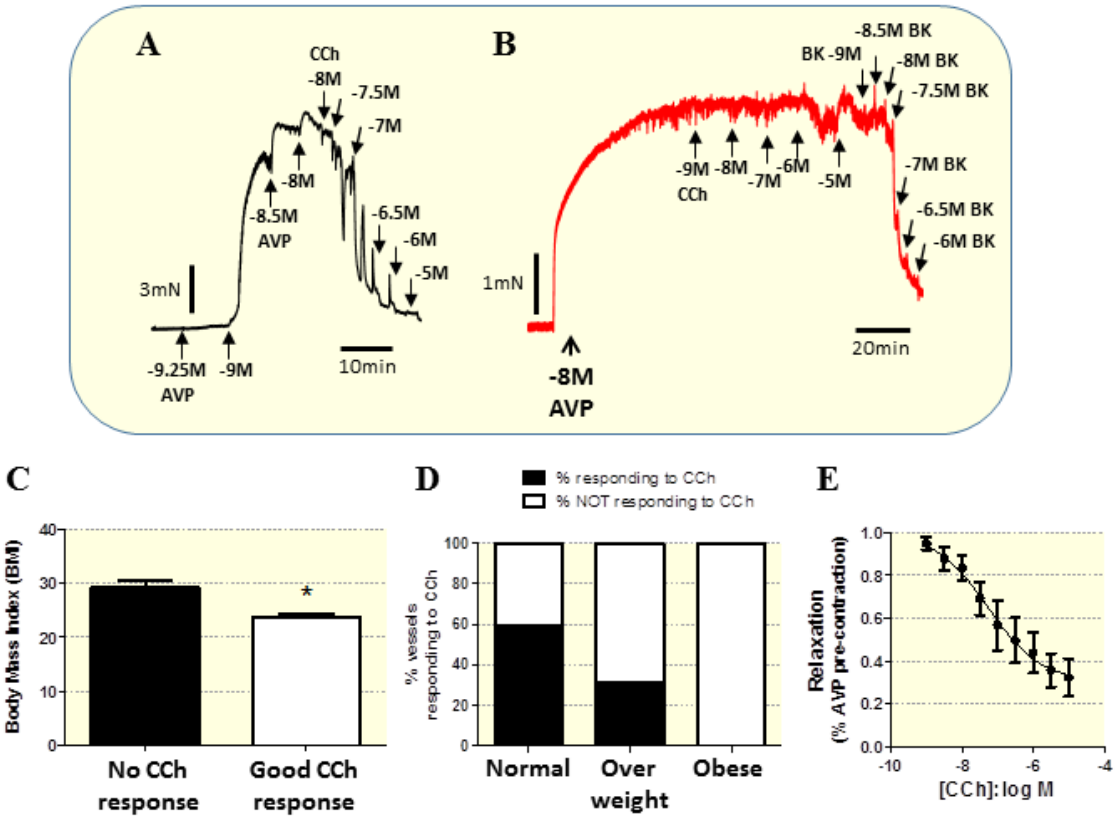

A
CCh
-8M
-7.5M
-7M
-8M
-8.5M
AVP
-6.5M
-6M
-5M
3mN
-9.25M
AVP
-9M
10min
B
-8.5M BK
BK -9M
-8M BK
-7.5M BK
-9M
CCh
-8M
-7M
-6M
-5M
-7M BK
-6.5M BK
-6M BK
1mN
20min
-8M
AVP
C
Body Mass Index (BMI)
40
30
20
10
0
*
No CCh response
Good CCh response
D
% responding to CCh
% NOT responding to CCh
% vessels responding to CCh
100
80
60
40
20
0
Normal
Over weight
Obese
E
Relaxation (% AVP pre-contraction)
1.0
0.8
0.6
0.4
0.2
0.0
-10
-8
-6
-4
[CCh]: log M

Figure 4

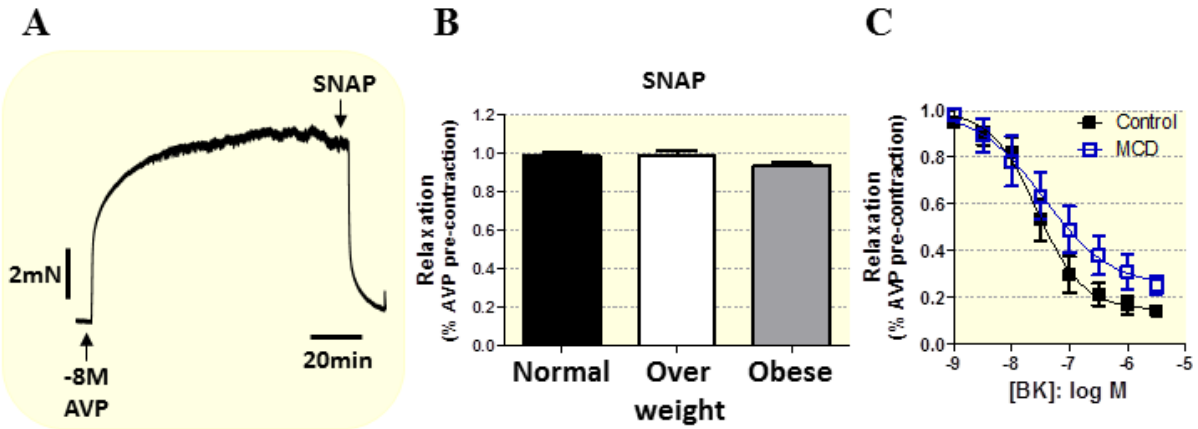

A
SNAP
2mN
-8M
AVP
20min
B
SNAP
Relaxation
(% AVP pre-contraction)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Normal
Over
weight
Obese
C
Relaxation
(% AVP pre-contraction)
1.0
0.8
0.6
0.4
0.2
0.0
-9
-8
-7
-6
-5
[BK]: log M
Control
MCD

Figure 5

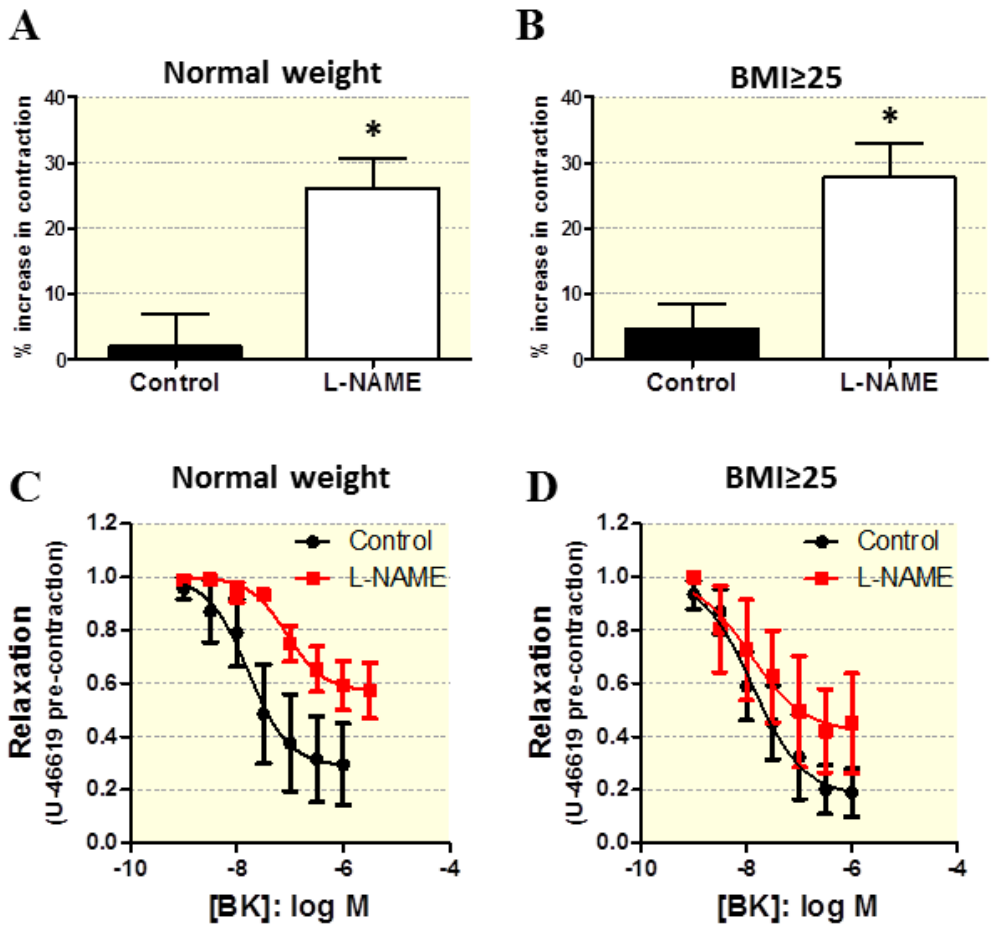

A
Normal weight
% increase in contraction
40
30
20
10
0
*
Control
L-NAME
B
BMI≥25
% increase in contraction
40
30
20
10
0
*
Control
L-NAME
C
Normal weight
Relaxation
(U-46619 pre-contraction)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-10
-8
-6
-4
[BK]: log M
Control
L-NAME
D
BMI≥25
Relaxation
(U-46619 pre-contraction)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-10
-8
-6
-4
[BK]: log M
Control
L-NAME

Figure 6

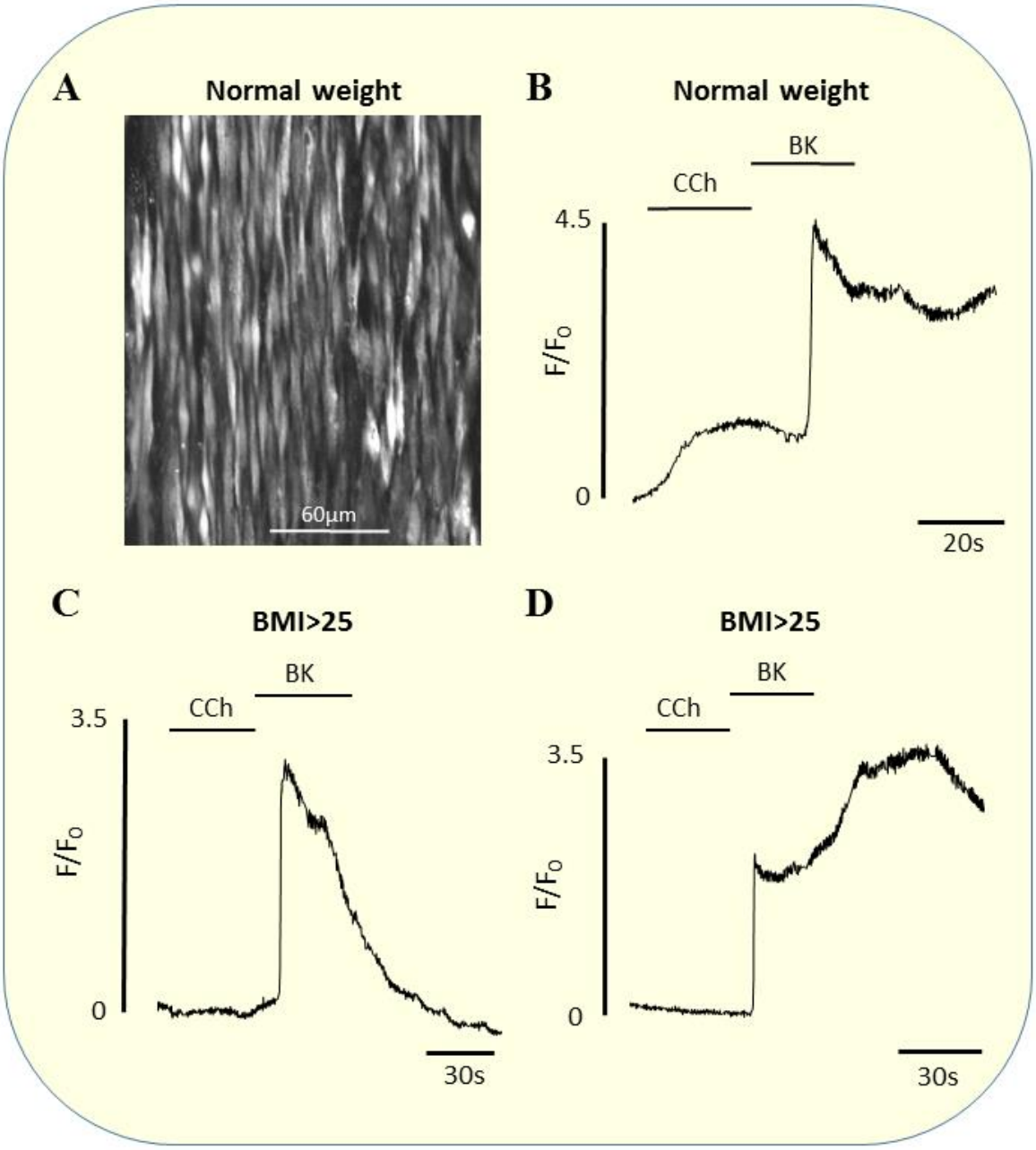
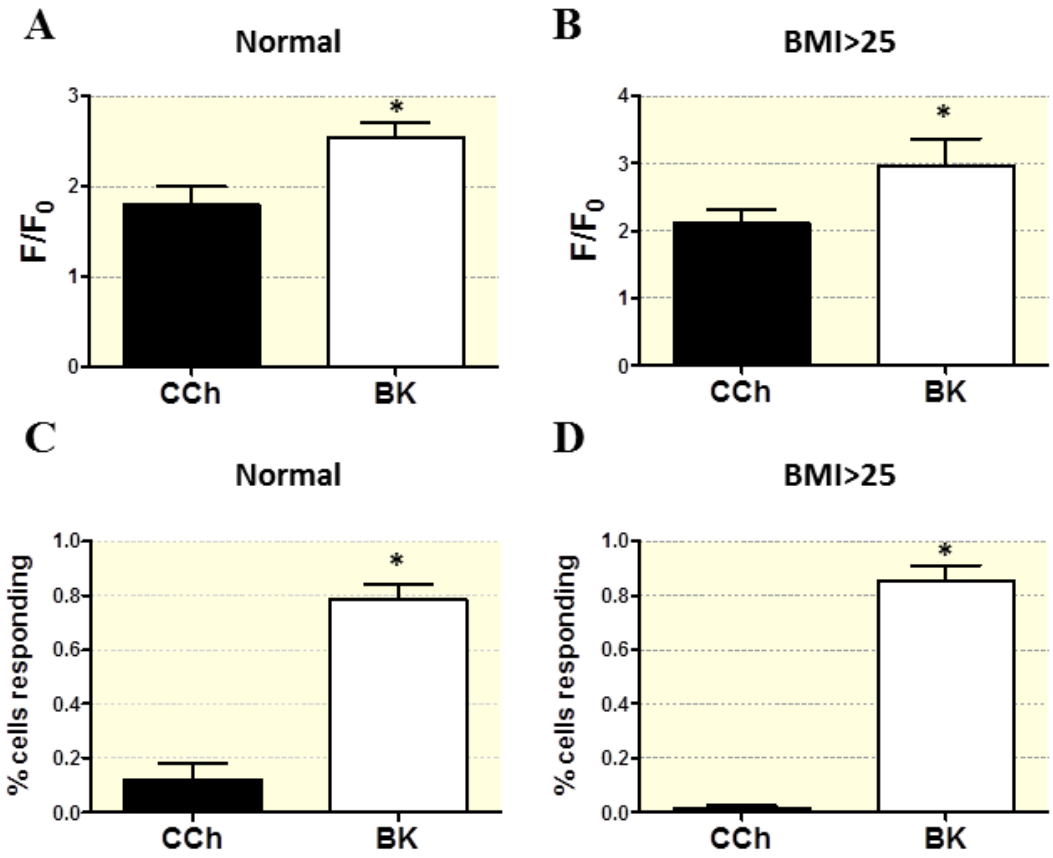

A
Normal weight
60µm
B
Normal weight
BK
CCh
4.5
F/F0
0
20s
C
BMI>25
BK
CCh
3.5
F/F0
0
30s
D
BMI>25
BK
CCh
3.5
F/F0
0
30s

Figure 7


A
Normal
F/F0
3
2
1
0
*
CCh
BK
B
BMI>25
F/F0
4
3
2
1
0
*
CCh
BK
C
Normal
% cells responding
1.0
0.8
0.6
0.4
0.2
0.0
*
CCh
BK
D
BMI>25
% cells responding
1.0
0.8
0.6
0.4
0.2
0.0
*
CCh
BK