

Original Article

Endothelial overexpression of endothelin-1 modulates aortic, carotid, iliac and renal arterial responses in obese mice

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Abstract

Endothelin-1 (ET-1) is essential for mammalian development and life, but it has also been implicated in increased cardiovascular risk under pathophysiological conditions. The aim of this study was to determine the impact of endothelial overexpression of the prepro-endothelin-1 gene on endothelium-dependent and endothelium-independent responses in the conduit and renal arteries of lean and obese mice. Obesity was induced by high-fat-diet (HFD) consumption in mice with Tie-1 promoter-driven, endothelium-specific overexpression of the prepro-endothelin-1 gene (TET^{het}) and in wild-type (WT) littermates on a C57BL/6N background. Isometric tension was measured in rings (with endothelium) of the aorta (A), carotid (CA) and iliac (IA) arteries as well as the main (MRA) and segmental renal (SRA) arteries; all experiments were conducted in the absence or presence of L-NAME and/or the COX inhibitor meclofenamate. The release of prostacyclin and thromboxane A₂ was measured by ELISA. In the MRA, TET^{het} per se increased contractions to endothelin-1, but the response was decreased in SRA in response to serotonin; there were also improved relaxations to acetylcholine but not insulin in the SRA in the presence of L-NAME. HFD per se augmented the contractions to endothelin-1 (MRA) and to the thromboxane prostanoid (TP) receptor agonist U46619 (CA, MRA) as well as facilitated relaxations to isoproterenol (A). The combination of HFD and TET^{het} overexpression increased the contractions of MRA and SRA to vasoconstrictors but not in the presence of meclofenamate; this combination also augmented further relaxations to isoproterenol in the A. Contractions to endothelin-1 in the IA were prevented by endothelin-A receptor antagonist BQ-123 but only attenuated in obese mice by BQ-788. The COX-1 inhibitor FR122047 abolished the contractions of CA to acetylcholine. The release of prostacyclin during the latter condition was augmented in samples from obese TET^{het} mice and abolished by FR122047. These findings suggest that endothelial TET^{het} overexpression in lean animals has minimal effects on vascular responsiveness. However, if comorbid with obesity, endothelin-1-modulated, prostanoid-mediated renal arterial dysfunction becomes apparent.

Keywords: endothelin-1; renal arteries; contraction; relaxation; thromboxane prostanoid receptors; serotonin; acetylcholine; isoproterenol; cyclooxygenase; endothelium-derived contracting factors; prostacyclin; obesity

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Introduction

The potent vasoconstrictor peptide endothelin-1 (ET-1)^[1] is essential for mammalian development and life^[2] but it has also been implicated in increased cardiovascular risk under pathophysiological conditions^[1, 3–8]. The production of ET-1 is encoded by the prepro-ET-1 gene and is succeeded by the production of prepro-ET-1 and subsequently big ET-1^[1, 9]. The latter is converted to the vasoactive ET-1 peptide, which

induces contractions of vascular smooth muscle upon activation of endothelin-A (ET_A) as well as endothelin-B (ET_B) receptors, both of which can be blocked individually with specific receptor antagonists^[1, 9–12]. Obesity augments ET-1-mediated ET_A receptor-dependent vasoconstrictor tone in humans *in vivo*^[4, 7, 8] and increases prepro-ET-1 gene expression in the arteries of mice fed a high-fat diet^[13]. However, the impact of overexpressing the prepro-ET-1 gene in the endothelium (the main source and origin of the vasoconstrictor peptide^[1, 14]) on changes in vascular reactivity that accompany obesity is unclear.

The renal circulation is crucial for the regulation of systolic

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arterial blood pressure, which increases when the blood flow to the kidneys is reduced^[15, 16]. In obese mice^[17] as well as in type I^[18] and type II diabetic rodents^[19], the renal vasculature is hyperresponsive to exogenous vasoconstrictor prostanoids. However, the role of endogenous endothelin-1 and endogenous prostanoids in the ability of renal arteries to constrict or dilate in this context are unclear.

Therefore, the effect of endothelial overexpression of the prepro-ET-1 gene was determined on prostanoid-mediated contractions and endothelium-dependent relaxations of isolated renal arteries from lean and obese mice; the findings in these small renal arteries were compared to results obtained in the iliac artery [large peripheral conduit blood vessel; to the best of our knowledge not explored in terms of the impact of obesity on its responsiveness], the carotid artery [standard preparation to investigate endothelium-dependent contractions in the mouse^[13, 20, 21]] and the aorta [standard preparation to investigate nitric oxide (NO)-mediated relaxations in the mouse^[3]].

Materials and methods

Animal studies

After weaning at the age of four weeks and genotyping to detect the presence or absence of the transgene^[22], male mice with heterozygous overexpression of the murine prepro-endothelin-1 gene (TET^{het}) and their wild-type (WT) littermates on a C57BL/6N background^[17] were randomized to either standard chow (13% kcal from fat, D5053, Lab Diet, Purina Mills, Richmond, IN, USA) or a high-fat diet (41% kcal from fat, D12079B, Research Diets Inc, New Brunswick, NJ, USA) for 8–9 months. The animals were housed at constant temperature (22°C on average) under a 12-h light-dark cycle with *ad libitum* access to food and water. On the day of experiments, the mice that were fasted overnight were anesthetized with an intraperitoneal injection of fentanyl citrate (0.4 mg/kg) and fluanisone (12.5 mg/kg; Janssen Pharmaceutica, Beerse, Belgium) plus midazolam (6.25 mg/kg; Roche, Basel, Switzerland) and sacrificed by exsanguination *via* cardiac puncture after reassurance of adequate anesthesia by the absence of a reaction to tail pinching. Blood was collected into chilled EDTA tubes and centrifuged at 2000 r/min at 4°C for 20 min, and 100 µL aliquots of plasma were stored at -80°C. Vascular tissues (aorta, carotid, iliac, as well as main and segmental renal arteries) were harvested for isometric tension recording experiments^[17, 20]. All procedures were approved by the institutional Committee on the Use of Live Animals for Teaching and Research (CULATR, project 2574-11) of the University of Hong Kong in line with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals issued by the US Institute of Laboratory Animal Research (ILAR, Eighth Edition, 2011).

Arterial blood pressure and heart rate

At the end of the study, arterial blood pressure and heart rate were measured non-invasively in conscious animals by using the tail-cuff method (Four channel BP-2000 Blood Pressure Analysis System, Visitech Systems Inc, Raleigh, NC, USA)^[23].

Body mass and fat content

Body and fat mass were determined in conscious lean and obese mice at the end of the study by time-domain nuclear magnetic resonance spectroscopy (Minispec model LF90II, Bruker Instruments, Billerica, MA, USA).

Plasma levels of metabolic parameters and endothelin-1

Fasting plasma glucose levels were measured at the time of sacrifice with a portable glucometer (Roche Diagnostics, Mannheim, Germany). Insulin was assayed with an *in house* high-sensitivity mouse insulin immunoassay kit (#32270; Antibody and Immunoassay Services AIS of the University of Hong Kong). Endothelin-1 levels were determined in plasma samples that were thawed on ice using the Quantikine ELISA kit (DET100; R&D Systems Inc, Minneapolis, MN, USA).

Vascular reactivity

Carotid arteries and the aorta with the renal and iliac arteries attached were excised and immediately placed into ice-cold modified Krebs-Ringer bicarbonate solution (pH 7.4) of the following composition (in mmol/L): NaCl, 129; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄, 1.17; NaHCO₃, 14.9; glucose, 5.5; calcium disodium EDTA, 0.026; and CaCl₂, 2.5 (control solution)^[20]. The vessels were cut into rings (2–3 mm in length) under a dissection microscope after removal of the adherent adipose and connective tissues. Arterial rings were suspended between jaws of four channel Halpern-Mulvany myographs (models 610M and 620M; Danish Myo Technology A/S, Aarhus, Denmark) using either pins (aorta) or 40 µm stainless steel wire (carotid, iliac, and renal arteries) for the measurement of isometric tension [in mN (9.81 mN=1 g)]. The optimal resting tension was determined for each preparation by repeated exposure to 60 mmol/L high potassium (high K⁺) depolarizing solution as previously described^[20]. These submaximal responses to high K⁺ were used as reference contractions. Due to the limited availability of renal arterial tissue in mice, some vasoconstrictors (ET-1, U46619) were examined in the main branches, while others (phenylephrine, serotonin) were explored in segmental renal arterial rings, taking advantage of the higher number per animal of the latter preparation compared to the main renal arteries. The relaxations to acetylcholine and insulin were also performed in the segmental renal arterial rings.

Prostanoid measurements

Aliquots (200 µL) of myograph chamber solution were collected after exposure of the carotid arterial rings to a final concentration (10⁻⁴ mol/L) of acetylcholine in the absence of presence of either BQ-123 or FR122047 (both 10⁻⁶ mol/L) in addition to L-NAME (3×10⁻⁴ mol/L). The aliquots were stored at -80°C until determination of the concentrations of 6-keto prostaglandin F_{1α} and thromboxane B₂ (the stable metabolites of prostacyclin and thromboxane A₂, respectively) in undiluted aliquots could be performed using commercially available ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions^[20, 24].

Drugs

Acetylcholine chloride, (cyclo(-D-Trp-D-Asp(ONa)-Pro-D-Val-Leu)) (BQ-123), BQ-788, endothelin-1, (-)-isoproterenol hydrochloride, *N*^o-nitro-*L*-arginine methyl ester (*L*-NAME), meclofenamic acid sodium salt (meclofenamate), *DL*-norepinephrine hydrochloride, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), *R*-(-)-phenylephrine hydrochloride, prazosin hydrochloride, *DL*-propranolol hydrochloride, and sodium nitroprusside dihydrate were purchased from Sigma-Aldrich (St Louis, MO, USA); phentolamine hydrochloride was obtained from Ciba (Basel, Switzerland); and 9,11-dideoxy-9 α ,11 α -methanoepoxy prostaglandin F_{2 α} (U46619) was acquired from Enzo Life Sciences (Farmingdale, NY, USA). FR122047 hydrochloride was purchased from Tocris Bioscience (Bristol, UK). Bosentan was graciously provided by Actelion (Allschwil, Switzerland), and S18886 was a kind gift from the Institut de Recherche Servier (Suresnes, France). Insulin was used in the soluble form of Actrapid (Novo Nordisk A/S, Bagsværd, Denmark) as described^[25]. All drugs were dissolved in distilled water except U46619 and insulin, which were prepared in ethanol (10⁻² mol/L stock) and diluted in control solution (from 6 \times 10⁻⁴ mol/L stock), respectively; and stock solutions (10⁻² mol/L) of ODQ and S18886 were prepared in dimethyl sulfoxide (DMSO). Final concentrations did not exceed 0.1% for either DMSO or ethanol. Concentrations are stated as the final molar concentration in the myograph chamber solution.

Calculations and statistical analysis

The results are shown as the mean \pm standard error of the mean (SEM), whereby *n* equals the number of mice per group. Contractions are expressed as the percent of the reference contraction to high K⁺ (60 mmol/L potassium chloride with equimolar substitution of sodium by potassium) obtained at the beginning of the experiment, and relaxations as the percentage of pre-contractile response to either phenylephrine or U46619. Distribution normality was tested with the D'Agostino-Pearson test (GraphPad Software, San Diego, CA, USA). Concentrations causing half maximal responses (EC₅₀) were calculated and compared using nonlinear regression and expressed as negative logarithms (pD₂ values). The calculated maximal responses (*E*_{max}) are presented as percent-

ages of the reference contraction. Either one-way ANOVA or the Kruskal-Wallis test followed by Bonferroni or Dunn's analysis, respectively, were used to analyze significant differences between unpaired groups as appropriate. Two-way ANOVA with repeated measurements were used for multiple comparisons of concentration-response curves followed by Bonferroni *post hoc* analysis where appropriate. Student's *t*-test was used for direct comparisons of samples showing a normal distribution, while the Mann-Whitney *U* test was applied for non-parametric samples. *P* values less than 0.05 were considered to indicate statistically significant differences.

Results

Physiological and plasma parameters

Mice consuming a high-fat diet for 8–9 months increased their body weight compared to controls; the relative fat mass doubled irrespective of the genotype. The plasma levels of ET-1 were comparable in lean and obese WT and TET^{het} mice. Fasting insulin and fasting glucose levels were higher in obese mice compared to lean animals, but all mice remained in the normoglycemic range. Similarly, the systolic arterial blood pressure values were within normal limits in all the groups. Heart rate increased with obesity, significantly more so when combined with TET^{het} overexpression. These parameters are summarized in Table 1.

Contractions to endothelin-1

Experiments with vasoconstrictor agents were performed in arteries incubated with *L*-NAME (3 \times 10⁻⁴ mol/L, 30-min pre-incubation)^[17] to determine the potential of ET-1^[26, 27]. In main renal arteries, the response to endothelin-1 (10⁻¹¹ to 10⁻⁷ mol/L) was shifted to the left in rings from lean mice with endothelial overexpression of the prepro-endothelin-1 (ET-1) gene (TET^{het}) compared to corresponding preparations from their WT littermates (Figure 1). Obesity augmented the pD₂ values of contractions to ET-1 in the renal arteries of WT but not TET^{het} mice (Figure 1). This was the only difference in the presence of the cyclooxygenase inhibitor meclofenamate (10⁻⁶ mol/L)^[20], which equilibrated the renal arterial responses to ET-1 among the other groups (Figure 1). Carotid arterial rings from the four experimental groups contracted similarly to increasing,

Table 1. Physiological and plasma parameters. Physiological and plasma parameters in lean and obese TET^{het} mice and wild type (WT) littermates. Values are means \pm SEM, numbers of experiments are indicated for each parameter individually. **P*<0.05 versus WT littermates. #*P*<0.05 versus lean controls.

Parameter (unit)	Lean		Obese	
	WT	TET ^{het}	WT	TET ^{het}
Body weight [<i>n</i> =18–23 (g)]	34.8 \pm 1.0	33.3 \pm 0.8	47.2 \pm 0.9 [#]	49.6 \pm 0.9 [#]
Fat mass [<i>n</i> =18–23 (%)]	15.5 \pm 1.0	14.9 \pm 0.8	29.2 \pm 0.9 [#]	31.3 \pm 0.6 [#]
Fasting insulin [<i>n</i> =13–18 (ng/mL)]	1.4 \pm 0.3	0.9 \pm 0.2	4.8 \pm 0.5 [#]	4.3 \pm 0.3 [#]
Fasting glucose [<i>n</i> =12–17 (mmol/L)]	4.6 \pm 0.3	4.4 \pm 0.1	5.5 \pm 0.2 [#]	5.5 \pm 0.3 [#]
Systolic blood pressure [<i>n</i> =12–17 (mmHg)]	107 \pm 1	109 \pm 2	112 \pm 2 [#]	115 \pm 3
Heart rate [<i>n</i> =12–17 (beat/min)]	620 \pm 13	652 \pm 11	664 \pm 8 [#]	695 \pm 9 [#]
Plasma endothelin-1 [<i>n</i> =13–18 (pg/mL)]	0.9 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1

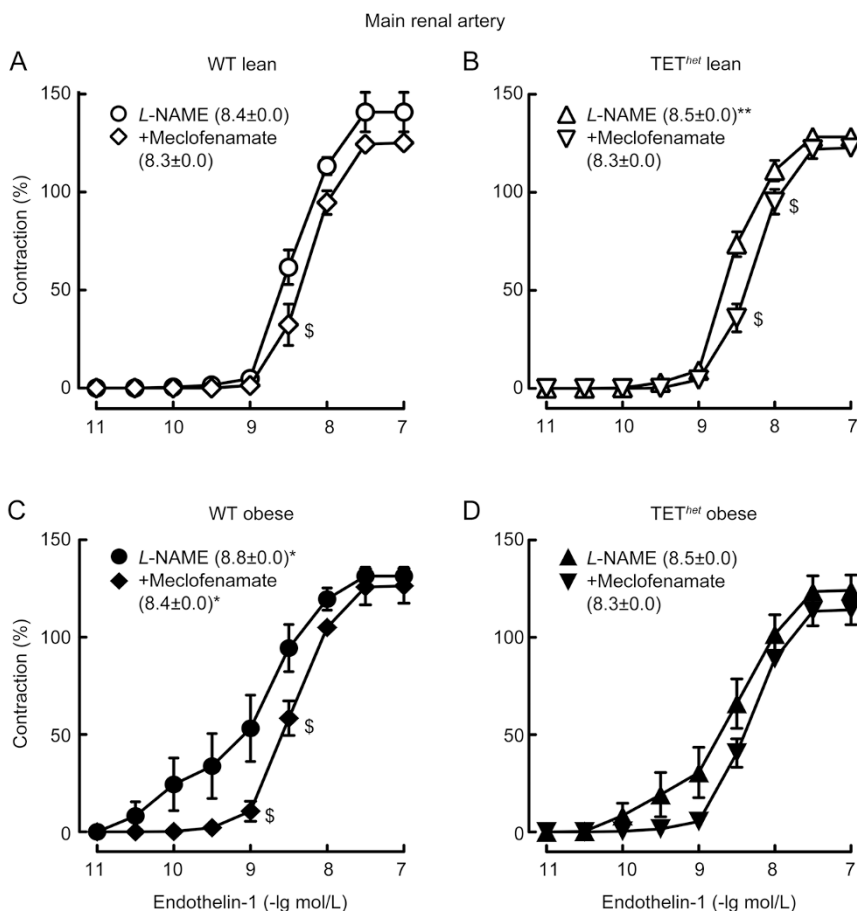


Figure 1. Contractions to endothelin-1 in isolated main renal arteries of lean wild-type (WT; A, $n=6$), lean heterozygous prepro-endothelin-1 gene overexpressing (TET^{het}, B, $n=9$), obese WT (C, $n=5$) and obese TET^{het} mice (D, $n=5$). All experiments were performed in the presence of L-NAME (3×10^{-4} mol/L) and either without or with meclofenamate (10^{-6} mol/L) as indicated. The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD₂ values in brackets for each group) are given as the mean ± SEM. * $P < 0.05$, ** $P < 0.01$ vs WT lean. § $P < 0.05$ vs corresponding experiments in the absence of meclofenamate in each individual group.

cumulative concentrations of ET-1 without differences in the pD₂ values for the responses to the vasoconstrictor peptide (WT lean 8.6 ± 0.1 , TET^{het} lean 8.7 ± 0.1 , WT obese 8.6 ± 0.1 , TET^{het} obese 8.8 ± 0.2).

In the *iliac* arterial rings, contractions to ET-1 were of a larger amplitude (E_{\max} WT lean $168\% \pm 23\%$, TET^{het} lean $164\% \pm 11\%$, WT obese $171\% \pm 10\%$, and TET^{het} obese $102\% \pm 10\%$ high K⁺) than in the carotid preparations (E_{\max} WT lean $27\% \pm 2\%$, TET^{het} lean $22\% \pm 1\%$, WT obese $23\% \pm 1\%$, and TET^{het} obese $26\% \pm 3\%$ high K⁺) and were comparable between preparations from both lean genotypes (Figure 2). However, responses to the highest concentrations were lower in the *iliac* arteries of obese TET^{het} compared to WT mice and lean controls (Figure 2D). Due to the overall high amplitude of the contractions to ET-1 in the *iliac* artery, this preparation was selected to compare the effects and efficacy of endothelin receptor antagonists across all of the groups. Therefore, concentration-response curves to ET-1 were obtained in *iliac* arteries in the presence of the endothelin-A (ET_A) receptor antagonist BQ-123^[11], the endothelin-B

(ET_B) receptor antagonist BQ-788^[12] or the dual ET_{A/B} receptor antagonist bosentan^[10] (all 10^{-6} mol/L) in addition to L-NAME. The endothelin-A (ET_A) receptor blocker BQ-123 prevented contractions in all *iliac* preparations (Figure 2A–2D), while the ET_B-selective antagonist BQ-788 shifted the ET-1 concentration-response curve to the right only in the arterial preparations from obese animals (Figure 2C and 2D). The dual ET_{A/B} receptor antagonist bosentan was equally effective as BQ-123 in preventing contractions in response to ET-1 (Figure 2A–2D). Therefore, the ET_A receptor-selective antagonist was chosen for further experiments examining ET-1-modulated vasoconstrictor responses.

Thromboxane prostanoid receptor activation

Contractions of main *renal* arteries to the full thromboxane prostanoid (TP) receptor agonist U46619 (10^{-11} to 3×10^{-6} mol/L) were augmented by obesity (Figure 3), confirming earlier findings^[17]. The cyclooxygenase inhibitor meclofenamate reduced the response to U46619 in all the groups (Figure 3),

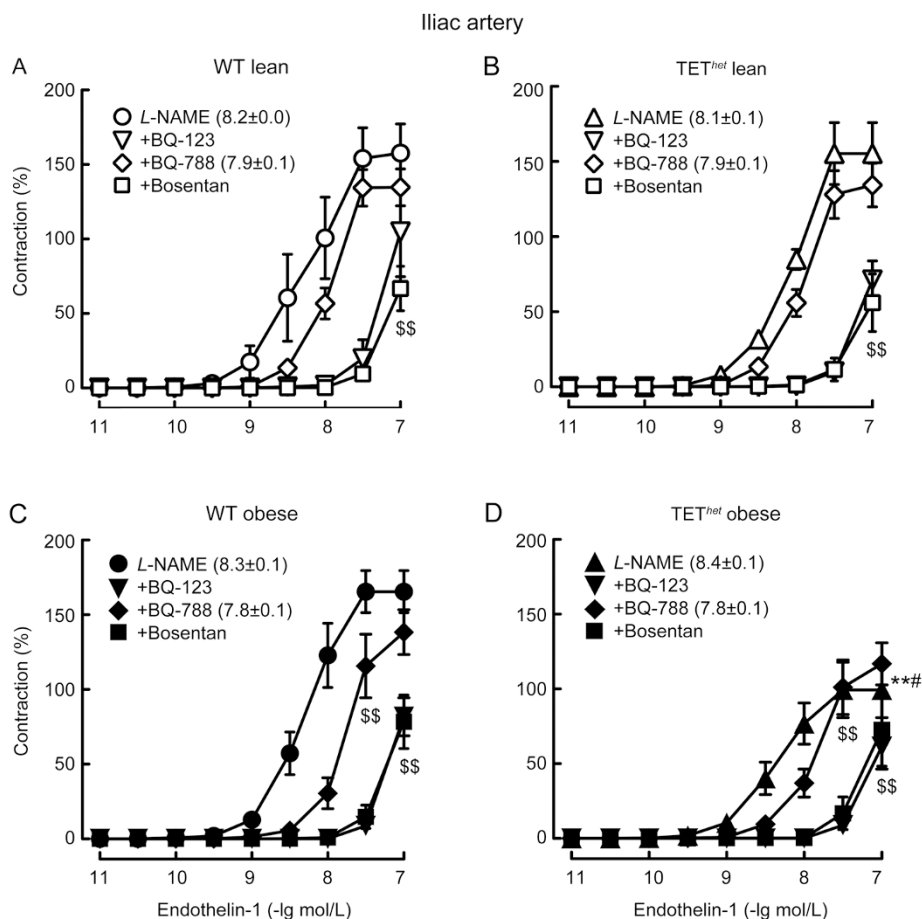


Figure 2. Contractions to endothelin-1 (ET-1) in iliac arterial rings from lean (A and B) and obese (C and D) wild-type (WT) mice and mice with heterozygous endothelial overexpression of the prepro-ET-1 gene (TET^{het}). All the experiments were performed in the presence of L-NAME (3×10^{-4} mol/L) and BQ-123, BQ-788, or bosentan (all 10^{-6} mol/L). The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD₂ values in brackets where calculation possible) are given as the mean ± SEM; n=6–9. **P<0.01 vs WT obese. #P<0.05 vs lean controls. \$\$P<0.01 vs experiments in the presence of L-NAME only in each individual group.

but foremost in preparations from obese TET^{het} mice in which responses were shifted to the right even compared to WT littermates fed a high-fat diet (Figure 3). Contractions to the TP receptor agonist in the obese WT group remained augmented during cyclooxygenase inhibition, while they were not different in obese TET^{het} mice compared to their lean controls in the presence of meclufenamate (Figure 3). By contrast to renal arteries, the leftward shift of TP receptor agonist-induced responses in the carotid arteries of obese mice in the presence of L-NAME only [pD₂: WT and TET^{het} obese 7.9 ± 0.1 compared to lean controls (WT lean 7.5 ± 0.1 and TET^{het} lean 7.8 ± 0.1; n=4–7, P<0.01)], remained during additional cyclooxygenase inhibition with meclufenamate (pD₂ WT and TET^{het} lean 7.4 ± 0.1, WT obese 7.6 ± 0.1, and TET^{het} obese 7.5 ± 0.1; n=7–12, P<0.05) irrespective of the genotype.

Serotonin

Segmental renal arterial contractions to serotonin (10^{-10} to 3×10^{-5} mol/L) in the presence of L-NAME only were shifted to the

right by TET^{het} overexpression in lean mice. Obesity shifted the response to the left in TET^{het} but not WT mice. These differences were absent in the additional presence of meclufenamate (Figure 4).

Phenylephrine

In the presence of L-NAME only, contractions to phenylephrine (10^{-10} to 10^{-4} mol/L) in segmental renal arterial rings were not different between lean TET^{het} and WT mice, while they were shifted to the left in obese TET^{het} compared to WT mice (Figure 5). Addition of the cyclooxygenase inhibitor meclufenamate shifted the vasoconstrictor response to α_1 -adrenoceptor activation to the right in corresponding preparations from all animals except in those of lean WT mice. Meclufenamate lowered the sensitivity to phenylephrine in the rings from obese versus lean animals irrespective of the genotype (Figure 5).

Relaxation responses to acetylcholine

Relaxations [during pre-contraction with phenylephrine

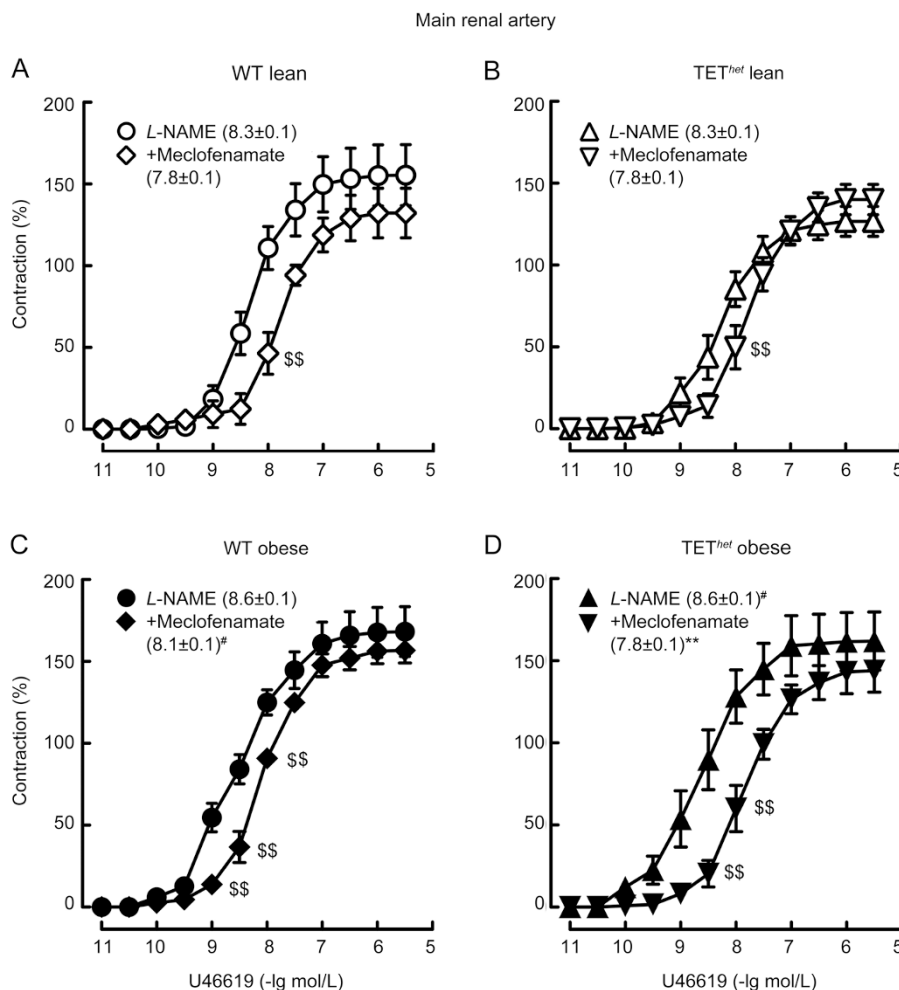


Figure 3. Contractions to the full TP receptor agonist U46619 in main renal arterial rings from lean wild-type (WT; A, $n=5$) and heterozygous pre-endothelin-1 gene overexpressing (TET^{het} ; B, $n=6$), and obese WT (C, $n=9$) and TET^{het} mice (D, $n=6$). All experiments were performed in the presence of L-NAME (3×10^{-4} mol/L) and without or with meclofenamate (10^{-6} mol/L) as indicated. The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the means \pm SEM. $^{*}P < 0.01$ vs WT obese. $^{\#}P < 0.05$ vs lean controls; $^{**}P < 0.01$ vs experiments in the presence of L-NAME only in each individual group.

(3×10^{-8} to 2×10^{-6} mol/L to obtain approximately 50% of high K^+ reference contractions) and in the presence of meclofenamate] of segmental renal arteries were examined in the absence or presence of the nitric oxide synthase inhibitor L-NAME (3×10^{-4} mol/L). In the presence of meclofenamate only, preparations from lean TET^{het} mice were more sensitive to the endothelium-dependent muscarinic agonist compared to corresponding rings from WT littermates. This difference was less pronounced in the renal arteries of obese animals since WT preparations exhibited an improved response in obesity (Figure 6). Irrespective of the body weight, L-NAME attenuated the relaxations to acetylcholine (10^{-10} to 10^{-4} mol/L) only in rings from WT but not in those from TET^{het} mice, where these relaxations were largely preserved (Figure 6). By contrast, maximal nitric oxide-dependent relaxations to acetylcholine in the aorta (phenylephrine-contracted to 64%

to 77% of high K^+) were similar across the groups in the presence (Figure 7A) or absence of meclofenamate (Figure 7B). Likewise, in aortic rings precontracted (74% to 108% of high K^+) with U46619, relaxations to norepinephrine [10^{-10} to 10^{-4} mol/L; in the presence of prazosin and propranolol (both 10^{-6} mol/L) in addition to meclofenamate to prevent activation of α_1 - and β -adrenoceptors, respectively; Figure 7C] did not differ between the groups; relaxations to norepinephrine under these experimental conditions are comparable to the release of nitric oxide upon activation of endothelial α_2 -adrenoceptors^[28].

At higher concentrations (10^{-6} to 10^{-4} mol/L) of acetylcholine, a secondary increase in tension was observed in the absence but not in the presence of meclofenamate (Figure 7), indicative of the occurrence of endothelium-dependent contractions^[13, 29]. These secondary contractions were larger in the aortic rings from obese TET^{het} mice (Figure 7B).

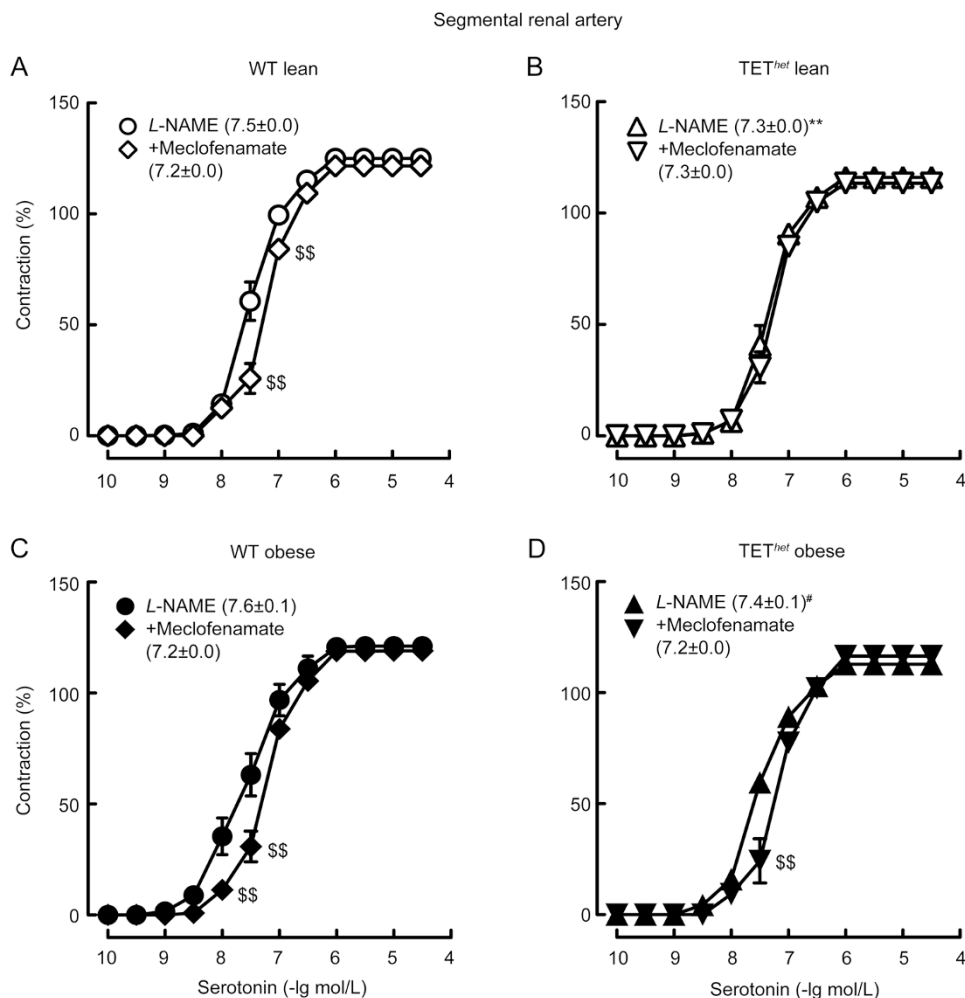


Figure 4. Contractions to serotonin in segmental renal arterial rings from lean wild-type (WT; A, $n=6$), lean heterozygous prepro-endothelin-1 gene overexpressing (TET^{het} ; B, $n=9$), obese WT (C, $n=7$) and obese TET^{het} mice (D, $n=4-5$). All the experiments were performed in the presence of *L*-NAME (3×10^{-4} mol/L) and without or with meclofenamate (10^{-6} mol/L) as indicated. The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the mean \pm SEM. ** $P < 0.01$ vs WT obese. # $P < 0.05$ vs lean controls. \$\$ $P < 0.01$ vs experiments in the presence of *L*-NAME only in each individual group.

Relaxations to sodium nitroprusside, isoproterenol, and insulin

Endothelium-independent relaxations to sodium nitroprusside (10^{-11} to 10^{-6} mol/L, in the presence of *L*-NAME) and the β -adrenergic agonist isoproterenol [10^{-10} to 3×10^{-6} mol/L, in the presence of meclofenamate, oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 10^{-5} mol/L; inhibitor of soluble guanylyl cyclase) were obtained in aortic preparations contracted to a similar degree across the groups with phenylephrine (84% to 92% of the response to high K^+); the same procedure was performed with phentolamine (10^{-5} mol/L; a non-selective α -adrenoceptor antagonist) and U46619 (80% to 98% of high K^+), respectively. Relaxations to sodium nitroprusside in the *aorta* were not different between the groups (Figure 8A), demonstrating an absence of an impact of the genotype on the sensitivity of vascular smooth muscle to nitric oxide. Paralleling the observed *in vivo* changes in heart rate, the aortic relaxations to

the β -adrenoceptor agonist isoproterenol were facilitated by obesity in WT mice, especially in those of the TET^{het} genotype (Figure 8B).

Insulin in phenylephrine-contracted (10^{-7} to 9×10^{-7} mol/L to obtain approximately 50% of high K^+ reference contractions) segmental *renal* arterial rings (all in the presence of meclofenamate) caused concentration-dependent decreases in tension that were comparable in all the groups irrespective of the absence or presence of *L*-NAME (Figure 9).

Prostanoid-mediated contractions to acetylcholine

Quiescent rings from *carotid* arteries were exposed to increasing concentrations of acetylcholine in the presence of *L*-NAME. The contractions were comparable in preparations from lean WT and TET^{het} mice (Figure 10A). Obesity increased the contractions in preparations from both genotypes but more so in

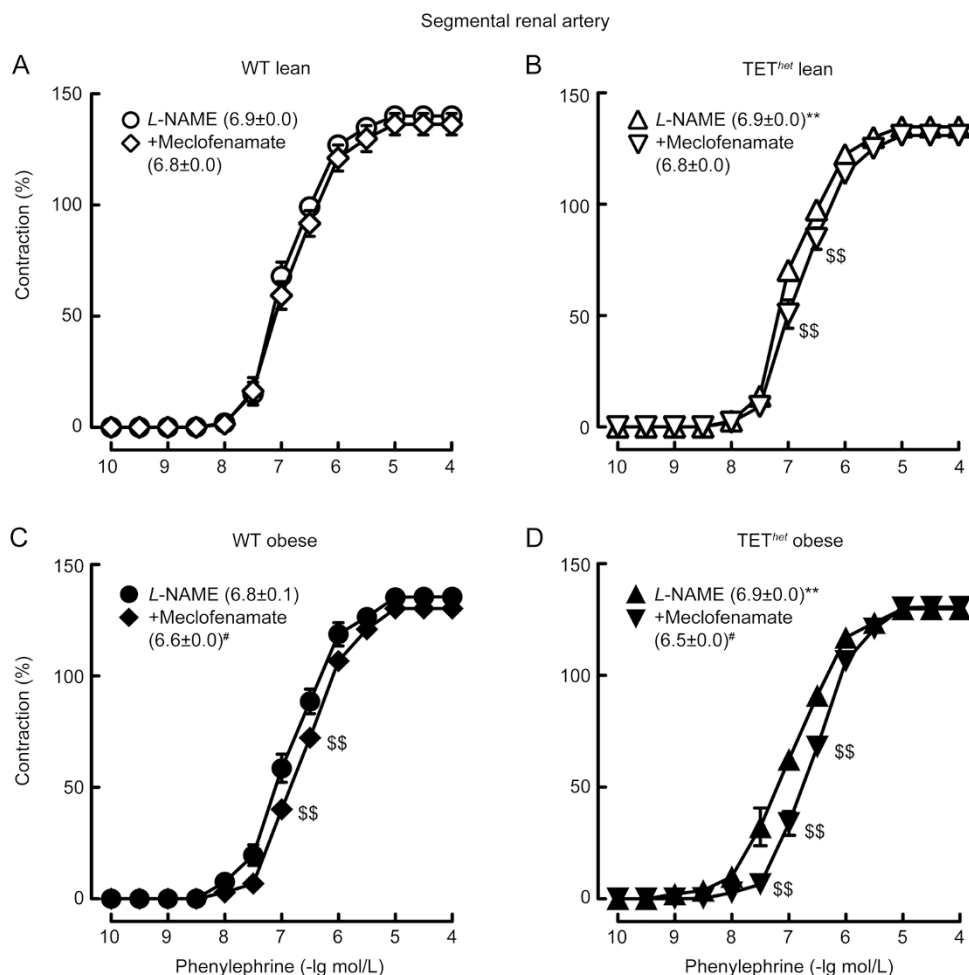


Figure 5. Contractions to the α_1 -adrenoceptor agonist phenylephrine in segmental renal arterial rings from lean wild-type (WT; A, $n=6$), lean heterozygous prepro-endothelin-1 gene overexpressing (TET^{het} ; B, $n=9$), obese WT (C, $n=7$) and obese TET^{het} mice (D, $n=4-5$). All the experiments were performed in the presence of L -NAME (3×10^{-4} mol/L) and without or with meclofenamate (10^{-6} mol/L) as indicated. The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the means \pm SEM. ** $P < 0.01$ vs WT obese. # $P < 0.05$ vs lean controls. \$\$ $P < 0.01$ vs experiments in the presence of L -NAME only in each individual group.

rings from obese TET^{het} mice (Figure 10B). In the four groups, the contractions to acetylcholine were abolished by either the selective COX-1 inhibitor FR122047 (10^{-6} mol/L) or by the TP receptor antagonist S18886 (10^{-7} mol/L; $E_{max} 0 \pm 0\%$ high K^+), which confirmed earlier findings in the rat aorta^[30,31].

The acetylcholine-stimulated release of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) was increased in *carotid* rings from obese TET^{het} mice under control conditions, and BQ-123 especially reduced the relative release of 6-keto $PGF_{1\alpha}$ in these preparations (Figure 10C), with the levels becoming comparable across the groups (Table 2). However, the addition of the ET_A receptor-selective antagonist did not significantly alter the contractions to acetylcholine across the groups (Figure 10). Only very low levels of the prostanoid were detected after incubation with FR122047. The levels of thromboxane B_2 (TXB_2) released by the *carotid* arteries were similarly low in all the groups with values around the detection limit of the assay

(Table 2).

Discussion

The present results demonstrate the following:

a) *Tie-1* promoter-driven heterozygous overexpression of the prepro-ET-1 gene (TET^{het})^[17,22,32] has marginal effects on vascular reactivity under lean conditions, such as slightly increased responses to ET-1 itself, a decreased sensitivity to serotonin and preserved relaxations to acetylcholine during nitric oxide synthase inhibition in renal arteries.

b) High-fat diet consumption leading to an augmented body weight and an increase in plasma insulin levels was associated with a higher contractility to ET-1 in renal arteries from WT mice in addition to elevated thromboxane prostanoid (TP) receptor responsiveness. Contractions to ET-1 in iliac arteries were partially endothelin-B receptor-dependent, and aortic relaxations to acetylcholine, norepinephrine, and sodium

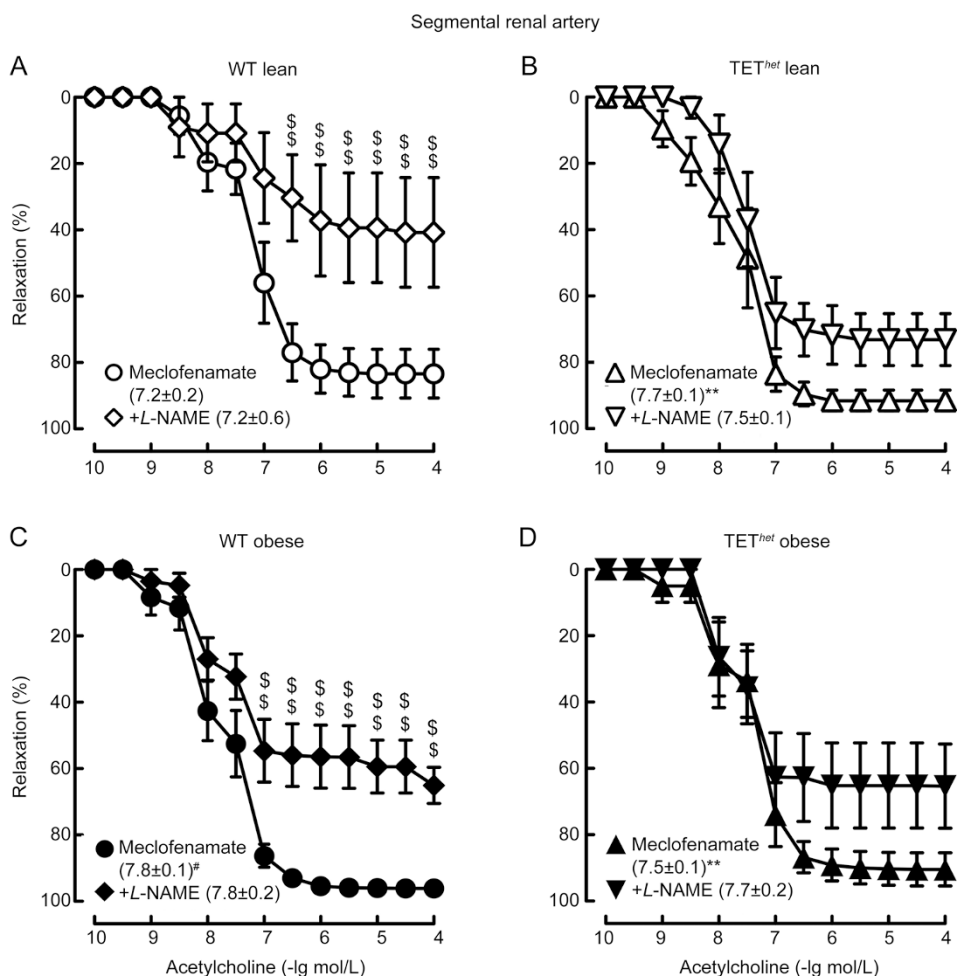


Figure 6. Relaxations to the endothelium-dependent agonist acetylcholine in segmental renal arterial rings from lean wild-type (WT; A, $n=5$), lean heterozygous prepro-endothelin-1 gene overexpressing (TET^{het} ; B, $n=5$) mice, and obese WT (C, $n=9$) and TET^{het} mice (D, $n=5-6$). All experiments were performed in the presence of meclofenamate (10^{-6} mol/L) and without or with L-NAME (3×10^{-4} mol/L) as indicated. The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the means \pm SEM. [#] $P < 0.01$ vs WT obese. ^{**} $P < 0.05$ vs lean controls. ^{§§} $P < 0.01$ vs experiments in the presence of meclofenamate only in each individual group.

nitroprusside were preserved in preparations from obese animals. Furthermore, obesity increased the heart rate, which was paralleled by facilitated relaxations to the β -adrenoceptor agonist isoproterenol in aortic rings.

c) These findings were aggravated in obese TET^{het} mice, in which augmented renal arterial contractions were consistently curtailed in the presence of the cyclooxygenase inhibitor meclofenamate. Isolated prostanoid-mediated contractions to acetylcholine examined in carotid arteries revealed a higher production/release of the endothelium-derived contracting factor (EDCF) prostacyclin in preparations from TET^{het} mice with diet-induced obesity.

The high-fat diet imposed in the present study increased the body mass due to excess adipose tissue. Since the animals remained normoglycemic and normotensive, their obesity can be regarded as an independent cardiovascular risk factor^[33] rather than a feature of the metabolic syndrome^[34].

In line with earlier studies^[35], obesity increased the heart rate, especially in TET^{het} mice. This is in accordance with the regulatory role of ET-1 on cardiac sympathetic innervation^[36]. The plasma ET-1 levels determined in the present study are similar to those reported for lean C57BL/6 WT control mice^[14, 37, 38]. In such animals, 3 weeks of high-fat diet elevates ET-1 in the circulation^[37], but the present experiments show that this effect is lost after 30 weeks of diet-induced obesity. This observation is in line with the absence of plasma ET-1 elevation following 10 weeks of high-fat feeding^[39]. By contrast to the homozygous TET-1 mice^[32], the heterozygous TET^{het} mice used in the present study (which were generated by several backcrosses of TET-1 mice onto a C57BL/6N background) had no increase in plasma ET-1 in response to either diet.

Endothelin-A (ET_A) receptor blockade by BQ-123 was equally effective in iliac preparations from all the groups in preventing contractions to ET-1, confirming the general

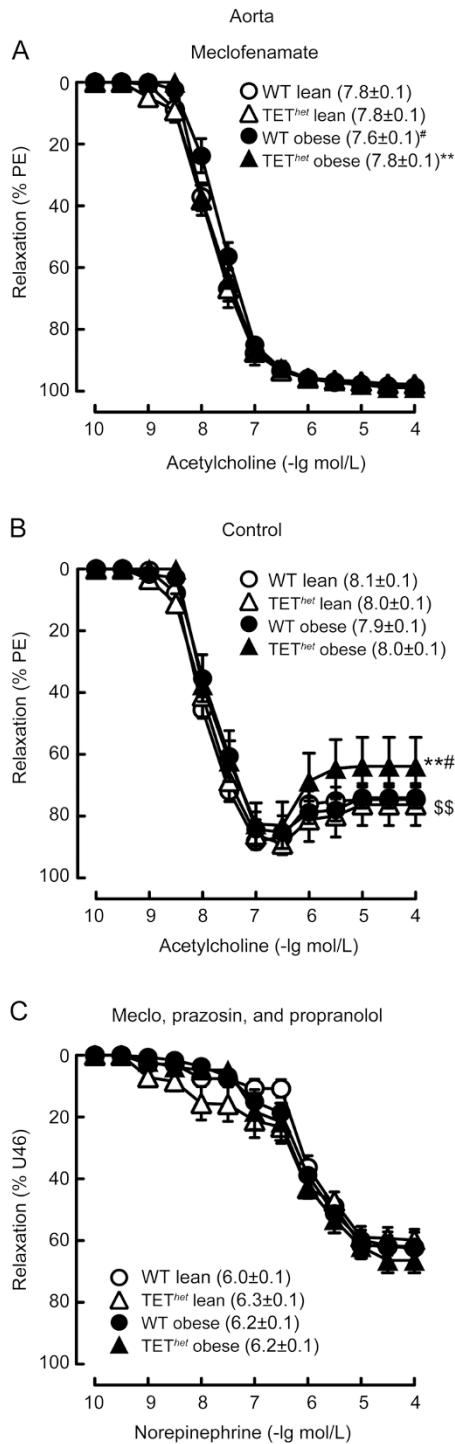


Figure 7. Nitric oxide-mediated relaxations to the endothelium-dependent agonists acetylcholine (A and B) and norepinephrine (C) in aortic rings from lean and obese wild-type (WT) and TET^{het} mice in the presence of meclufenamate (Meclo), prazosin and propranolol (all 10^{-6} mol/L) or in the absence/presence of meclufenamate alone as indicated; $n=5-10$. The data are expressed as changes in tension relative to the pre-contractions to phenylephrine (PE) or U46619 (U46), and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the means \pm SEM. ^{**} $P<0.01$ vs WT obese. [#] $P<0.05$ vs lean controls. ^{\$\$} $P<0.01$ vs experiments in the presence of meclufenamate only in each individual group.

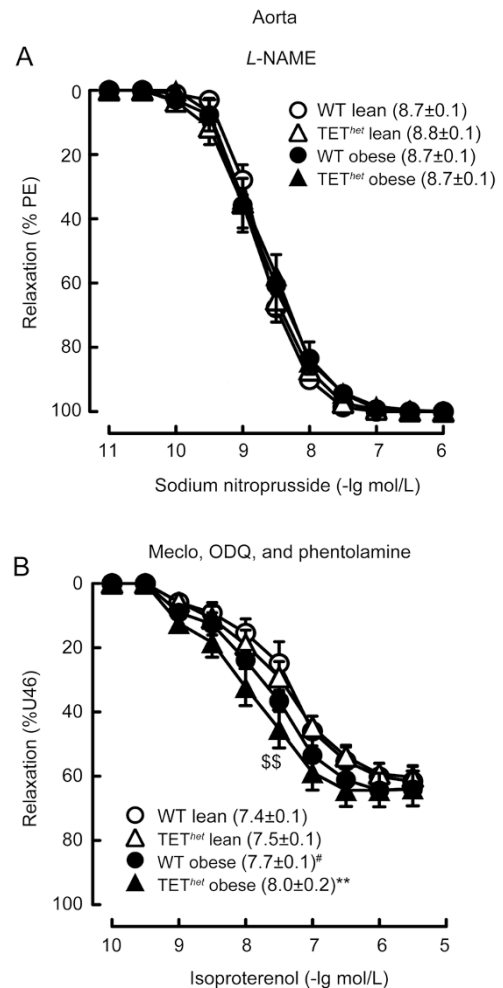


Figure 8. Relaxations to the nitric oxide donor sodium nitroprusside (A) and the β -adrenoceptor agonist isoproterenol (B) in aortic rings from lean and obese wild-type (WT) and TET^{het} mice in the presence of L-NAME (3×10^{-4} mol/L) or meclufenamate (Meclo, 10^{-6} mol/L), ODQ, and phentolamine (both 10^{-5} mol/L, respectively, as indicated; $n=6-10$). The data are expressed as changes in tension relative to pre-contractions to phenylephrine (PE) or U46619 (U46), and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the means \pm SEM. ^{**} $P<0.01$ vs WT obese. [#] $P<0.05$ vs lean controls. ^{\$\$} $P<0.01$ vs experiments in the presence of L-NAME only in each individual group.

dependency on this receptor subtype in the vasoconstrictor response to this peptide^[40]. However, a functional relevance of ET_B receptors in vascular smooth muscle was revealed in iliac arteries from obese mice, where the ET_B receptor-selective antagonist BQ-788 attenuated contractions to ET-1 in line with previous studies in hypercholesterolemic animals^[41, 42]. Similar to arteries of transgenic mice with endothelium-selective^[32, 38, 43] or global overexpression of the prepro-ET-1 gene^[44], maximal contractions to ET-1 were attenuated in iliac rings from obese TET^{het} mice. Unlike in systemic conduit vessels such as carotid and iliac arteries, contractions to ET-1 were augmented in renal arteries from obese animals. This highlights the poten-

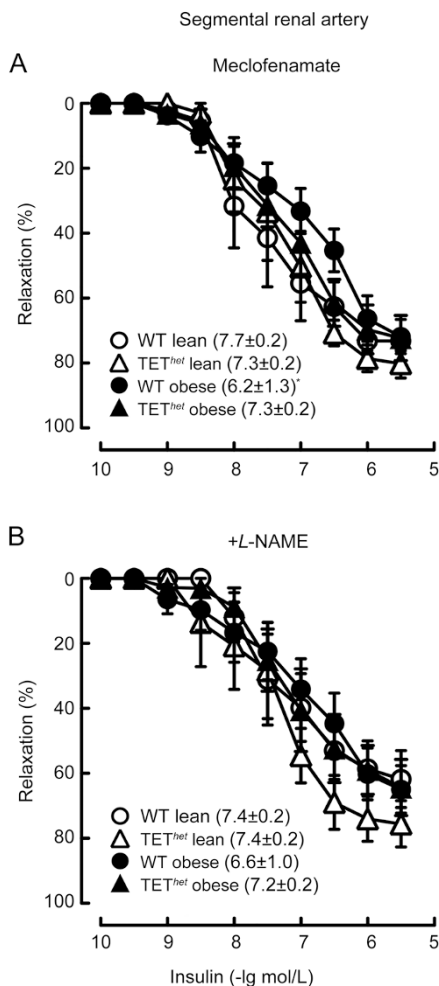


Figure 9. Relaxations to insulin in segmental renal arterial rings from lean wild-type (WT; $n=5$), lean heterozygous prepro-endothelin-1 gene overexpressing (TET^{het} ; $n=5$), obese WT ($n=9$) and obese TET^{het} mice ($n=6$). All the experiments were performed in the presence of meclofenamate (10^{-6} mol/L) and without (A) or with *L*-NAME (B; 3×10^{-4} mol/L) as indicated. The data are expressed as changes in tension relative to pre-contractions to phenylephrine, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the means \pm SEM. * $P < 0.05$ vs WT lean.

tial of the vasoconstrictor peptide to contribute to an increased vascular risk in the renal circulation. The aggravation by obesity was partially cyclooxygenase-dependent, suggesting the contribution of endogenously released vasoconstrictor prostanooids as demonstrated in the response to ET-1 in the aortae of spontaneously hypertensive rats^[6]. Cyclooxygenase inhibition also attenuated contractions to the peptide in renal arteries from lean mice, but its effect was marginal in obese TET^{het} mice. This permits the conclusion that moderate endothelial overexpression of the prepro-ET-1 gene does not potentiate the cyclooxygenase-dependent aspect of the response to exogenously added peptide in mouse renal arteries. Moreover, the lack of augmentation of contractions to ET-1 in renal arteries

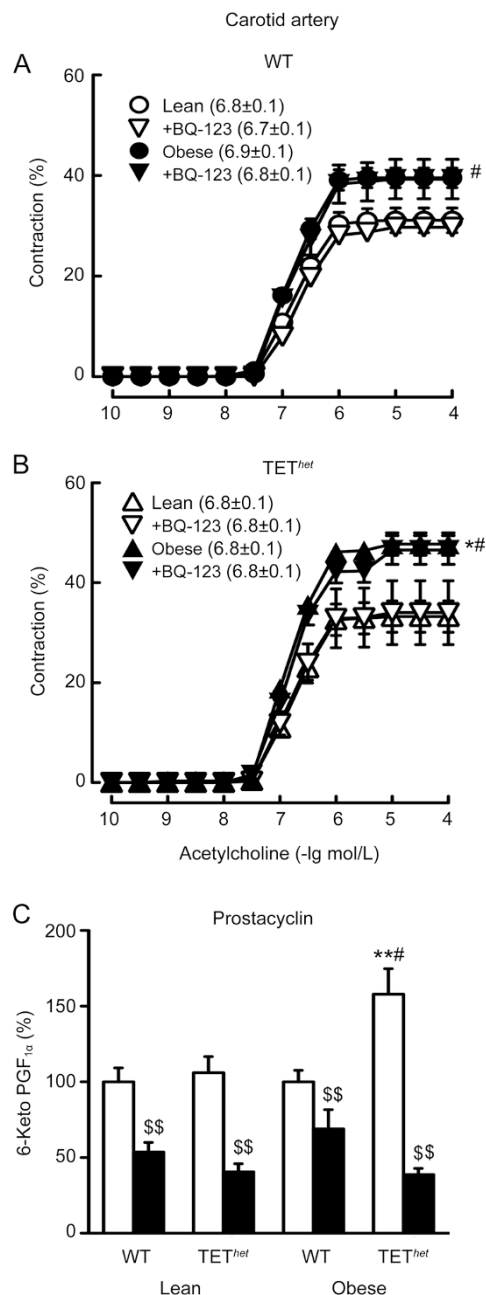


Figure 10. Contractions to acetylcholine in carotid arterial rings (with endothelium) from lean (A) and obese (B) wild-type (WT) and TET^{het} mice. All the experiments were in the presence of *L*-NAME (3×10^{-4} mol/L; $n=14-18$) as well as the endothelin-A receptor antagonist BQ-123 (10^{-6} mol/L; $n=7-13$). The data are expressed as the percentage of reference contractions to a 60 mmol/L high potassium solution, and the negative logarithms of the calculated concentrations that cause a half maximal responses (pD_2 values in brackets for each group) are given as the mean \pm SEM. The release of the stable prostacyclin breakdown product 6-keto prostaglandin $F_{1\alpha}$ (C; 6-keto $PGF_{1\alpha}$) from carotid arterial rings from lean and obese WT and TET^{het} mice from these experiments in the presence of either *L*-NAME only (3×10^{-4} mol/L; white bars) or with BQ-123 (10^{-6} mol/L; black bars) is shown. The data are expressed in percentage of WT controls and are shown as the mean \pm SEM. *** $P < 0.01$ vs WT obese. * $P < 0.05$ vs lean controls. ** $P < 0.01$ vs experiments in the presence of *L*-NAME only in each individual group.

Table 2. Release of prostanoid metabolites from carotid artery rings exposed to acetylcholine. Data are normalized for one milliliter, expressed in pg/mL and shown as mean±SEM. **P*<0.05 vs obese WT littermates; #*P*<0.05 vs lean control. †*P*<0.05 vs experiments in the presence of L-NAME only in each individual group.

Antagonist/inhibitor	Lean		Obese	
	WT	TET ^{het}	WT	TET ^{het}
6-keto Prostaglandin F _{1α} (pg/mL)				
L-NAME only	652±60 [†]	691±69	562±43	887±94 ^{*#}
+BQ-123 (10 ⁻⁶ mol/L)	349±42 [§]	280±37 [§]	388±71 [§]	345±35 [§]
+FR122047 (10 ⁻⁶ mol/L)	18±4 [§]	25±4 [§]	56±16 [§]	40±17 [§]
Thromboxane B ₂ (pg/mL)				
L-NAME only	12±3	13±3	14±2	10±1
+BQ-123 (10 ⁻⁶ mol/L)	16±3	14±3	16±3	17±4
+FR122047 (10 ⁻⁶ mol/L)	1±1	5±2	4±1	6±2

Release of the stable prostacyclin and thromboxane A₂ breakdown products 6-keto prostaglandin F_{1α} and thromboxane B₂ from carotid arterial rings of lean and obese TET^{het} mice and WT littermates measured in samples collected after the final acetylcholine concentration (10⁻⁴ mol/L) and assayed by ELISA, *n*=4–18. All experiments were in the presence of L-NAME (3×10⁻⁴ mol/L). Measurements from five milliliters myograph chamber solution samples for thromboxane B₂ around the detection limit of the assay (1.6 pg/mL).

from obese TET^{het} mice can be seen in the context of desensitization to the peptide itself as observed in the iliac arteries of these animals and as indicated in other models of ET-1 overexpression^[32, 38, 43, 44].

The attenuation of contractions to direct TP receptor activation by U46619 during cyclooxygenase inhibition by meclofenamate across the groups is indicative of the contribution of endogenous prostanoids to these vasoconstrictor responses. Thereby, activation of endothelial TP receptors can lead to the release of endogenous prostanoids^[45, 46]. The latter appears to be of the utmost importance with regard to obesity augmented contractions to the TP receptor agonist^[17] in TET^{het} mice, where the difference in responses between the renal arteries from lean and obese mice was abolished by meclofenamate. By contrast, in WT animals, contractions to U46619 remained amplified by obesity during cyclooxygenase inhibition. Therefore, this may then be seen as an increased TP receptor responsiveness of vascular smooth muscle cells, assuming that the contribution of endogenous prostanoids can be excluded in the presence of meclofenamate. In line with this interpretation, in conduit arteries such as the carotid arteries of obese mice in the presence of meclofenamate, the potencies of the TP receptor agonist were increased compared to preparations from lean control animals irrespective of the genotype. These observations highlight the relevance of endogenous prostanoids in augmented contractions upon TP receptor activation in the renal arteries with regard to an up-regulated ET-1 system in obesity. The same was true regarding the

augmented segmental renal arterial contractions to serotonin that were described in obese TET^{het} mice^[17] with attenuated responses in preparations from obese animals to the same levels as in lean controls during cyclooxygenase inhibition. These findings indicate that serotonin releases vasoconstrictor prostanoids from the renal arteries of obese TET^{het} mice with endothelial prepro-ET-1 gene overexpression, which was similar to that demonstrated in the kidney vasculature of spontaneously hypertensive rats^[47]. Given the confirmed high reactivity of renal arteries to serotonin [possibly involving decreased desensitization^[48]] and taking into consideration the possible potentiating effects of local ET-1 peptide on such vasoconstrictor agents^[49], the monoamine may contribute to arterial blood pressure regulation^[50, 51] in obesity. Moreover, the potentiation of phenylephrine-induced contractions in segmental renal arterial rings from obese TET^{het} mice compared to WT mice was fully sensitive to cyclooxygenase inhibition. Therefore, meclofenamate decreased the responses to the α₁-adrenoceptor agonist, particularly in preparations from obese animals. This indicates that adrenergic stimulation may also lead to the release of prostanoids under pathophysiological conditions such as obesity. Prostanoid release by phenylephrine is not as well documented as for serotonin^[47, 52] and ET-1^[5, 6, 9, 26], but experiments in aortic rings from diabetic rats have suggested a possible involvement of vasoconstrictor prostanoids in contractions to the α₁-adrenoceptor agonist^[53]. The apparent potentiation of the response to phenylephrine in preparations from obese TET^{het} mice can be attributed to endogenous ET-1 aggravating adrenergic contractions (similar to serotonin responses) as shown with norepinephrine in human arteries^[49].

With all of the increases in the vasoconstrictor agonist-induced renal arterial responses, it is tempting to hypothesize why obese mice, particularly those of the TET^{het} genotype, did not present hypertensive levels in their systolic arterial blood pressures considering the pivotal role of kidneys in regulating blood pressure^[54]. Moreover, an increased vasomotor tone in renal arteries caused by continuous vasoconstrictor signaling *in vivo* would functionally have a similar effect as that described for renal artery stenosis^[15, 16]. A possible explanation for the absence of hypertension *in vivo* can be seen in the largely preserved renal arterial relaxations to insulin. This hormone relaxes canine carotid arteries irrespective of the presence or absence of endothelial cells^[55], while responses in rat mesenteric arteries and aortae appear to be largely endothelial NOS-dependent^[56]. In the present study, the contribution of vasodilator prostaglandins, as suggested by hemodynamic investigations in the perfused rat kidney^[57], are unlikely to be involved in the relaxations to insulin based on the results obtained during cyclooxygenase inhibition. The observations in murine segmental renal arterial rings are in line with findings in rat renal arteries in which insulin caused comparable relaxations in preparations from diabetic and non-diabetic animals with the response being largely NO-independent in the latter^[58]. In addition, insulin given to diabetic animals *in vivo* subsequently facilitates NO-mediated relaxations of small resistance arteries *ex vivo*^[59]. In obese mice with higher circu-

lating levels of insulin, this could counteract the augmented vasoconstrictor responses in the kidney vasculature *in vivo*.

ET-1 not only promotes the release and action of vasoconstrictor prostanoids^[6, 9] but also that of NO in isolated arteries, including those from spontaneously hypertensive rats^[60], and can induce endothelium-dependent hyperpolarization (EDH)-mediated relaxation in isolated mesenteric arteries^[61]. This alternative mechanism of relaxation may indeed be involved in the renal vasculature as demonstrated in human interlobar arteries^[62]. In the present study, endothelium-dependent relaxations to acetylcholine in segmental renal arteries from animals in all the groups were similar in the absence and presence of L-NAME, indicating preserved NO- and EDH-mediated responses in these preparations, respectively. Since nitric oxide synthase inhibition attenuated the relaxations in preparations from WT rather than TET^{het} mice, facilitated hyperpolarization by ET-1^[61] cannot be excluded for these renal arterial preparations. Preserved nitric oxide-mediated relaxations in the aortae of obese TET^{het} mice are in line with the findings in endothelial ET-1 overexpressing ApoE-deficient mice fed a high-fat diet^[63].

The present study confirms that diet-induced obesity enhances endothelium-dependent contractions to acetylcholine^[13]. The predominance of prostacyclin production compared to the marginal levels of thromboxane A₂ illustrates its primary role as an endothelium-derived contracting factor in rodent blood vessels^[5, 6, 9, 20, 24, 30, 64]. Since ET_A receptor blockade with BQ-123 reduced prostacyclin release in all the groups, particularly in carotid arterial rings from obese TET^{het} mice, the present study confirms that ET-1 promotes the release of endothelium-derived vasoconstrictor prostanoids^[5, 6, 9, 26, 65], which is compatible with endothelium-selective overexpression in the TET^{het} model. This augmented prostacyclin production may partially explain the potentiated prostanoid-mediated contractions in obese TET^{het} mice, in which the effect of BQ-123 in reducing prostanoid production was the largest. However, the contractions to the muscarinic agonist were unaltered in the presence of BQ-123, indicating that the responsiveness to prostanoids remains augmented in obesity irrespective of the amounts released. Furthermore, prostaglandin E₂ may contribute to vasoconstrictor responses and can be augmented by ET-1^[65]. In the renal circulation of larger rodents, prostaglandin E₂ indeed has vasoconstrictor activity^[66]. The present study does not allow for the definitive identification of the prostanoid that is mainly responsible for the increased vasoconstrictor tone in renal arteries, particularly in response to U46619, where the most pronounced shifts by cyclooxygenase inhibition were observed. In the human renal circulation, high concentrations of both prostacyclin and prostaglandin E₂ have vasoconstrictor activity due to activation of TP receptors^[67].

Collectively, the present findings underscore a novel and direct role for endothelial ET-1 in regulating cyclooxygenase-dependent vasoconstrictor prostanoid formation associated with obesity, especially in the renal circulation. The finding that augmented prostanoid-mediated contractions due to ET-1 require a pathological condition such as obesity is in accor-

dance with studies demonstrating that ET-1 could induce the release of endothelium-derived prostanoids in the arteries from spontaneously hypertensive rats but not in those from normotensive rats^[6]. Similarly, ET-1 stimulates prostanoid release from regenerated but not native porcine coronary endothelium^[5]. Thus, the current findings lend further support to the concept that the endothelin system plays a minor role in regulating vascular function under physiological conditions but becomes more important during pathophysiological processes and/or vascular cell injury^[9, 68, 69].

In conclusion, the present study demonstrates that endothelial overexpression of the prepro-ET-1 gene potentiates prostanoid-mediated, endothelium- and TP receptor-dependent vasoconstriction in obesity. This can be largely attributed to an augmented release of prostanoids, specifically prostacyclin, which has repeatedly been identified as a major endothelium-derived contracting factor in rodents^[20, 24, 30, 64]. By contrast, contractions to the vasoconstrictor peptide itself can be attenuated in ET-1 overexpressing mice, and endothelium-dependent relaxations to acetylcholine may be preserved if not even improved with obesity. This could explain the lack of an exacerbation of the cardiovascular phenotype in obese TET^{het} mice.

As the release of the prostanoid prostacyclin was reduced by ET_A receptor blockade, and since therapeutic approaches of cyclooxygenase inhibition are associated with an increased cardiovascular risk, alternative strategies are necessary. Because ET_A receptor blockade also possibly curtails prostanoid formation in obese or overweight humans, the tandem of ET-1 and prostanoid-mediated vasoconstriction appears relevant to the understanding of vascular dysfunction in obese patients and thus may help to delineate new strategies for the therapy and/or prevention of cardiovascular disorders associated with obesity. Because the increased vasoconstrictor responses were observed in renal arteries from obese normotensive animals, renal arterial dysfunction could be a crucial step in the initiation of blood pressure elevation that may occur once counter regulatory mechanisms decline in function during disease progression. Cyclooxygenases consistently appeared to be involved in this hyperresponsiveness, particularly in preparations from obese TET^{het} mice. This suggests that renal arteries of obese TET^{het} mice may have a higher propensity to release endogenous prostanoids upon stimulation by other vasoconstrictors. Therefore, although ET-1 does not directly contribute to hypertension, it could potentially function as a modulator in promoting prostanoid production and augmenting the reactivity of vascular smooth muscle, all of which could become critical in causing blood pressure elevation, especially if this phenomenon occurs within the renal vasculature.

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Author contribution

All authors designed this study by helping to develop the experimental design. Oliver BARETELLA performed all of the experiments, analyzed the data and wrote the manuscript. Sookja K CHUNG contributed transgenic animals. Aimin XU and Paul M VANHOUTTE edited the manuscript. And all the authors reviewed and approved the manuscript.

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