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Mirabegron relaxes arteries from human visceral adipose tissue through antagonism of α_1-adrenergic receptors

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ABSTRACT

Aim: As inadequate perfusion has emerged as a key determinant of adipose tissue dysfunction in obesity, interest has grown regarding possible pharmacological interventions to prevent this process. Mirabegron has proved to improve insulin sensitivity and glucose homeostasis in obese humans via stimulation of β_3-adrenoceptors which also seem to mediate endothelium-dependent vasodilation in disparate human vascular beds. We characterized, therefore, the vasomotor function of mirabegron in human adipose tissue arteries and the underlying mechanisms.

Methods: Small arteries (116–734 μm) isolated from visceral adipose tissue were studied ex vivo in a wire myograph. After vessels had been contracted, changes in vascular tone in response to mirabegron were determined under different conditions.

Results: Mirabegron did not elicit vasorelaxation in vessels contracted with U46619 or high-K^+ (both $P > 0.05$). Notably, mirabegron markedly blunted the contractile effect of the α_1-adrenergic receptor agonist phenylephrine ($P < 0.001$) either in presence or absence of the vascular endothelium. The anti-contractile action of mirabegron on phenylephrine-induced vasoconstriction was not influenced by the presence of the selective β_3-adrenoceptor blocker L-748,337 ($P < 0.05$); lack of involvement of β_3-adrenoceptors was further supported by absent vascular staining for them at immunohistochemistry.

Conclusions: Mirabegron induces endothelium-independent vasorelaxation in arteries from visceral adipose tissue, likely through antagonism of α_1-adrenoceptors.

1. Introduction

Adequate perfusion has emerged as a key player in the remodeling of AT occurring in obesity and aging to accommodate an increased fat burden [1,2]. In addition to angiogenesis, the ability of AT nutritive arteries to dilate in response to vasoactive stimuli seems important to prevent the formation of a hypoxic environment, resulting in increased production of inflammatory cytokines and favoring, in turn, the occurrence of metabolic abnormalities [3]. Better comprehension of the mechanisms involved in the vasodilator reactivity of AT arteries, therefore, is important to devise effective strategies for improving AT perfusion, hence preventing the metabolic derangement in these patients.

As β_3-adrenoceptors mediate lipolysis in white AT and thermogenesis in brown AT, targeting these receptors has been proposed as an effective therapeutic strategy to reduce obesity and improve lipid and glucose homeostasis [4]. In fact, recent clinical studies have reported that chronic administration of the β_3-adrenoceptor agonist mirabegron is able to increase brown fat, HDL cholesterol, insulin sensitivity, β-cell function and oral glucose tolerance, even in the absence of any effect on body weight or fat mass [5,6]. Interestingly, in addition to its favorable metabolic properties, stimulation of β_3-adrenoceptors has demonstrated

Abbreviations: AT, adipose tissue; BK, bradykinin; CCRC, cumulative concentration-response curve; ET-1, endothelin 1; L-NMMA, L-N^G-nitro-arginine methyl ester; NO, nitric oxide; PE, phenylephrine; PSS, physiological salt solution; VAT, visceral adipose tissue; PVAT, perivascular adipose tissue.

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beneficial vascular actions in both animal models and human arteries. Thus, NO-mediated vasorelaxation has been observed in the rat aorta following exposure to a preferential β_3-adrenoceptor agonist [7]. Similarly, β_3-adrenoceptor stimulation has been shown to restore the NO/redox balance and improve endothelial function in a rabbit model of hyperglycemia [8]. Importantly, presence of β_3-adrenoceptors has also been detected in the endothelium, but not in the smooth muscle, of human coronary arteries, where their stimulation results in vasodilation mediated by both release of NO and hyperpolarization [9].

Even though mirabegron has been predominantly identified as a β_3-adrenoceptor agonist, it may exert some biological effects by acting on other adrenergic receptors or amine transporters [10]. Thus, it has been shown to relax urethral smooth muscle [11] and inhibit neurogenic smooth muscle contraction in the human prostate [12] via α_1-adrenoceptor antagonism. In addition, it has been proven able to antagonize noradrenaline-induced contraction in the rat aorta, but not in the rat spleen [11].

Whether mirabegron exerts vasorelaxing actions in arteries of human VAT has never been investigated. The present study, therefore, assessed the effects of mirabegron on resistance-sized arteries isolated from human VAT and characterized the subtype of adrenoceptor(s) involved.

2. Methods

2.1. Chemicals and solutions

U46619 (Tocris Bioscience, Bristol, United Kingdom), mirabegron (Tocris Bioscience), doxazosin mesylate (Tocris Bioscience), nadolol (Sigma-Aldrich, St. Louis, MO, USA), L-748,337 (Tocris Bioscience) and bupranolol (Toronto Research Chemicals, Toronto, Canada) were dissolved in dimethyl sulfoxide. Isoproterenol hydrochloride (Sigma-Aldrich), BK (Sigma-Aldrich), clonidine (Sigma-Aldrich), yohimbine (Sigma-Aldrich), L-NMMA (Sigma-Aldrich), ET-1 (Calbiochem, St. Louis, MO, USA) and PE (Sigma-Aldrich) were dissolved in distilled H_2O. Physiological salt solution (PSS) contained (mmol L^{-1}): NaCl 115; $NaHCO_3$ 25; K_2HPO_4 2.5; $MgSO_4$ 1.2; glucose 5.5; HEPES 10; and $CaCl_2$ 1.3 (pH 7.4). High-K^+ solution was obtained as a mix of PSS and a solution containing NaCl 20 mmol L^{-1} and KCl 95 mmol L^{-1}, to obtain a K^+ concentration of 32 mmol L^{-1} in the organ chamber. Buffers were continuously aerated with 5% CO_2 in air at 37 °C. All the indicated concentrations of the pharmacological agents added to the bathing solution represent their final concentration in the organ chamber.

2.2. Participants

VAT biopsies were collected from 33 patients (18 males, 15 females) undergoing laparoscopic surgery (cholecystectomy, hernial repair, metabolic surgery or colectomy). The clinical characteristics of the participants and the results of the biochemical analyses performed on blood samples collected in a fasting state before surgery are reported in Table 1. The study protocol was approved by the Institutional Review Board of the University Tor Vergata (approval number 13/2016), and all the participants gave written informed consent before their participation in the study. A list of the participants in the different protocols is reported in Table in the Data Supplement.

2.3. Adipose tissue collection and vessel preparation

VAT biopsies were collected intraoperatively during planned surgery, placed in ice-cold PSS, and either immediately processed or stored overnight in 30 mL PPS at 4 °C. Arteries were isolated from the tissue in PSS at room temperature and cleaned of surrounding fat and connective tissue, according to a procedure reported elsewhere [13]. The obtained vessels (lumen diameter ranging from 116 to 734 μm, average 360 ± 79 μm) were then cut into 2 to 4 segments of ≈ 2 mm length and mounted into the chambers of a wire myograph (DMT, Aarhus, Denmark) containing 5 mL of PSS at 37 °C. Afterwards, the arteries were kept for 15–30 min at 37 °C to perform the force calibration, with the arterial segments stretched to obtain a diameter and a wall tension corresponding to a transmural pressure of 100 mmHg. The internal diameter of the arteries was calculated, their viability was tested by addition of the high-K^+ solution, and the presence of intact endothelium was assessed by testing relaxation to BK (1 μmol L^{-1}). Segments that failed to contract or relax were discarded. A software (Lab Chart Pro; AD instruments, Dunedin, New Zeeland) was used to record and analyze the vasomotor response.

Table 1
Clinical characteristics of the participants.

Variable	
Sex, M/F	18/15
Age, years (range)	55 (20–73)
Body mass index, kg m^{-2}	31.4 ± 1.3
Weight, kg	93.2 ± 5.1
Height, m	1.72 ± 0.02
Systolic blood pressure, mmHg	135.4 ± 3.7
Diastolic blood pressure, mmHg	79.6 ± 2.1
Glucose, mg dL^{-1}	103.6 ± 5.1
Fibrinogen, mg dL^{-1}	363 ± 18
Creatinine, mg dL^{-1}	0.89 ± 0.04
AST, mg dL^{-1}	25.3 ± 1.7
ALT, mg dL^{-1}	26.2 ± 2.4
Antihypertensive drugs, %	33
Oral antidiabetes drugs, %	15
Lipid-lowering drugs, %	36
Anti-platelet drugs, %	22

Data are reported as means ± SEM.

2.4. Evaluation of the vasomotor responses to mirabegron in arteries contracted with different vasoconstrictor agents

Arteries obtained from VAT biopsies were prepared as described in the paragraph 2.3 and, after a washout period to re-establish the baseline tone, were contracted using either the stable synthetic analog of the endoperoxide prostaglandin PGH_2 U46619 (1 μmol L^{-1}; n = 3), ET-1 (3 nmol L^{-1}; n = 3) or high-K^+ solution (32 mM; n = 3). After 5 to 10 min, changes in the vascular tone in response to increasing concentrations of mirabegron (10^{-7} to 10^{-4} mol L^{-1}) were determined. In an attempt to evaluate the effects of mirabegron on α-adrenergic receptor-mediated vasoconstriction, vessels (n = 3) were challenged with the α-adrenergic receptor agonist PE (10 μmol L^{-1}). The contraction induced by PE, however, was not sustained, fading over a short time, probably due to activation of endothelial α-adrenergic receptors leading to release of NO [14]. The effect of mirabegron on PE-induced contraction, therefore, was assessed by preincubating arteries (n = 4) for 15 min with L-NAME (100 μmol L^{-1}), before constricting them with PE (10 μmol L^{-1}) and then constructing a CCRC to mirabegron. Alternatively, the effect of mirabegron on PE-induced contraction was assessed by comparing the CCRC to PE (10^{-8} to 10^{-4} mol L^{-1}) before and after incubation of arteries (n = 6) with mirabegron (10 μmol L^{-1}).

2.5. Involvement of endothelium in the vascular response to mirabegron

To investigate the possible role of endothelium-derived relaxing substances different from NO in the vascular response to mirabegron, vessels (n = 4) were denuded of endothelium by surface abrasion, gently inserting and removing a nylon suture (Ethilon 8–0; Ethicon, Somerville, NJ, USA) for 50× in the lumen, before mounting them in the myograph chamber; the success of the endothelial removal procedure was confirmed by absent relaxation of vessels to 1 μmol L^{-1} BK following precontraction with U46619 (1 μmol L^{-1}). Afterwards, arteries were contracted with PE (10 μmol L^{-1}) and the CCRC to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) was constructed in each segment.

2.6. Investigation of role of β-adrenergic receptors in the vasorelaxing effect of mirabegron

To evaluate the role of β-adrenergic receptors in the vasorelaxation elicited by mirabegron in arteries obtained from VAT, the CCRC to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) was determined in vessels priorly incubated with L-NAME (100 μmol L^{-1} for 15 min) and then contracted with PE (10 μmol L^{-1}), in the absence or presence of either the β_1/β_2-adrenoceptor blocker nadolol (10 μmol L^{-1}; n = 4) or the $\beta_1/\beta_2/\beta_3$-adrenergic receptor blocker bupranolol (10 μmol L^{-1}; n = 4), each of them preincubated for 5 min. In addition, we assessed the effect of mirabegron (10 μmol L^{-1}) on the CCRC to PE (10^{-8} to 10^{-4} mol L^{-1}), either in the absence or presence of the selective β_3-adrenoceptor blocker L-748,337 (10 μmol L^{-1} preincubated for 5 min; n = 3).

2.7. Characterization of the mechanisms of PE-induced vasoconstriction in VAT arteries

In order to gain more insights into the mechanisms involved in the PE-induced vasoconstriction, experiments were performed using the selective α_1-adrenergic receptor antagonist doxazosin or the nonselective α_1/α_2 adrenergic receptor antagonist yohimbine. To this end, CCRCs to PE (10^{-8} to 10^{-4} mol L^{-1}) were constructed in the absence or presence of either doxazosin (1 μmol L^{-1}; n = 3) or yohimbine (1 μmol L^{-1}; n = 3), each preincubated for 5 min. Furthermore, because of the relevant contribution of α_2-adrenoceptors to the vascular response to yohimbine observed in previous studies in other vascular beds [15], in order to ascertain their possible vasoconstrictor role in VAT arteries, studies were performed by use of the α_2-adrenoceptor agonist clonidine. Thus, following removal of the endothelium to abrogate the possible vasodilator effect of endothelial α_2-adrenoceptors [14], the vasoconstrictor responses to PE (10 μmol L^{-1}; n = 3) and to the selective α_2-adrenergic receptor agonist clonidine (10 μmol L^{-1}; n = 3) were compared. Finally, to ascertain the possible counteracting effect of β-adrenergic receptor stimulation on the vasoconstrictor response to PE of VAT arteries, CCRCs to PE (10^{-7} to 10^{-4} mol L^{-1}) were compared in the absence or presence of the β_1/β_2-adrenergic receptor agonist isoproterenol (10 μmol L^{-1}, preincubated for 5 min; n = 3).

2.8. Comparison of the effects of mirabegron and doxazosin on ET-1-induced contraction

To obtain further hints about the mechanism(s) involved in the effect of mirabegron on ET-1-mediated vasoconstriction, experiments were performed using the selective α_1-adrenergic receptor antagonist doxazosin. To this aim, vessels were contracted with ET-1 (3 nmol L^{-1}) and, after 5 to 10 min, changes in the vascular tone in response to increasing concentrations of mirabegron (10^{-7} to 10^{-4} mol L^{-1}; n = 3) or doxazosin (10^{-7} to 10^{-4} mol L^{-1}; n = 3) were compared.

2.9. Histology

In order to ascertain whether mirabegron might exert indirect anticontractile actions through release of vasorelaxing substances from PVAT [16], segments of a sample vessel were formalin fixed and stained with hematoxylin-eosin for histological analysis, to confirm that no residual PVAT had been left in place after the procedure used before mounting the vessels in the wire myograph.

2.10. Immunohistochemistry

Samples (n = 5) were formalin-fixed and embedded in paraffin. Serial sections were used for the immunohistochemistry staining to study the expression of β_3-adrenergic receptors. Paraffin sections (3 μm thick) were treated with citrate buffer pH 7.8 during 20 min at 95 °C for antigen retrieval. Afterwards, sections were incubated with an anti-β_3-adrenergic receptor polyclonal rabbit antibody (AB5122, Sigma Aldrich) for 60 min. Reactions were detected using a HRP-DAB Detection Kit (UCS Diagnostic, Roma, Italy). An Axioscope 5 light microscope (Zeiss, Oberkochen,

Germany) was used to evaluate the immunohistochemical reactions as number of β_3-adrenoceptor positive cells. Reactions were set-up by use of specific positive and negative control tissues (in particular, kidney tissue and visceral adipose tissue were used as a positive control and a tissue microarray section of brain, prostate, breast, and thyroid as a negative control).

2.11. Statistical analysis

For the CCRC experiments, differences between the dose-tension relations were analyzed using 2-way ANOVA or 2-way ANOVA for repeated measures, followed by Bonferroni post-hoc test for multiple comparisons, as appropriate. Changes in the dose-tension relations from baseline were analyzed by one-way ANOVA for repeated measures. Other comparisons (for experiments performed in arterial segments from the same patients) were tested by paired *t*-test. Potency ($-\log_{10}$ EC_{50}) and efficacy (E_{max}) of inhibition of the CCRC to PE by mirabegron, doxazosin or yohimbine were calculated by non-linear regression analysis and compared by 2-way ANOVA and 1-way ANOVA, respectively. A P value <0.05 was considered statistically significant. CCRCs are reported as percent of relaxation or as tension (N m^{-1}). All statistical analyses were performed by use of the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Data are reported as means ± SEM.

3. Results

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3.1. Comparison of the vascular response to mirabegron in arteries precontracted with different vasoconstrictor agents

Mirabegron (10^{-7} to 10^{-4} mol L^{-1}) resulted in a concentration-dependent relaxation in arteries contracted with ET-1 (3 nmol L^{-1}), with a maximal relaxation reaching approximately 60% of the starting tension (P = 0.001 vs. baseline). By contrast, no significant vasorelaxing response to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) was observed in arteries contracted with U46619 (1 μmol L^{-1}) or high-K^+ solution (32 Mm) (both P > 0.05 vs. baseline), without differences between these 2 vasoconstrictors (P > 0.05). As a result, the vasorelaxing effect of mirabegron was significantly higher in those vessels contracted with ET-1 (3 nmol L^{-1}) than in those contracted with either U46619 (1 μmol L^{-1}) or high-K^+ (32 mM) (Fig. 1, panel A).

Challenge of VAT arteries with PE (10 μmol L^{-1}) did not result in a sustained vasoconstrictor response from baseline after 5 min (a representative tracing is shown in Fig. 1, panel B, top); by contrast, vessels preincubated with the NO synthase inhibitor L-NMMA (100 μmol L^{-1}) showed a significant vasoconstrictor response 5 min after exposure to PE (a representative tracing is shown in Fig. 1, panel B, bottom); the vasoconstrictor effect of PE (10 μmol L^{-1}), therefore, resulted significantly greater in vessels preincubated with L-NMMA (100 μmol L^{-1}) than in those untreated (Fig. 1, panel C).

Arteries exposed to increasing concentrations of PE (10^{-8} to 10^{-4} mol L^{-1}) had a concentration-dependent vasoconstrictor response; the degree of PE-induced contraction, however, was significantly lower in vessels preincubated with mirabegron (10 μmol L^{-1}) than in those untreated (Fig. 1, panel D).

3.2. Effect of endothelium removal on mirabegron-induced vasorelaxation

As expected, bradykinin (BK, 1 μmol L^{-1})-induced relaxation was

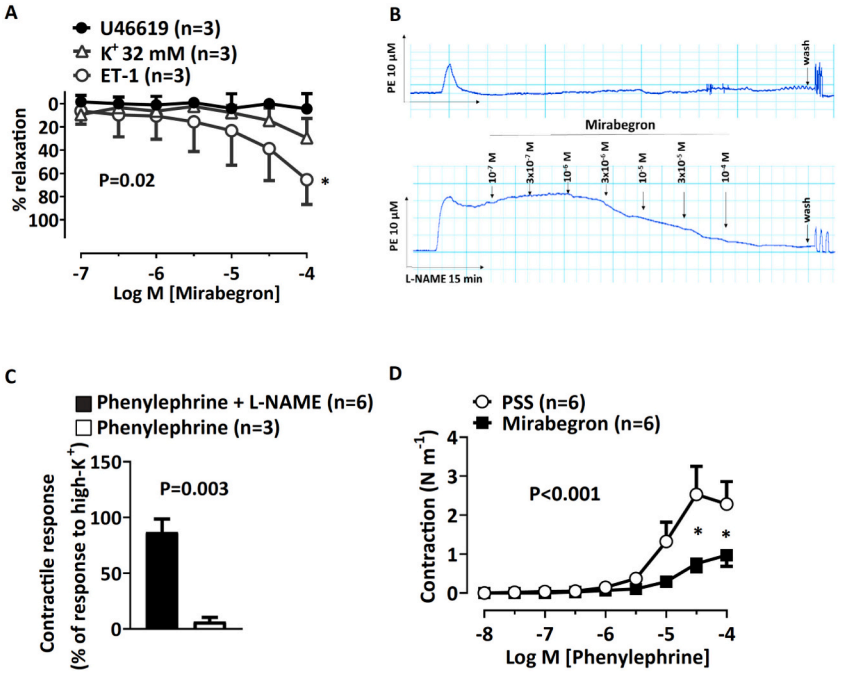

Fig. 1. A - Cumulative concentration-response curves (CCRCs) to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) in arteries precontracted with U46619 (1 µmol L^{-1}), ET-1 (3 nmol L^{-1}) or a high-K^+ solution (32 mM). B - Representative tracings of: Top, response to phenylephrine (10 µmol L^{-1}) in an arterial segment not previously incubated with L-NAME; bottom, contractile response to phenylephrine (10 µmol L^{-1}) in an arterial segment preincubated with L-NAME (100 µmol L^{-1}), followed by the CCRC to mirabegron. C - Comparison of the vasoconstrictor response to phenylephrine (10 µmol L^{-1}) in the presence or absence of L-NAME (100 µmol L^{-1}). D - CCRCs to phenylephrine (10^{-8} to 10^{-4} mol L^{-1}) in the absence or presence of mirabegron (10 µmol L^{-1}). Data are means±SEM. The P values indicate the significance level of differences at the 2-way ANOVA, paired *t*-test or 1-way ANOVA for repeated measures as appropriate. * $P < 0.05$ at the post hoc analysis. PSS, physiological salt solution.

almost completely abolished in segments of arteries with denuded, as compared with those with intact endothelium (Fig. 2, panel A). By contrast, the vasorelaxing response to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) after contraction with PE (10 µmol L^{-1}) was not different between segments with intact endothelium preincubated with L-NAME (100 µmol L^{-1}) and those with denuded endothelium (Fig. 2, panel B).

3.3. β-adrenoceptors and vasorelaxing effect of mirabegron

In arteries preincubated with L-NAME (100 µmol L^{-1}) and contracted with PE (10 µmol L^{-1}), the vasodilator effect of mirabegron (10^{-7} to 10^{-4} mol L^{-1}) was not different in the absence or presence of the β_1/β_2-adrenergic receptor blocker nadolol (10 µmol L^{-1}, Fig. 3, panel A); under the same conditions, the vasodilator response to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) was similar in the presence or absence of the $\beta_1/\beta_2/\beta_3$-adrenergic receptor blocker bupranolol (10 µmol L^{-1}) [17] (Fig. 3; panel B). Also, preincubation of arteries with the selective β_3-adrenergic receptor blocker L-748,337 (10 µmol L^{-1}) [10] did not modify the cumulative concentration response curve to PE (10^{-8} to 10^{-4} mol L^{-1}, Fig. 3, panel C).

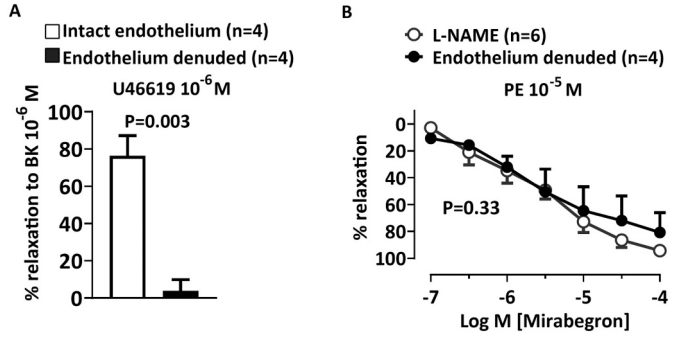

Fig. 2. A - Comparison of bradykinin (BK, 1 µmol L^{-1})-induced relaxation in arteries with either intact or denuded endothelium precontracted with U46619 (1 µmol L^{-1}). B - Comparison of the vasorelaxing responses to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) following contraction with phenylephrine (10 µmol L^{-1}) between segments with either intact endothelium preincubated with L-NAME (100 µmol L^{-1}) or denuded endothelium. Data are means±SEM. The P values indicate the significance level of differences at the paired *t*-test or the 2-way ANOVA, as appropriate.

3.4. Mechanisms of PE-induced vasoconstriction in VAT arteries

As expected, preincubation with the selective α_1-adrenergic receptor antagonist doxazosin (1 µmol L^{-1}) abolished the contractile response of VAT arteries to incrementing concentrations of PE (10^{-8} to 10^{-4} mol L^{-1},Fig. 4, panel A); similarly, the response to increasing concentrations of PE was abrogated in those segments preincubated with the $\alpha_{1/2}$-adrenergic receptor antagonist yohimbine (1 µmol L^{-1}) [18] (Fig. 4, panel B). The inhibitory effect on the CCRC to PE exerted by either doxazosin (log EC_{50} = -5.82, $E_{max} = 0.08 \pm 0.02$ N m^{-1}) and yohimbine (log EC_{50} = -5.95, $E_{max} = 0.03 \pm 0.02$ N m^{-1}), however, was higher compared to that evoked by mirabegron (log EC_{50} = -4.58, $E_{max} = 1.02 \pm 0.28$ N m^{-1}; $P < 0.001$ and $P = 0.004$, respectively). Following endothelium removal, challenging of arteries with the selective α_2-adrenergic receptor agonist clonidine (10 µmol L^{-1}) resulted in a negligible vasoconstrictor response, which was significantly lower than the one induced by an equimolar concentration of PE (10 µmol L^{-1}) (Fig. 4, panel C). Finally, prior incubation with the nonselective $\beta_{1/2}$-adrenergic receptor agonist isoproterenol (10 µmol L^{-1}) did not result in any significant change of the CCRC to PE (10^{-8} to 10^{-4} mol L^{-1}, Fig. 4, panel D).

3.5. Effects of doxazosin on ET-1-induced vasoconstriction as compared to mirabegron

Similar to mirabegron (10^{-7} to 10^{-4} mol L^{-1}), doxazosin (10^{-7} to 10^{-4} mol L^{-1}) resulted in a concentration-dependent relaxation in arteries contracted with ET-1 (3 nmol L^{-1}), with no significant difference between the vasorelaxing effect of the 2 drugs (Fig. 5).

3.6. Histology

Hematoxylin-eosin staining of a sample vessel confirmed that the procedure used before mounting arterial segments in the myograph had left no residual PVAT (Fig. 6, panels A and B).

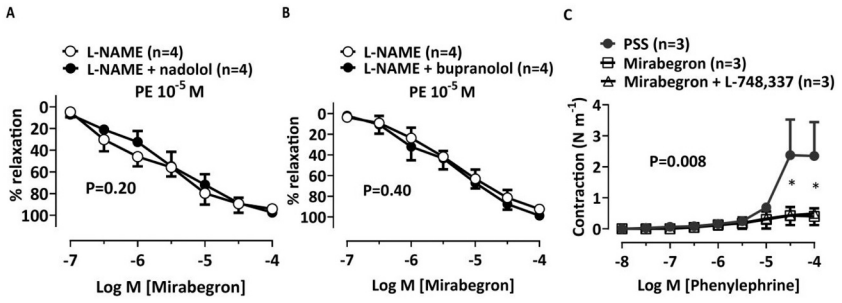

Fig. 3. A - Comparison of the vasorelaxing response to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) in the absence or presence of the β_1/β_2-adrenoceptor blocker nadolol (10 μmol L^{-1}) in arteries contracted with phenylephrine (10 μmol L^{-1}) following incubation with L-NAME (100 μmol L^{-1}). B - Comparison of the vasorelaxing response to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) in the absence or presence of the nonselective $\beta_1/\beta_2/\beta_3$-adrenoceptor blocker bupranolol (10 μmol L^{-1}) in arteries contracted with phenylephrine (10 μmol L^{-1}) following incubation with L-NAME (100 μmol L^{-1}). C - Cumulative dose-response curves to phenylephrine (10^{-8} to 10^{-4} mol L^{-1}) in the absence or presence of mirabegron (10 μmol L^{-1}), either alone or in combination with the selective β_3-adrenoceptor blocker L-748,337 (10 μmol L^{-1}). Data are means±SEM. The P value indicates the significance level of differences at the 2-way ANOVA for repeated measures. * $P < 0.05$ at the post hoc analysis. PSS, physiological salt solution.

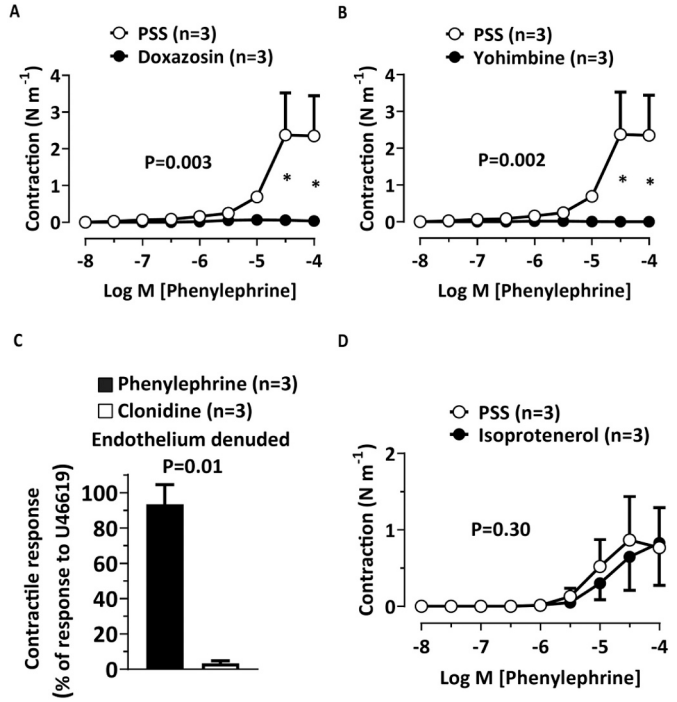

Fig. 4. A - Cumulative concentration-response curves (CCRCs) to phenylephrine (10^{-8} to 10^{-4} mol L^{-1}) in absence or presence of the selective α_1-adrenergic receptor antagonist doxazosin (1 μmol L^{-1}). B - CCRCs to phenylephrine (10^{-8} to 10^{-4} mol L^{-1}) in absence or presence of the nonselective α_1/α_2-adrenergic receptor antagonist yohimbine (1 μmol L^{-1}). C - Comparison of the contractile responses to phenylephrine (10 μmol L^{-1}) and clonidine (10 μmol L^{-1}) in arteries with denuded endothelium. D - CCRCs to phenylephrine (10^{-8} to 10^{-4} mol L^{-1}) in the absence or presence of the β-adrenergic receptor agonist isoproterenol (10 μmol L^{-1}). Data are means±SEM. The P values indicates the significance level of differences at the 2-way ANOVA for repeated measures or paired *t*-test, as appropriate. * $P < 0.05$ at the post hoc analysis. PSS, physiological salt solution.

3.7. Immunohistochemistry

Positive staining for β_3-adrenergic receptors was found in epithelial cells of the renal tubule (Fig. 5, panels A and B) and in perivascular adipose tissue (Fig. 5, panels C and D), which were used as positive controls. Analysis of intact vessels, by contrast, did not demonstrated any β_3-adrenergic receptor staining, in either endothelial or smooth muscle cells of all the examined samples (Fig. 7, panels E and F).

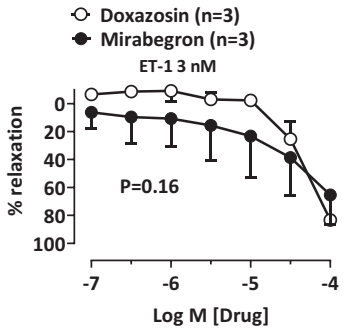

Fig. 5. Comparison of the cumulative concentration-response curves (CCRCs) to mirabegron (10^{-7} to 10^{-4} mol L^{-1}) and doxazosin (10^{-7} to 10^{-4} mol L^{-1}) in arteries precontracted with ET-1 (3 nmol L^{-1}). Data are means±SEM. The P value indicates the significance level of difference at the 2-way ANOVA.

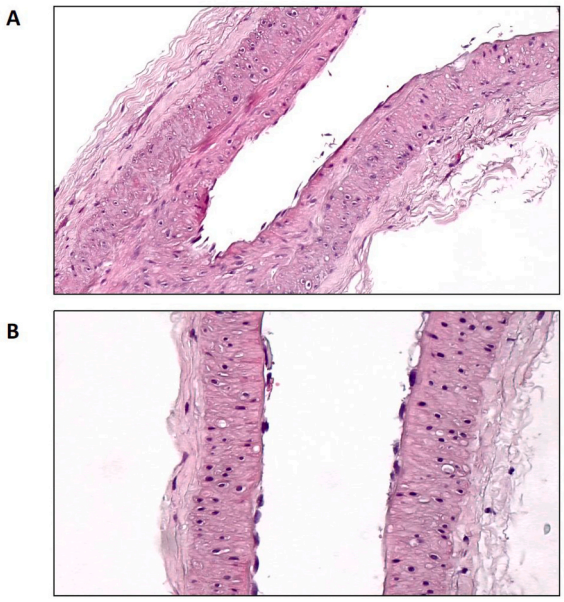

Fig. 6. Histology. A and B – Cross-sectional and longitudinal section of a sample artery obtained from VAT following hematoxylin-eosin staining, demonstrating the absence of any residual PVAT after the procedure used before mounting the arterial segments in the wire myograph.

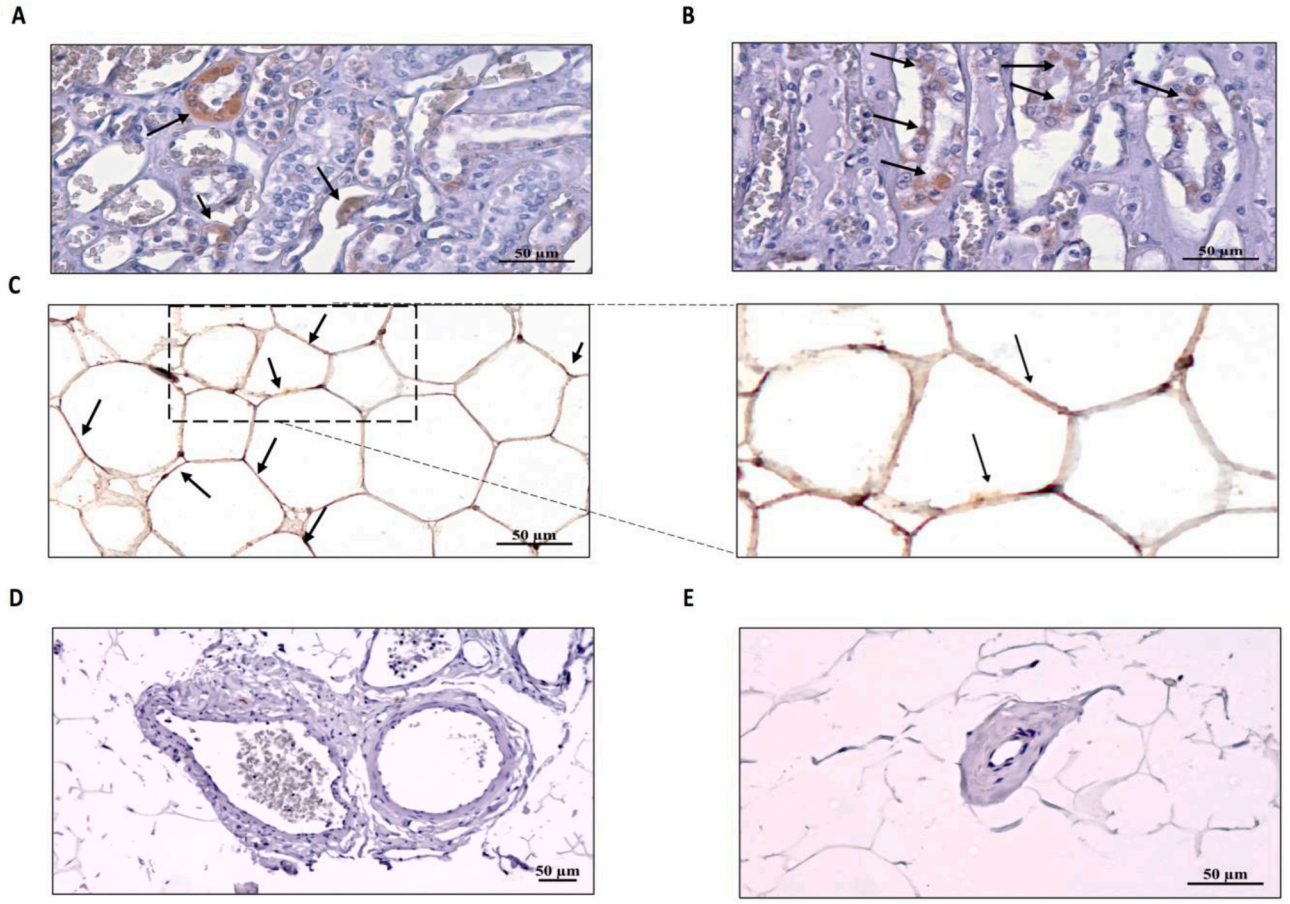

Fig. 7. Immunohistochemistry. A and B - Expression of β_3-adrenergic receptors in epithelial cells of the renal tubule (arrows), at lower (A) and higher (B) magnification. C and D - Representation of expression of β_3-adrenoceptor-positive perivascular visceral adipose tissue cells (arrows). E and F - Representation of absent immunostaining for β_3-adrenoceptors of endothelial and smooth muscle cells of visceral adipose tissue arteries.

4. Discussion

The main finding of the present study is that mirabegron exerts a vasorelaxing action in arteries of human VAT, likely through inhibition of α_1-adrenergic receptors.

Interestingly, mirabegron-induced vasorelaxation was observed in arteries contracted with PE or ET-1, but not in those contracted with U46619 or high-K^+. This finding suggests that mirabegron interferes with selective mechanisms of vascular smooth muscle contraction. It might be explained by the role of receptor-operated calcium channels, including those activated by adrenoceptors, in the sustained phase of ET-1-mediated vasoconstriction [19], by overlapping of intracellular signal transduction pathways involved in vascular smooth muscle contraction [20], or even by antagonism of mirabegron on ET_A receptors. The latter mechanism was indirectly tested in our study by use of doxazosin, an established α1-adrenoceptor antagonist with no effect to block ET_A receptors. Our observation that mirabegron shares with doxazosin a similar inhibitory effect on ET-1-mediated contraction argues against an involvement of ET_A receptors in its vascular action.

Of note, in our study PE failed to elicit sustained vasoconstriction in those arterial segments that had not previously incubated with the NO synthase inhibitor L-NAME. This observation hints toward the presence of functional α-adrenergic receptors mediating NO release in human VAT arteries and is in keeping with the notion that constrictor amines, such as norepinephrine, can release endothelium-derived vasodilator substances that act as physiological antagonists of the smooth muscle contractile responses [21]. Finally, an additional characteristic of the mirabegron-induced vasorelaxation evidenced by our study is its independence from endothelial release of NO or other endothelium-derived vasodilators, as the vasorelaxing effect of mirabegron was preserved in vessels that had been preincubated with L-NAME or that had undergone endothelium removal. Taken together, these observations imply that the drug must act directly on vascular smooth muscle cells, having ruled out a role of endothelium in the vasodilator response to mirabegron.

A number of studies have reported the presence of β_3-adrenoceptors mediating a variety of biological function in the heart and the vasculature [22]. In particular, studies in human internal mammary artery have demonstrated the presence of β_3-adrenoceptor messenger ribonucleic acid and protein, as well as positive immunostaining for β_3-adrenoceptors; also, stimulation of β_3-adrenoceptors induced endothelium-dependent relaxation in PE-precontracted rings, an effect that was greatly blunted by selective antagonism of β_3-adrenoceptors [23]. More specifically, β_3-adrenoceptor-mediated, endothelium-dependent vasodilation to mirabegron has been observed in arteries of the cremaster muscle from Sprague-Dawley rats after β_1/β_2-adrenoceptor blockade [24]. In our study, having excluded an involvement of β_1- and β_2-adrenoceptors in the mirabegron-induced vasorelaxation by use of nadolol, we tested the possible role of β_3-adrenoceptors using 2 complementary approaches. We observed that either the non-selective β_3-adrenergic bupranolol or the selective β_3-adrenoceptor blocker L-748,337 do not impact the vasorelaxing/anti-contractile actions of mirabegron on PE-induced vasoconstriction. Taken together, these results definitely argue against a role of β_3-adrenoceptors in the mirabegron-induced vasorelaxation in human VAT arteries, a view further strengthened by the results of our immunohistochemistry analyses, showing absent

immunostaining for β_3-adrenergic receptors in the arterial endothelium and smooth muscle; expression of these receptors, by contrast, was found in the epithelial cells of the renal tubule and in white adipocytes. In this regard, our results are at odds with previous observations indicating the presence of β_3-adrenoceptors mediating vasodilation in human arteries [9,23]. Even though the reasons for these discrepancies are not entirely clear, differences between vascular beds and the vessel size may provide a likely explanation. More in general, the results of our study depose against a functional role of the β-adrenergic receptors in VAT arteries, given that, when these arteries were preincubated with the β_1/β_2-adrenergic receptor agonist isoproterenol, the contractile effect of PE was unaffected. This finding is in contrast with previous observations indicating that either isoproterenol or the selective β_2-adrenoceptor agonist salbutamol, when infused into the brachial artery, may induce forearm vasodilation by a combination of endothelium-dependent and independent mechanisms [25,26]. The smaller size of the vessels used in our study compared to the forearm vasculature might, again, be postulated as a possible explanation for these divergent results.

A previous study has shown that some β_3 adrenoceptor ligands may concurrently exert competitive antagonism on α_1-adrenoceptors in rat pulmonary arteries [27]. Also, mirabegron has been demonstrated to relax smooth muscle in urethra, vas deferens and prostate by a dual mechanism, involving a combination of β_3-adrenoceptor activation and α_1-adrenoceptor blockade [11]. In our study, mirabegron exerted an inhibitory action on the α-adrenoceptor-mediated vasoconstrictor response to PE, similar to that induced by the selective α_1-adrenoceptor blocker doxazosin or the non-selective α_1/α_2-adrenoceptor antagonist yohimbine. The inhibitory effect of mirabegron on PE-induced contraction, however, was minor than that of the other 2 agents, suggesting that it has lower intrinsic antagonistic activity on α-adrenoceptors, even though use of a single dose, rather that multiple concentrations, of each α-adrenoceptor antagonist prevent us from drawing firm conclusions in this regard. In addition, our observation that the selective α_2-adrenoceptor agonist clonidine did not elicit a vasoconstrictor response argues against a contractile role of these receptors in human VAT arteries, thereby hinting toward α_1-adrenoceptor antagonism as the most likely mechanism of the vasorelaxing effect of mirabegron in those vessels. This conclusion is in keeping with the previous observation in the smooth muscle of the urinary tract [11] and this accordance helps to overcome a potential limitation of our study, related to the small number of some experimental series.

4.1. Perspectives

Prospective clinical trials have demonstrated that long-term stimulation of β_3-adrenoceptors by mirabegron increases the mass of brown AT, favors the browning of white AT, improves insulin sensitivity and decreases cardiovascular risk [6,7], thereby sparking growing interest about its potential in the treatment of obesity and related cardiometabolic disease. The results of the current study expand those previous observations by showing that, in addition to the effects mediated by β_3 adrenoceptor stimulation, mirabegron has the ability to relax nutritive arteries of the VAT through antagonism of α_1-adrenoceptors. This action suggests that mirabegron might effectively improve VAT perfusion, thereby favoring a healthy AT remodeling and preventing, in turn, some of the unwanted cardiometabolic consequences of obesity and aging. A note of caution in the interpretation of our results stems from the consideration that the circulating concentrations of mirabegron effective to relax VAT arteries might be higher than those achievable in vivo following its oral administration. On the other hand, however, it cannot be excluded that chronic treatment with mirabegron might induce vasorelaxation at doses lower than those proven effective in our ex-vivo vessel preparations; also, in intact vessels in vivo β_3-adrenoceptor stimulation of perivascular adipocytes by mirabegron might constitute an additional mechanism of vasoralaxation through increased fat cell-derived NO [16]. The latter mechanism has been ruled out in our study by careful removal of PVAT after the procedure used before mounting the vessels, but, taken together, these considerations underscore the need of larger, specifically-designed clinical studies to test the efficacy of this approach in human disease.

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CRediT authorship contribution statement

Alessandro De Stefano: Methodology, Software, Data curation. **Francesca Schinzari:** Writing – review & editing. **Nicola Di Daniele:** Writing – review & editing. **Giuseppe Sica:** Resources. **Paolo Gentileschi:** Resources. **Giuseppina Vizioli:** Writing – original draft. **Carmine Cardillo:** Conceptualization, Validation, Supervision, Funding acquisition. **Manfredi Tesauro:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2022.107094.

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