



# Altered cyclooxygenase-1 and enhanced thromboxane receptor activities underlie attenuated endothelial dilatory capacity of omental arteries in obesity

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## ABSTRACT

**Aims:** Obesity is a risk factor for endothelial dysfunction, the severity of which is likely to vary depending on extent and impact of adiposity on the vasculature. This study investigates the roles of cyclooxygenase isoforms and thromboxane receptor activities in the differential endothelial dilatory capacities of arteries derived from omental and subcutaneous adipose tissues in obesity.

**Main methods:** Small arteries were isolated from omental and subcutaneous adipose tissues obtained from consented morbidly obese patients ( $n = 65$ , BMI  $45 \pm 6 \text{ kg m}^{-2}$  [Mean  $\pm$  SD]) undergoing bariatric surgery. Relaxation to acetylcholine was studied by wire myography in the absence or presence of indomethacin (10  $\mu\text{M}$ , cyclooxygenase inhibitor), FR122047 (1  $\mu\text{M}$ , cyclooxygenase-1 inhibitor), Celecoxib (4  $\mu\text{M}$ , cyclooxygenase-2 inhibitor), N $\omega$ -Nitro-L-arginine methyl ester (L-NAME, 100  $\mu\text{M}$ , nitric oxide synthase inhibitor) or combination of apamin (0.5  $\mu\text{M}$ ) and charybdotoxin (0.1  $\mu\text{M}$ ) that together inhibit endothelium-derived hyperpolarizing factor (EDHF). Contractions to U46619 (thromboxane A<sub>2</sub> mimetic) were also studied.

**Key findings:** Acetylcholine relaxation was significantly attenuated in omental compared with subcutaneous arteries from same patients ( $p < 0.01$ ). Indomethacin ( $p < 0.01$ ) and FR122047 ( $p < 0.001$ ) but not Celecoxib significantly improved the omental arteriolar relaxation. Cyclooxygenase-1 mRNA and U46619 contractions were both increased in omental compared with subcutaneous arteries ( $p < 0.05$ ). L-NAME comparably inhibited acetylcholine relaxation in both arteries, while apamin + charybdotoxin were less effective in omental compared with subcutaneous arteries.

**Significance:** The results show that the depot-specific reduction in endothelial dilatory capacity of omental compared with subcutaneous arteries in obesity is in large part due to altered cyclooxygenase-1 and enhanced thromboxane receptor activities, which cause EDHF deficiency.

## 1. Introduction

Obesity is a global epidemic and a significant risk factor for type 2 diabetes, hypertension and cardiovascular disease. Qatar has one of the highest rates of both childhood and adult obesity in the world [1], which correlate with increased incidence of type 2 diabetes and cardiovascular disease [2]. Common to these comorbidities is endothelial dysfunction, which together with increasing state of insulin resistance predisposes the obese individuals to adverse cardiovascular events and

increased mortality and morbidity [3].

Although most fat in the body are stored in the subcutaneous depot, the subcutaneous adipose tissue (SAT) is less metabolically active compared with visceral adipose tissue, which includes the omental adipose tissue (OAT) [4]. The metabolic differences between SAT and OAT are amplified by obesity with OAT considered more pathogenic due to the capacity to secrete more proinflammatory cytokines [5,6], which could adversely impact vasomotor function. However, neither the severity of such an impact nor its molecular determinants are well

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understood in a relatively young but morbidly obese population such as in Qatar.

Recently, Farb et al. [7] suggested that a malfunction of the cyclooxygenase (COX) enzyme in the endothelium might contribute to omental endothelial dysfunction in obesity. COX catalysis the first step in the conversion of arachidonic acid into prostanoids, which includes prostacyclin (PGI<sub>2</sub>, a vasodilator) and thromboxane A<sub>2</sub> (TXA<sub>2</sub>, a vasoconstrictor) [8]. Although in most arteries, the balance is normally in the favour of dilator prostanoids, this could change in diseases, such as hypertension and diabetes, leading to vascular dysfunctions [9,10]. Since obesity is commonly associated with both hypertension and diabetes, an increase in constrictor prostanoid activity is therefore a potential risk factor for endothelial dysfunction in affected individuals.

Two isoforms of the enzyme (COX-1 and COX-2) are found in the vascular endothelium but are differentially regulated [11,12]. COX-1 is expressed constitutively [13], while COX-2 is in large part induced by shear stress [13,14] and inflammatory stimuli [15]. Mice with disruption of COX-1 activity lost constitutive but not inducible prostaglandin (PG) synthesis [16] and displayed a complete inhibition of endothelium-dependent contractions in isolated aortic rings [17]. On the other hand, COX-2-derived prostanoids were implicated in abnormal vasoconstrictor responses in diabetic animals [18,19]. Thus, both COX-1 [9] and COX-2 derived prostanoids can alter the balance in prostanoid activity [12] and cause endothelial dysfunction. This study tested the hypothesis that differences in COX isoforms and thromboxane A<sub>2</sub> TP receptor activities are the drivers of the depot-specific differences in endothelial dilatory capacities of OAT and SAT arteries in morbid obesity.

## 2. Materials and methods

### 2.1. Patient recruitment

Obese patients undergoing laparoscopic bariatric surgery for weight loss were recruited from the pre-operative clinics of Al Emadi and Hamad general hospitals in Doha, Qatar. The study was approved by the institutional (Anti-Doping Lab Qatar [SCH-ADL-070] and Hamad medical corporation Qatar [SCHJOINT- 111]) and national (Shafallah Medical Genetic Centre [2011-013]) ethics committees and conducted in accordance with institutional guidelines and principles outlined in the World Medical Association Declaration of Helsinki. All participants provided written consents. Patients with coronary artery disease, malignancy or terminal illness, connective tissue disease, immune-compromised conditions and those with substance abuse were excluded.

### 2.2. Anthropometric measurements

Physical measurements of height and weight as well as blood pressure were taken at pre-surgery clinic visits. Body mass index (BMI) was calculated as the weight (kg) divided by the square of the height (m<sup>2</sup>).

### 2.3. Blood and adipose tissue collection

On the day of surgery and following an overnight fast, blood samples were obtained.

During surgery, samples of SAT and OAT were collected into a serum-free medium (Cellgro, Mediatech Manassas, VA). Both blood and adipose tissue (AT) samples were quickly transported to the laboratory where the blood was separated into plasma and arteries were isolated from within the ATs and assessed for reactivity. The remaining ATs were digested with collagenase to obtain the stromal vascular fraction (SVF) as previously described [20]. Plasma, SVF and arteries (not used for reactivity tests) were stored at -80 °C until analyzed for lipids, insulin and glucose in plasma and COX-1 and COX-2 mRNA in SVF and arteries.

**Table 1**  
Patients' characteristics.

parameter	Value
No. of patients (Male/Female)	22/44
Age (year)	35 (10)
BMI (Kg.m <sup>-2</sup> )	45.1 (6.3)
Systolic blood pressure (mmHg)	128 (15)
Diastolic blood pressure (mmHg)	72 (10)
Hypertension %	18.2
Triglyceride (mg/dl)	112 (80-149)
Total Cholesterol (mg/dl)	186 (38)
HDL cholesterol (mg/dl)	47 (43-51)
LDL cholesterol (mg/dl)	107 (32)
Fasting plasma Glucose (mg/dl)	117.0 (7.2)
Diabetes Mellitus %	12.1
Insulin (mIU/L)	11.7 (7.2-18.7)
HOMA-IR	3.3 (2.1-6.4)

Data are presented as Mean (SD) or Median (interquartile range).

### 2.4. Assessment of vascular reactivity

#### 2.4.1. Vessel preparation

Small arteries (244 ± 96 μm, normalized internal diameter) were isolated from the ATs under a dissecting microscope and cut into segments (~2 mm long). They were then mounted on wires (40 μm thick) in an automated dual-wire myograph (510 A; JP Trading, Aarhus, Denmark) containing normal physiological salt solution (NPSS). The NPSS contained (in mM) 112 sodium chloride, 5 potassium chloride, 1.8 calcium chloride, 1 magnesium chloride, 25 sodium hydrogen carbonate, 0.5 potassium dihydrogen phosphate, 0.5 sodium dihydrogen phosphate, and 10 glucose (bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> to pH 7.4). Segments were continuously aerated at 37 °C and pre-tensioned to an equivalent of 100 mmHg (13.3 kPa). The normalized luminal diameter of each segment was obtained as described previously [21,22]. An equilibration period of at least 1 h was allowed during which time the vessels were contracted separately with potassium chloride (90 mM) and noradrenaline (10 μM) to optimize vessel response.

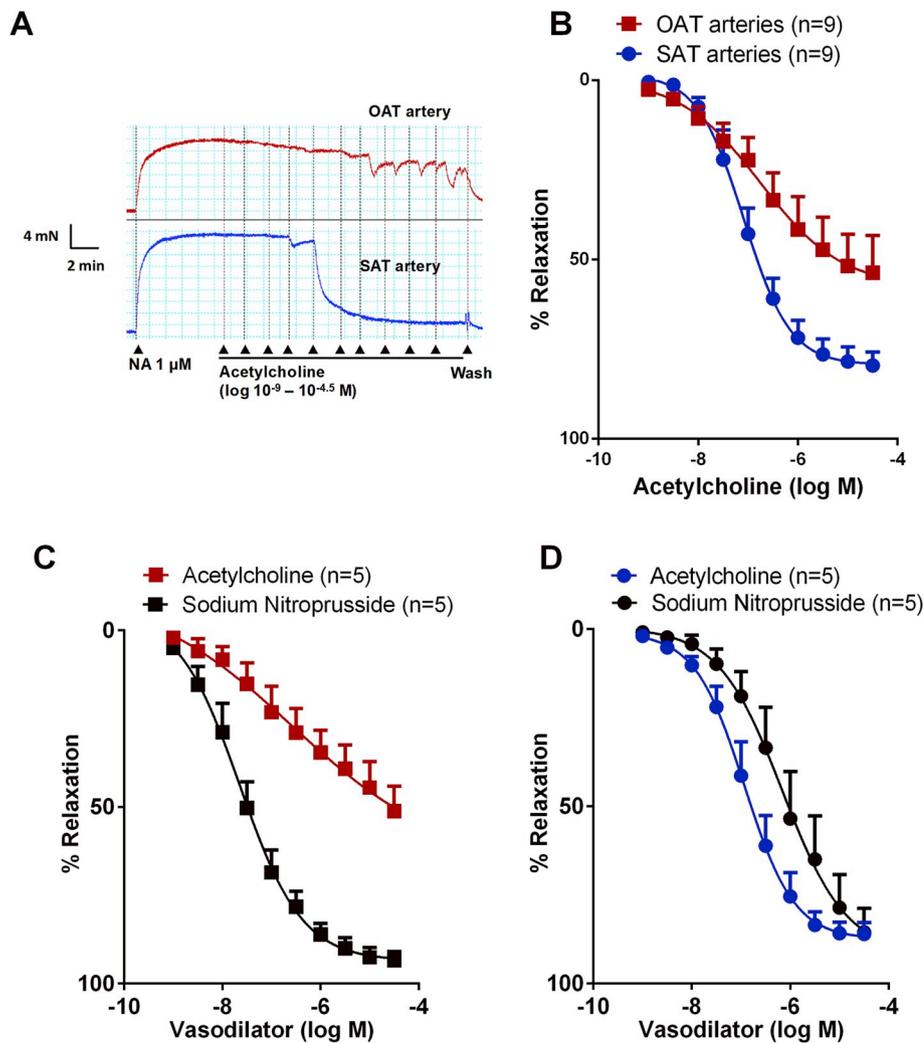
#### 2.4.2. Experimental protocol

**2.4.2.1. Assessment of endothelium-dependent vasorelaxation.** Following equilibration, segments were stimulated with noradrenaline (1-5 μM) to build initial tone. When the tone was stable, acetylcholine (ACh, 10<sup>-9</sup> - 10<sup>-4.5</sup> M), the classical endothelium-dependent dilator was added cumulatively starting with the lowest concentration, and relaxation responses were recorded.

**2.4.2.2. Assessment of endothelium-independent vasorelaxation.** To measure the ability of the segments to relax independently of the endothelium, ACh was replaced with sodium Nitroprusside (SNP, 10<sup>-9</sup> - 10<sup>-4.5</sup> M), a direct nitric oxide (NO) donor and applied cumulatively to the same segments.

The generation of curves for both ACh and SNP on the same segments allowed for comparison between endothelium-dependent and -independent relaxation of same artery.

**2.4.2.3. Determination of roles of eNOS, COX and EDHF in the endothelium-dependent relaxation of SAT and OAT arteries.** Endothelial nitric oxide synthase (eNOS), cyclooxygenase enzyme (COX), and endothelium-derived hyperpolarizing factor (EDHF) are the 3 main mediators of endothelium-dependent relaxation. To determine their contributions to ACh relaxation of SAT and OAT arteries, curves were generated in the presence of 100 μM L-NAME, 10 μM indomethacin or a combination of 0.5 μM apamin + 0.1 μM charybdotoxin, respectively. L-NAME and indomethacin were added at least 30 min before ACh curves were generated. Apamin + charybdotoxin were applied at least



**Fig. 1.** Concentration-relaxation curves for acetylcholine or sodium nitroprusside in human omental (OAT) and subcutaneous (SAT) adipose tissue arteries. Artery segments were pre-contracted with 1–5  $\mu\text{M}$  noradrenaline and agonists doses added in a cumulative fashion once the contractions had plateaued. (A) Typical recording of Acetylcholine relaxation in OAT and SAT arteries from same patient, which clearly demonstrates attenuated endothelium-dependent relaxation in OAT compared with SAT arteries. (B) Relaxation curves for acetylcholine in both arteries. Acetylcholine relaxation was significantly attenuated in OAT compared with SAT arteries from same patients ( $p = 0.0179$  between the curves,  $n = 9$ , 2-way ANOVA). (C) Comparison between Acetylcholine and Sodium nitroprusside relaxation in the same OAT arteries. Acetylcholine curve was significantly shifted to the right of the curve for Sodium nitroprusside ( $p = 0.0104$ , between the curves,  $n = 5$ , 2-way ANOVA). (D) In contrast to OAT arteries, SAT arteries were slightly more responsive to Acetylcholine compared with Sodium nitroprusside, although the curves were not statistically different from each other ( $n = 5$ ). Data are expressed as % relaxation (mean  $\pm$  SEM) of the initial tone induced by noradrenaline and were fitted with GraphPad Prism software.

10 min prior to the construction of ACh curve.

**2.4.2.4. Assessment of thromboxane  $A_2$  TP receptor-mediated contractile responsiveness.** Following equilibration, cumulative concentration-contraction curves were generated separately for U46619 (thromboxane  $A_2$  analogue,  $10^{-9}$  -  $10^{-5.5}$  M) and noradrenaline ( $10^{-9}$  -  $10^{-4.5}$  M) on the same segment with a washout period of at least 30 min between the curves. This protocol allowed for comparison between thromboxane  $A_2$  TP receptor-mediated vs. noradrenergic receptor-mediated contractile responsiveness of the same arterial segment.

## 2.5. COX mRNA expression in stromal vascular fractions of SAT and OAT by RT<sup>2</sup>-profiler PCR array analysis

SVF was obtained from SAT and OAT as previously described [23]. RNA was extracted using Trizol and following manufacturer's instructions (Invitrogen, Carlsbad, CA USA). One microgram of RNA was used to synthesize cDNA and gene expression profiling was determined using RT<sup>2</sup> Profiler human hypertension PCR arrays (Human hypertension PAHS-037Z, Qiagen Sciences, Maryland, USA), which included COX-1 (PTGS1) and COX-2 (PTGS2) genes. Data were normalized to  $\beta$ -actin and expressed as ratio of target gene Ct/ $\beta$ -actin Ct.

### 2.5.1. COX isoforms mRNA expression in arteries isolated from within SAT and OAT by single Real-Time-PCR analysis

SAT and OAT arteries were ground in liquid nitrogen and RNA

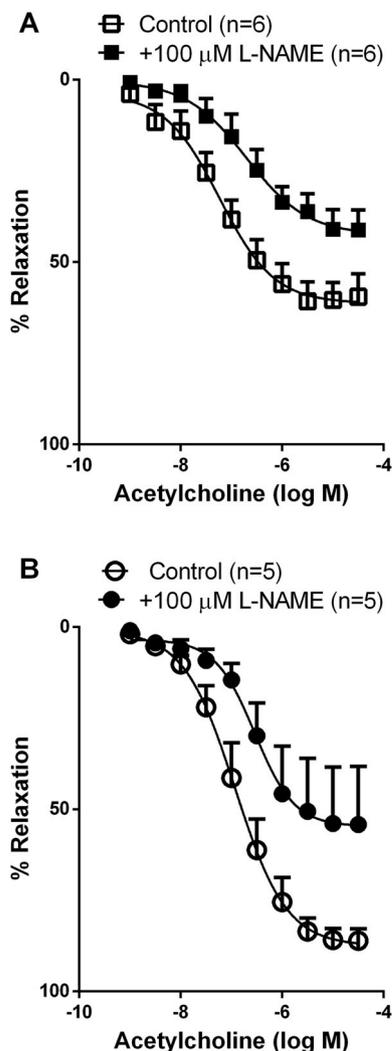
extracted using Trizol following manufacturer's instructions (Invitrogen, Carlsbad, CA USA). cDNA was synthesized from 500 ng total RNA using Reverse Transcription Reagent Kit (Applied Biosystems, Maryland USA). The mRNA expressions of COX-1/2 genes were determined by Real-time PCR performed on ViiA7 real-time PCR system (Applied Biosystems™, Foster City, CA USA) using the following primer sequences (Invitrogen, Carlsbad, CA USA): COX-1: 5'- CATTCTGGCA GAGACCTGTGG -3' and 5'- CGTTACTTCTCTGCCAGC -3' and COX-2: 5'- ACACAACCCAAATTCCCAGGTTT -3' and 5'- GCCTATGTGCTAG CCCACAAAGAA -3'. Data were normalized to  $\beta$ -actin and expressed as ratio of target gene Ct/ $\beta$ -actin Ct.

## 2.6. Assays

Fasting plasma glucose (FPG) and lipids were measured using a routine chemistry analyser (Cobas; Roche Diagnostics, Mannheim, Germany) [20]. Insulin was measured using commercially available ELISAs. Insulin resistance was calculated using the homeostatic model assessment (HOMA), where  $\text{HOMA} = (\text{glucose in mmol/L} \times \text{insulin in mIU/L}) / 22.5$  [24].

## 2.7. Data and statistical analysis

Arterial reactivity data were recorded and analyzed using the Lab Chart software (DMT, Denmark). In response to each dose, peak values of vessel tension for contractile agonists and lowest value for relaxing agonists were recorded. Increases in tension were calculated as peak



**Fig. 2.** Concentration-relaxation curves for acetylcholine in the absence or presence of 100  $\mu$ M L-NAME in human omental (OAT) and subcutaneous (SAT) adipose tissue arteries. Artery segments were pre-contracted with 1–5  $\mu$ M noradrenaline and acetylcholine ( $10^{-9}$  -  $10^{-4.5}$  M) added in a cumulative fashion once the contractions had plateaued. L-NAME significantly inhibited acetylcholine relaxation in both OAT arteries (A,  $p = 0.0035$  between the curves, 2-way ANOVA,  $n = 6$ ) and SAT arteries (B,  $p = 0.0329$  between the curves, 2-way ANOVA,  $n = 5$ ). Data are expressed as % relaxation (mean  $\pm$  SEM) of the initial tone induced by noradrenaline and were fitted with GraphPad Prism software.

values (minus baseline) divided by vessel diameter (mN/ $\mu$ m). Decreases in tension were calculated as percentage relaxation with reference to initial tone due to noradrenaline.

Data were analyzed using GraphPad Prism software version 6.02 (GraphPad Inc. La Jolla, CA USA) and expressed as Mean (SD or SEM) or Median (interquartile range) depending on normality of distributions. Agonist log –concentration response curves were constructed on log scale and fitted using the sigmoidal fitting routine. The concentration of agonist causing 50% of maximum response is expressed as Mean  $-\log EC_{50}$  ( $pEC_{50}$  for contractile agonists) or Mean  $-\log IC_{50}$  ( $pIC_{50}$  for relaxant agonist) and used as a measure of sensitivity to the agonists. Comparisons were made using Student's paired and unpaired t-tests or 2-way ANOVA with post-hoc correction as appropriate. Significance was defined as  $p < 0.05$ .  $n =$  number of samples or experiments.

## 2.8. Drugs/Reagents

Noradrenaline hydrochloride, acetylcholine, L-NAME, indomethacin and collagenase were purchased from Sigma (Poole, Dorset, UK). U46619, Apamin and charybdotoxin were purchased from Tocris (Bristol, UK). All stock solutions were made up in distilled water except indomethacin that was prepared in DMSO.

Insulin ELISA was from Mercodia Diagnostics (Uppsala, Sweden). RT<sup>2</sup> Profiler human hypertension PCR arrays and cDNA synthesis kits were from Applied Biosciences (Qiagen Sciences, Maryland, USA) and Qiagen (Hilden, Germany). Trizol and Single RT-PCR primers for COX isoforms were obtained from Invitrogen (Carlsbad, CA USA).

## 3. Results

### 3.1. Patients characteristics

Samples were obtained from a total of 66 obese patients (67% females). The patients' characteristics are summarized in Table 1. Notably, the patients were morbidly obese with body mass index (BMI > 40; obese BMI > 30 kg m<sup>-2</sup>), insulin-resistant (HOMA index of insulin resistance >2) and hyperinsulinemic (plasma insulin >7.0 mIU/L). Twelve (18%) of the patients had history of hypertension and 8 (12%) had history of type-2 diabetes. Overall, they had relatively normal lipid profile and blood pressure at the time of sampling.

### 3.2. Comparison of endothelium-dependent relaxation between arteries derived from OAT and SAT

Endothelial function was evaluated using magnitude of ACh relaxation (classical endothelium-dependent relaxation) recorded in OAT and SAT arteries. Typical recordings of ACh-induced relaxation in both arteries from same patient are shown in Fig. 1A. ACh relaxation was significantly attenuated in OAT compared with SAT arteries (Fig. 1B,  $p < 0.001$  between the curves,  $n = 9$ , 2-way ANOVA). Mean maximum relaxation to ACh was  $56.22 \pm 8.57\%$  in OAT arteries compared with  $79.54 \pm 3.76\%$  in SAT arteries ( $p < 0.05$ ,  $n = 9$ , Bonferroni test).

### 3.3. Investigation of endothelium-independent relaxation of OAT and SAT arteries

The ability of OAT or SAT arteries to relax independently of the endothelium was assessed by their relaxation to SNP (a NO donor and endothelium-independent dilator). SNP caused significantly greater relaxation of OAT arteries compared with ACh applied to the same segments (Fig. 1C,  $p = 0.0104$  between the curves,  $n = 5$ , 2-way ANOVA). In contrast, SAT arteries were slightly less responsive to SNP compared with ACh on the same segments (Fig. 1D), but the curves were not statistically different.

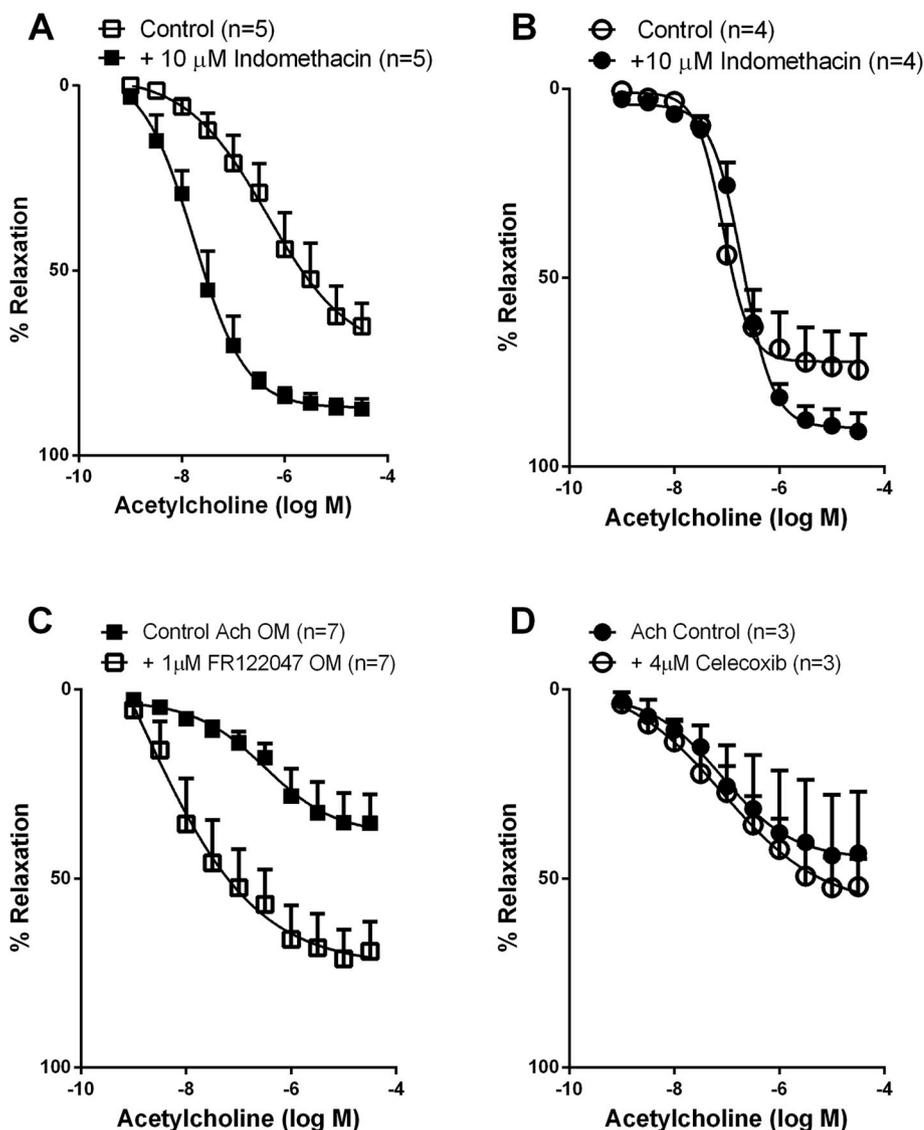
### 3.4. Roles of eNOS, COX and EDHF in the endothelium-dependent relaxation of OAT and SAT arteries

#### 3.4.1. Role of eNOS

Nitric oxide (NO) is a major mediator of endothelium-dependent vasodilation. Thus, we investigated NO contribution to ACh relaxation of both OAT and SAT arteries. In the presence of the NO synthase inhibitor, L-NAME (100  $\mu$ M), ACh relaxation was inhibited with significant rightward shifts in the curves for both OAT (Fig. 2A,  $p < 0.01$ , 2-way ANOVA) and SAT (Fig. 2B,  $p < 0.05$ , 2-way ANOVA) arteries. Maximum relaxation was decreased by 27% in OAT and by 37% in SAT arteries under these conditions.

#### 3.4.2. Role of COX

COX catalyzes the first step in the biosynthesis of prostanoids which contribute to endothelium-dependent regulation of vascular tone.



**Fig. 3.** Concentration-relaxation curves for acetylcholine in the absence or presence of 10  $\mu$ M indomethacin, 1  $\mu$ M FR122047 or 4  $\mu$ M Celecoxib in human omental (OAT) and subcutaneous (SAT) adipose tissue arteries. Artery segments were pre-contracted with 1–5  $\mu$ M noradrenaline and acetylcholine ( $10^{-9}$  -  $10^{-4.5}$  M) added in a cumulative fashion once the contractions had plateaued. Indomethacin enhanced acetylcholine relaxation in OAT arteries (A,  $p = 0.0087$  between the curves, 2-way ANOVA,  $n = 5$ ) but not in SAT arteries (B,  $n = 4$ ). FR122047 significantly enhanced acetylcholine relaxation in OAT arteries (C,  $p = 0.005$  between the curves, 2-Way ANOVA,  $n = 7$ ). In contrast, Celecoxib had no effect on acetylcholine relaxation in OAT arteries (D,  $n = 3$ ). Data are expressed as % relaxation (mean  $\pm$  SEM) of the initial tone induced by noradrenaline and were fitted with GraphPad Prism software.

**3.4.2.1. Effect of nonselective inhibition of COX.** In the presence of indomethacin (10  $\mu$ M), a non-selective COX inhibitor, ACh relaxation was significantly enhanced in OAT ( $p < 0.001$ , 2-way ANOVA, Fig. 3A) but not in SAT arteries (Fig. 3B), which displayed only a slight but insignificant improvement in maximum relaxation to ACh.

**3.4.2.2. Effect of selective inhibition of COX-1 or COX-2 in OAT arteries - role of COX isoforms.** Following the result with indomethacin, which showed that COX activity opposed rather than contributed to endothelium-dependent relaxation of OAT arteries, we investigated the roles of the 2 isoforms of the enzyme (COX-1 and COX-2) in this observation using selective inhibitors. In the presence of 1  $\mu$ M FR122047, a selective COX-1 inhibitor [25,26], ACh relaxation was significantly enhanced ( $p < 0.01$ ,  $n = 7$ , 2-way ANOVA, Fig. 3C) with maximum relaxation increased from  $38.2 \pm 7.3\%$  to  $73.2 \pm 11.9\%$  ( $p < 0.01$ ,  $n = 7$ , Bonferroni test). In contrast, 4  $\mu$ M Celecoxib, a selective COX-2 inhibitor [27,28] had no effect on the ACh curve (Fig. 3D).

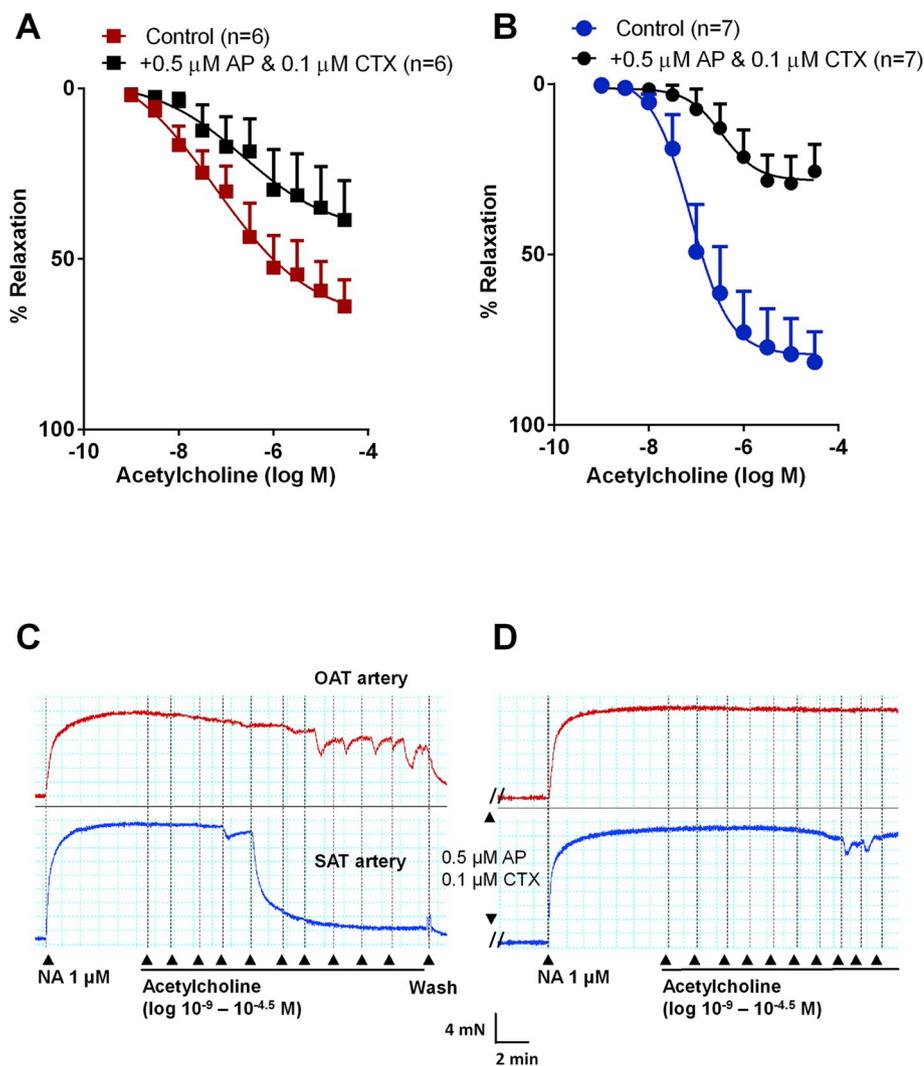
### 3.4.3. Role of endothelium-derived hyperpolarizing factor (EDHF)

Since eNOS and COX activities could not completely account for ACh relaxation in both OAT and SAT arteries, we extended the investigation to determine the role of EDHF. To achieve this, ACh curves were generated in the presence of a combination of 0.5  $\mu$ M apamin

(SKCa blocker) and 0.1  $\mu$ M charybdotoxin (IKCa blocker). Under these conditions, ACh curves for both OAT (Fig. 4A) and SAT (Fig. 4B) were significantly shifted to the right ( $p < 0.01$ ,  $n = 6-7$ , 2-way ANOVA). Maximum relaxation was reduced by 40% ( $63.94 \pm 7.78$  to  $38.56 \pm 11.55$ ,  $p < 0.001$ ,  $n = 6$ , Bonferroni test) in OAT arteries and by 64% ( $81.51 \pm 8.73$  to  $29.12 \pm 7.79$ ,  $p = 0.001$ ,  $n = 7$ , Bonferroni test) in SAT arteries. Typical recordings of ACh relaxation in the absence and presence of apamin and charybdotoxin in both arteries from the same patient are shown in Fig. 4C and D respectively. Sensitivity to ACh was unchanged by pretreatment with apamin and charybdotoxin in both arteries. The  $pIC_{50}$  values were  $7.104 \pm 0.593$  vs.  $5.294 \pm 1.454$  in OAT and  $6.727 \pm 0.382$  vs.  $6.290 \pm 0.145$  in SAT arteries before and after apamin+charybdotoxin treatment respectively.

### 3.5. Thromboxane $A_2$ TP and noradrenergic receptor-mediated contractile responses of OAT vs. SAT arteries

Since COX opposition to endothelium-dependent relaxation can be mediated by increased contractile prostanoid activity via thromboxane  $A_2$  TP receptor activation, we compared the responsiveness of OAT and SAT arteries to U46619, a thromboxane  $A_2$  (TXA<sub>2</sub>) analogue and potent thromboxane  $A_2$  TP receptor agonist. We also compared thromboxane  $A_2$  TP receptor- and noradrenergic receptor-mediated contractile



**Fig. 4.** Concentration-relaxation curves for acetylcholine in the absence or presence of a combination of 0.5  $\mu\text{M}$  apamin and 0.1  $\mu\text{M}$  charybdotoxin in human omental (OAT) and subcutaneous (SAT) adipose tissue arteries. Artery segments were pre-contracted with 1–5  $\mu\text{M}$  noradrenaline and acetylcholine ( $10^{-9}$  -  $10^{-4.5}$  M) added in a cumulative fashion once the contractions had plateaued. The combination of apamin and charybdotoxin significantly inhibited acetylcholine relaxation in both (A) OAT arteries ( $p = 0.0065$  between the curves,  $n = 6$ ) and (B) SAT arteries ( $p = 0.0028$  between the curves,  $n = 7$ ). Typical recordings of acetylcholine relaxation before (C) and after EDHF blockade with the combination of apamin and charybdotoxin (D) in both arteries. Data are expressed as % relaxation (mean  $\pm$  SEM) of the initial tone induced by noradrenaline and were fitted with GraphPad Prism software.

responsiveness of the same arteries.

U46619 contractions were significantly greater in OAT compared with SAT arteries ( $p < 0.01$ , 2-way ANOVA, Fig. 5A). On the other hand, NA contractions were slightly higher in SAT compared with OAT arteries, but there was no statistically significant difference between the curves (Fig. 5B).

Generally, both arteries were more responsive to U46619 compared with noradrenaline (Fig. 5C and D for OAT and SAT respectively). However, significant differences were recorded only in OAT arteries ( $p < 0.001$ , 2-way ANOVA, Fig. 5C), with mean log  $\text{EC}_{50}$  of  $-7.91 \pm 0.10$  and  $-6.67 \pm 0.14$  M for U46619 and noradrenaline respectively in these arteries.

### 3.6. Differential COX-isoforms mRNA expressions in OAT and SAT stromal vascular fractions and arteries

#### 3.6.1. In SVF

COX isoform expression in SVF was measured using RT<sup>2</sup> Profiler PCR arrays for human hypertension genes. COX-2 mRNA was significantly higher compared with COX-1 mRNA in SVFs derived from both OAT (Fig. 6A,  $n = 13$ – $15$ ,  $p = 0.0001$ ,  $t$ -test) and SAT (Fig. 6B,  $n = 10$ – $16$ ,  $p = 0.0001$ ,  $t$ -test).

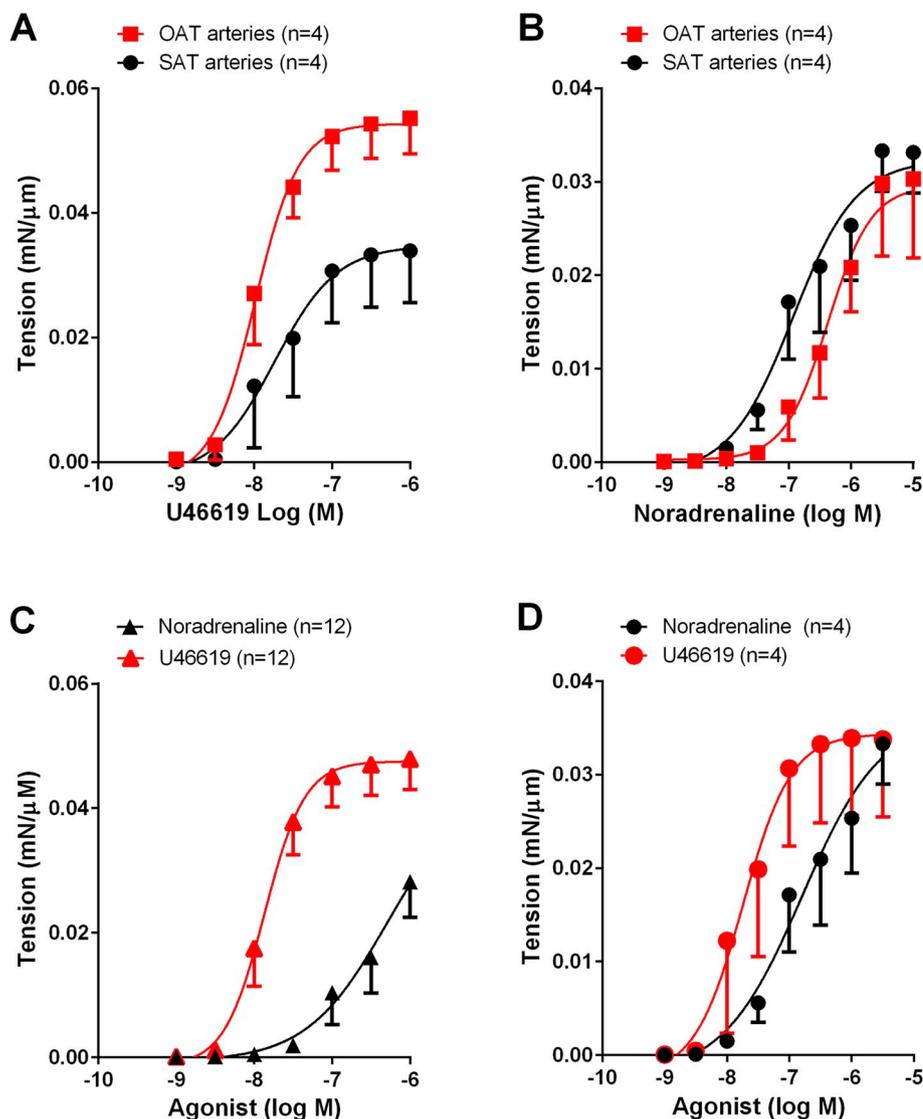
#### 3.6.2. In arteries

Since SVF is a mixture of cells of different origins, including immune cells, which might express COX isoforms differently from vascular

endothelial cells, we decided to specifically analyze the expression of these isoforms in arteries derived from OAT and SAT using single real time RT-PCR. The results showed that COX-1 mRNA was higher in both arteries compared with COX-2. However, significant upregulation of COX-1 was only seen in OAT arteries (Fig. 6C,  $n = 5$ ,  $p = 0.0317$ ,  $t$ -test) but not in SAT arteries (Fig. 6D,  $n = 4$ ).

## 4. Discussion

It is widely recognized that the microvascular circulation is a major target for obesity-related endothelial dysfunction [29]. Although the mechanism of this abnormality is not well understood, it is often attributed to reduction in NO availability [29]. Our data show for the first time that reduction in EDHF rather than NO per se accounts for decreased endothelial dilatory capacity of OAT compared with SAT arteries in morbid obesity. Our data also show that this situation is orchestrated by increased but altered COX-1 and thromboxane  $\text{A}_2$  TP receptor activities, which oppose endothelium-dependent relaxation of these arteries. The depot-specific difference in endothelial dilatory capacity between OAT and SAT arteries is in agreement with previous reports albeit for a different (North American) obese population [30] that also suggested that COX-1-derived vasoconstrictor prostanoids were partly responsible for this abnormality [7]. The current data has gone further to demonstrate that the OAT arteries, which suffer reduced endothelial dilatory capacity, also display enhanced thromboxane  $\text{A}_2$  TP receptor activity, demonstrated by hyper contractile response to the

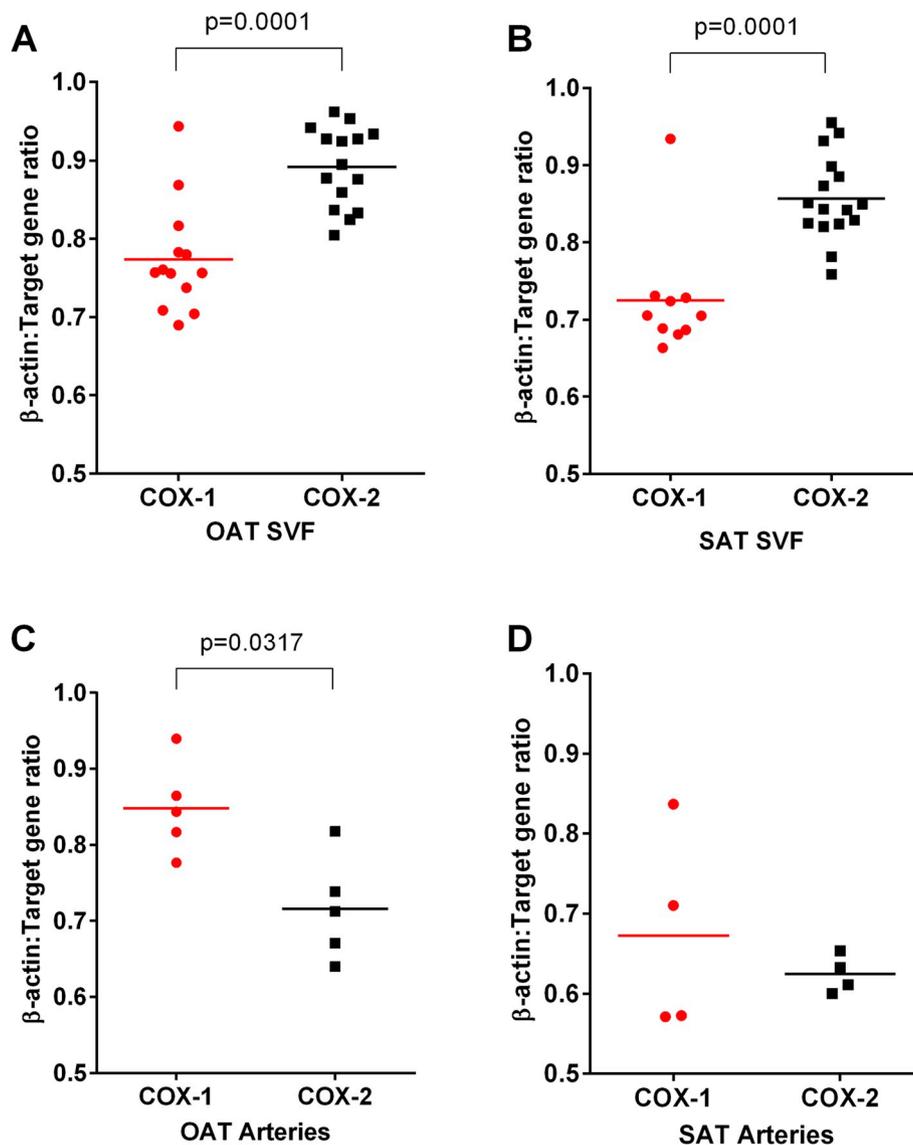


**Fig. 5.** Concentration-contraction curves for U46619 or noradrenaline in human omental (OAT) and subcutaneous (SAT) adipose tissue arteries. U46619 ( $10^{-9}$  -  $10^{-5.5}$  M) or noradrenaline ( $10^{-9}$  -  $10^{-4.5}$  M) were added in a cumulative fashion starting with the lowest dose. U46619 contractions were significantly greater in OAT arteries compared with SAT arteries (A,  $p = 0.002$  between the curves, 2-Way ANOVA,  $n = 4$ ). On the other hand, noradrenaline contractions were slightly greater in SAT arteries compared with OAT arteries, but the differences were not statistically significant (B,  $n = 4$ ). When contractions to both agonists were compared on the same artery segments, U46619 contractions were significantly greater compared with noradrenaline contractions in OAT arteries (C,  $p < 0.0001$  between the curves, 2-Way ANOVA,  $n = 12$ ) but not in SAT arteries (D,  $n = 4$ ). The curves were generated by fitting data with GraphPad Prism software.

potent and selective thromboxane  $A_2$  TP receptor agonist U46619 [31] compared with SAT arteries. This provides the first direct evidence for the greater vulnerability of human OAT arteries to exaggerated contractile prostanoid activity that has the potential to limit their endothelium-dependent dilatory capacity in morbid obesity. Moreover, both increased  $TXA_2$  gene expression and prostanoid-mediated vasoconstriction have been reported in obesity [32]. The data is also consistent with COX-1 being the primary isoform related to endothelium-dependent contractions [9,17,33,34] and enhanced thromboxane  $A_2$  TP receptor-dependent vascular contractions being associated with metabolic diseases [32,35]. Together, COX-1/ $TXA_2$  pathway has been shown to mediate vascular hypercontractility linked to endothelial dysfunction in obesity [32] and experimental model of hyperlipidemia [36]. Thromboxane  $A_2$  TP receptor is coupled to guanine nucleotide-binding protein  $G_q$  in vascular smooth muscle (VSM) [37]. Activation of the receptor stimulates membrane-bound  $G_q$ -dependent phospholipase C (PLC)  $\beta$  to hydrolyze Phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) to inositol triphosphate ( $IP_3$ ) and diacyl glycerol (DAG) [38].  $IP_3$  activates the mobilization of intracellular  $Ca^{2+}$ , and DAG activates PKC, to initiate and promote vasoconstriction respectively.

Unlike a previous report [30], the reduction in OAT arteriolar endothelial dilatory function in the current study is not due to absolute lack of endothelial NO. NO accounted for about 27% of OAT relaxation and 37% of SAT relaxation. The difference of 10% in NO involvement in

the relaxation of these arteries is substantially less than the difference (25%) between their ACh curves, which suggests that a reduction in NO does not account for a larger part of the reduction in endothelial dilatory capacity recorded in the OAT compared with SAT arteries. Moreover, NO role is known to decrease with vessel size. NO diffuses from the endothelium to the underlying vascular smooth muscle and activates the soluble Guanylyl cyclase (sGC)/cyclic guanine monophosphate (cGMP)/protein kinase G (PKG) pathway to cause relaxation [39]. Since NO alone could not fully account for ACh relaxation of these arteries, and COX activity opposed their relaxation, the role of EDHF, which is known to increase with smaller vessel size [40,41] was explored. The data confirmed that the differences in endothelial dilatory capacities between OAT and SAT arteries were largely due to differences in EDHF contributions, which were markedly reduced in OAT compared with SAT arteries. While NO and EDHF contributions could fully account for ACh relaxation of SAT arteries, they (EDHF in particular) fell significantly short in OAT arteries, resulting in marked reduction in endothelial dilatory capacity and consistent with EDHF being the major mediator of endothelial-dependent vasodilation in human small vessels [42]. Although it was not possible to obtain omental arteries from non-obese individuals to determine the extent of EDHF contribution to normal endothelial dilatory capacity of these vessels, Gillham et al. [43] did establish that a significant component of the endothelial vasodilatory capacity of omental arteries isolated from



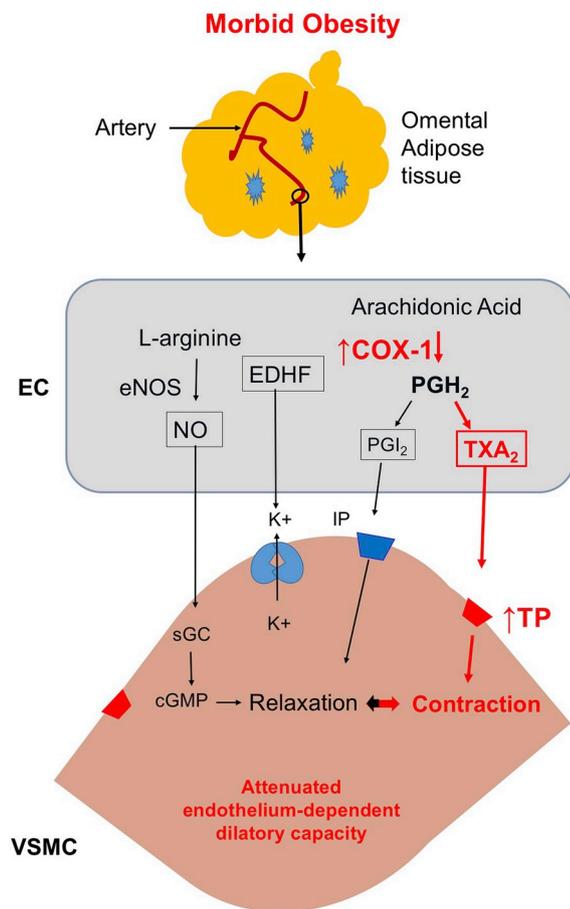
**Fig. 6. Tissue COX isoform gene expression profiles.** In the adipose tissue stromal vascular fractions (SVF), COX-2 mRNA expression was significantly increased in both (A) OAT ( $p = 0.0001$   $t$ -test,  $n = 15$ ) and (B) SAT ( $p = 0.0002$   $t$ -test,  $n = 16$ ) compared with COX-1 ( $n = 13$  and  $10$  for OAT and SAT respectively). On the other hand, the arteries expressed more COX-1 mRNA compared with COX-2, with significant differences in (C) OAT arteries ( $p = 0.0317$   $t$ -test,  $n = 5$ ) but not in (D) SAT arteries (D,  $n = 4$ ).

normal pregnant women is independent of NO/prostacyclin synthesis and attributable to EDHF.

Abnormal endothelial function could alter the mechanism as well as mediators of ACh relaxation. Both NO and EDHF mediate ACh relaxation of healthy human SAT arteries [42,44] and the current data show that the situation for SAT arteries remains relatively unchanged even in morbid obesity. The greater role played by EDHF in SAT arteries, which had better endothelial dilatory capacity compared with OAT arteries suggests that its role in these vessels was not a compensatory response to reduction in NO as had been reported under certain conditions [44,45]. Rather, the data suggest that the shortfall in OAT capacity is largely a direct consequence of a deficiency in EDHF itself and to a lesser extent to a reduction in NO bioavailability. Moreover, chronic exposure to proinflammatory mediators can downregulate EDHF formation in arteries [40] consistent with the current data. EDHF is released by the endothelium when endothelial cell calcium concentration rises and triggers the opening of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels of small ( $\text{SK}_{\text{Ca}}$ ) and intermediate ( $\text{IK}_{\text{Ca}}$ ) conductances [46]. The opening of these channels is followed by the release of hyperpolarizing factor(s) onto the underlying vascular smooth muscle cells to cause relaxation [46].

Incidentally, thromboxane  $\text{A}_2$  TP receptor stimulation is associated with loss of small-conductance  $\text{Ca}^{2+}$ -activated potassium ( $\text{SK}_{\text{Ca}}$ ) channel activity and decreased EDHF-mediated responses in the rat mesenteric artery [47]. Thus, both absolute EDHF deficiency and the opposing effect of increased thromboxane  $\text{A}_2$  TP receptor activity could explain the reduction in EDHF role in OAT compared with SAT arteries in the current study. Although charybdotoxin can also block the large conductance,  $\text{Ca}^{2+}$ -activated,  $\text{K}^+$  channel ( $\text{BK}_{\text{Ca}}$ ) [48], when used in combination with apamin as in this study, it completely blocks EDHF-mediated relaxation [43,48–51], which could not be achieved with iberiotoxin, another  $\text{BK}_{\text{Ca}}$  blocker [51,52]. The schema in Fig. 7 illustrates how the ability of EDHF to hyperpolarize the vascular smooth muscle could be limited by altered COX-1 and enhanced contractile thromboxane  $\text{A}_2$  TP receptor activities in morbid obesity.

The OAT depot is more heavily implicated in metabolic syndrome compared with SAT. Obese individuals with excess fat stored in visceral adipose depots generally suffer greater adverse metabolic consequences compared with similarly overweight subjects with excess fat stored predominantly in subcutaneous sites [53]. Although COX-2 mRNA was upregulated in the SVF of both arteries, it was more so in OAT



**Fig. 7. Scheme of proposed role of altered COX-1 and enhanced thromboxane A<sub>2</sub> TP receptor activities in mediating impaired omental endothelial dilatory capacity in morbid obesity.** As the omental adipose tissue expands beyond its homeostatic limit, its secretory and metabolic activities are dysregulated. As a consequence, it becomes pathogenic and adversely impacts the local vasculature resulting in the upregulation of the pathway highlighted in red. This includes (1) enhanced but altered COX-1 activity in the endothelial cell (EC) which favour the production of more contractile prostanoids, possibly TXA<sub>2</sub> and (2) enhanced thromboxane A<sub>2</sub> TP receptor activity in the underlying vascular smooth muscle cell (VSMC) which favour increased thromboxane A<sub>2</sub> TP receptor-mediated contractions. Both of these molecular changes lead to attenuated endothelium-dependent dilatory capacity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared with SAT. This is consistent with OAT being more extensively infiltrated with immune-inflammatory cells, such as macrophages and T-lymphocytes [6] and having the capacity to secrete more interleukin-6 (IL-6) [54] and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [29,55] which could modulate the deleterious impact of local fat on vasomotor function. TNF- $\alpha$  downregulates endothelial NO synthase (eNOS) expression at the posttranscriptional level [29,56] and IL-6 attenuates EDHF-mediated vasodilation [57] and can induce monocyte chemoattractant protein-1 (MCP-1) formation in the vascular smooth muscle via JAK (janus-activated kinase)/STAT (signal and transducers and activators of transcription) signaling pathway [58] to further disrupt vascular tone. Thus, as an important source of low-grade inflammation, visceral fat can directly contribute to the local development of endothelial dysfunction [29]. The current study shows that this micro environmental change alters endothelial COX-1 and VSM thromboxane A<sub>2</sub> TP receptor activity in such a way that leads to reduction in the endothelial dilatory capacities of local vessels as illustrated in Fig. 7.

#### 4.1. Conclusion

The current data show that the depot-specific reduction in endothelial dilatory capacities of omental compared with subcutaneous arteries in human obesity is in large part due to altered COX-1 and enhanced thromboxane A<sub>2</sub> TP receptor activities, which effectively cause EDHF deficiency, and therefore, reduction in endothelial function. Thus, it opens the possibility that selective thromboxane inhibition could be an effective means of preventing or ameliorating endothelial dysfunction in obesity.

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#### Declaration of competing interest

None.

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