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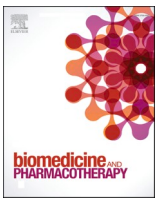

Chronic sarpogrelate treatment improves renal sympathetic hyperactivity in experimental diabetes

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ABSTRACT

Diabetes and derived complications, especially diabetic nephropathy and neuropathy annually cause great morbimortality worldwide. 5-hydroxytryptamine (5-HT) acts as a modulator of renal sympathetic input and vascular tone. In this line, 5-HT_2 receptor blockade has been linked with reduced incidence and progression of diabetic microvascular alterations. In this work, we aimed to determine, in diabetic rats, whether 5-HT_2 blockade ameliorates renal function and to characterize the serotonergic modulatory action on renal sympathetic neurotransmission. Diabetes was induced in male Wistar rats by alloxan administration (150 mg/kg, s.c.), and sarpogrelate (30 mg/kg·day, p.o.; 5-HT_2 antagonist) was administered for 14 days (DM-S). Normoglycemic and diabetic (DM) animals were maintained as aged-matched controls. At 28th day, DM-S animals were anesthetized and prepared for the *in situ* autoperfusion of the kidney. Renal vasoconstrictor responses were induced electrically or by i.a. noradrenaline (NA) administration. The role of 5-HT and selective 5-HT agonist/antagonist were studied on these renal vasopressor responses. Sarpogrelate treatment decreased renal sympathetic-induced vasopressor responses, reduced renal hypertrophy and kidney damage markers increased in DM. Intraarterial 5-HT inhibited the sympathetic-induced renal vasoconstrictions, effect reproduced by 5-CT, AS-19, L-694,247 and LY 344864 (5-$HT_{1/5/7}$, 5-HT_7, 5-HT_{1D} and 5-HT_{1F} receptor agonists, respectively). Blocking 5-$HT_{1D/1F/7}$ receptors completely abolished the 5-CT sympatho-inhibition. NA vasoconstrictions were not altered by any of the 5-HT agonists tested. Thus, in experimental diabetes, chronic sarpogrelate treatment reduces renal damage markers, kidney hypertrophy and renal sympathetic hyperactivity and modifies serotonergic modulation of renal sympathetic neurotransmission, causing a sympatho-inhibition by prejunctional 5-$HT_{1D/1F}$ and 5-HT_7 activation.

1. Introduction

Diabetes mellitus has emerged as a major global health challenge, affecting millions of people worldwide; moreover, it is expected to be the most common chronic disease in a few decades [1]. One of the most significant associated complications is the diabetic nephropathy, a progressive kidney condition that conduces to end-stage renal disease [2], to which various mechanisms (including inflammation, oxidative stress, altered signaling pathways) and the existence of other comorbidities [3,4] contribute. One of these concomitant pathologies is the diabetic neuropathy, characterized by a sympathetic imbalance, that is partly responsible of renal damage development [5].

Serotonergic system, due to its great variety of receptors [6] stands out not only by its central function in anxiety or depression (among others), but also due to its peripheral regulatory role in vascular function, platelet aggregation, and tissue remodeling. Recent studies have suggested a potential link between 5-hydroxytryptamine (5-HT) and the pathogenesis of diabetic nephropathy, outlining new possible therapeutic strategies consisting of targeting different 5-HT receptor type/-subtypes [7,8]. In fact, in renal vasculature, 5-HT exerts both direct actions [9–11] and indirect effects by modulating sympathetic renal innervation in normoglycemic and diabetic animals [7,12]. 5-HT_2 receptors, widely expressed in renal tissues, are of particular interest since they mediate deleterious effects such as vasoconstriction [10,13],

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inflammation, and fibrosis, which are key processes in the development and progression of diabetic nephropathy [8,14].

The prevalence of nephropathy as a diabetic complication continues to increase day by day. Hence, finding innovative therapeutic strategies to reduce final renal failure is a must in the XXI century. Sarpogrelate, a selective 5-HT_2 receptor antagonist [15], has emerged as a potential therapeutic drug for various vascular complications associated with diabetes [16]; thus, blocking 5-HT_2 receptor may also become a new possible target to modify/improve pathogenic mechanisms underlying diabetic nephropathy [17,18], closely related to other long-term diabetic complications, such microvascular alterations/damage or diabetic neuropathy.

In this line, our work analyzed the effect of chronic blockade of 5-HT_2 receptors on kidney dysfunction and renal adrenergic damage in diabetes, as well as determined the serotonergic profile involved in the modulation of renal sympathetic input in sarpogrelate-treated diabetic animals.

2. Material and methods

2.1. Ethical approval of the study protocol

The experimental protocol was approved by the Research Ethical Committee of University of Salamanca (ID number 0001003) according with regulation provided by the European Union of use of animals for scientific purposes and Spanish guidelines (Directive 2010/63/EU; R.D. 53/2013).

2.2. Drugs utilized

Each drug and its respectively supplier used in the experiment were: sarpogrelate hydrochloride from VulcanChem (Los Angeles, CA, US); heparin sodium from Rovi (Madrid, Spain); sodium pentobarbital (Dolethal®; Vetoquinol, Madrid, Spain); atropine sulphate (Scharlau, Barcelona, Spain); 1-phenylbiguanide (1-PBG), 5-hydroxytryptamine (5-HT) hydrochloride, alloxan monohydrate and noradrenaline (NA) bitartrate (Merck life Sciences, Madrid, Spain); 5-carboxamidotriptamine (5-CT), 8-OH-DPAT, AS-19, CGS 12066B dimaleate, cisapride, GR 127935 hydrochloride, L-694,247, LY 344864 hydrochloride, SB 258719 hydrochloride and SB 699551 (Tocris Bioscience, Bristol, UK).

Al these compounds were dissolved in saline at the experimentation time, except AS-19 (dissolved in ethanol, 5 %) and cisapride (0.01 M HCl). None of the vehicles exhibit properties to alter renal perfusion pressure (renal-PP), heart rate (HR) or systemic blood pressure (SBP).

2.3. General methods

Diabetes mellitus was induced in animals (male Wistar rats; 300 ± 25 g) by the administration of alloxan (150 mg/kg, s.c.) [7,19,20]. Diabetic animals were maintained for 28 days in the animal facilities of the University of Salamanca, with food and drink *ad libitum*, controlled temperature and humidity conditions (22 ºC and 50 %, respectively) in 12/12 h light-dark cycle.

Body weight and non-fasting glycemia were periodically controlled using a glucometer (*Accu-chek*®, Aviva, Roche Diagnostics; Barcelona, Spain). Glycemia over 250 mg/dL was set as diabetic-glycemia values. Animals with glycemia under this value were discarded.

From day 14, animals received sarpogrelate (30 mg/kg·day, p.o.; dissolved in drinking water) [19,21] to achieve the sarpogrelate-treated diabetic group (DM-S).

In parallel, a normoglycemic group of animals (NG) and a non-treated diabetic group of animals (DM) were maintained for 28 days as controls to perform complementary studies (Fig. 1)

2.4. Experimental protocols: renal sympathetic neurotransmission study in sarpogrelate-treated diabetic rats

After 28 days of diabetes induction, DM-S animals were anesthetized (pentobarbital, 60 mg/kg, i.p.) and prepared for the *in situ* autoperfusion of the left kidney as previously described by our group [7,12]. Jugular and femoral veins were cannulated for drugs i.v. administration. Both carotid arteries were cannulated, the right for the hemodynamic control of the animal and the left carotid artery to stablish renal autoperfusion

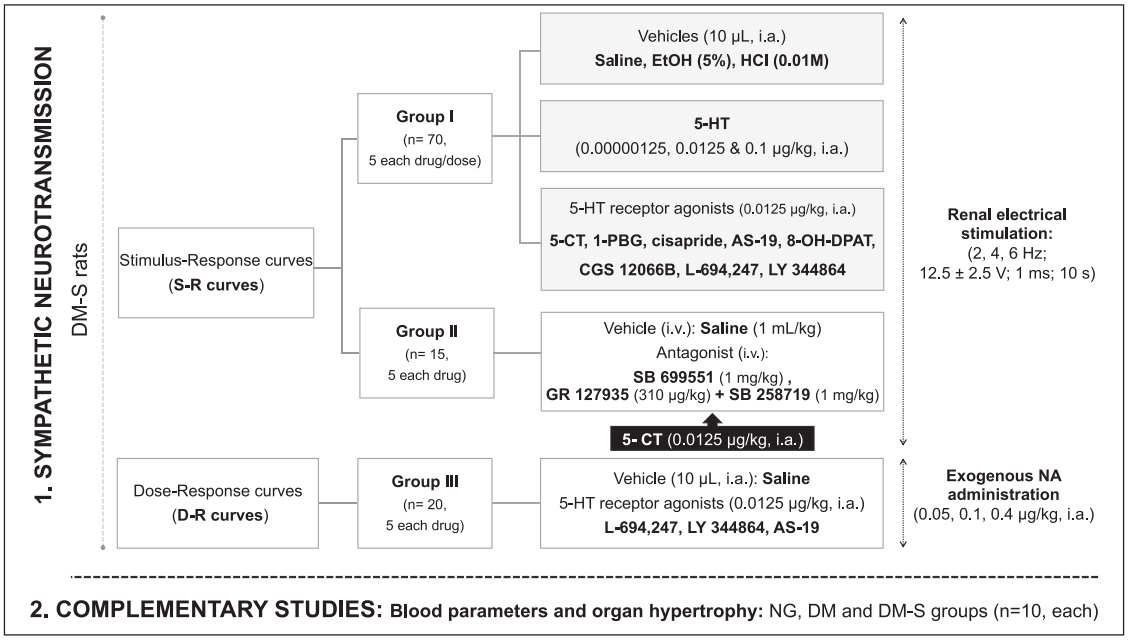

Fig. 1. Experimental design, including protocols and number of animals used. (1) The sympathetic neurotransmission study in sarpogrelate-treated diabetic group (DM-S) was divided into two main clusters, inducing the vasopressor responses by either (i) renal periarterial nerves electrical stimulation (S-R curve) or (ii) i.a. bolus noradrenaline (NA) administration (D-R curves). (2) The complementary studies were carried out in normoglycemic (NG), diabetic (DM) and DM-S rats, analyzing organ hypertrophy and blood renal parameters.

using an extracorporeal circuit to the cannulated left renal artery through a Gilson peristaltic pump [7,12]. Both carotid arteries were connected to different pressure transducers, ensembled to an e-corder 410 amplifier (Model ED410, Cibertec, Spain) to record the renal-PP, SBP and HR.

Once the circuit was stablished, heparin sodium (5 mg/kg, i.v.) was administered as a preventive blood clotting agent, and saline i.v. infusion was initiated at 2 mL/h rate (to maintain volemia in the animals). Atropine (1 mg/kg) was intravenously administered as a muscarinic blocker.

At the beginning of the experiment, flow was adjusted to equate renal-PP and SBP. Flow was kept constant until the end of the procedure, at a flow rate (from 2 to 2.9 mL/min) stablished through the peristaltic pump from left carotid artery to the renal vascular bed [7]. After 15 min of surgery, the hemodynamic condition was stable. At this time the baseline values of SBP, HR and renal-PP were recorded. Afterwards, the sympathetic renal nerves were electrically stimulated or the i.a. NA administration was performed (as indicated below).

At this point, the DM-S group (n=105; Fig. 1) was divided into two different clusters to investigate the effect of 5-HT-receptors agonists/antagonist on the vasopressor responses induced by (a) the electrical stimulation of periarterial renal nerves (stimulus-response (S-R) curves) or (b) the i.a. NA administration (dose-response (D-R) curves). The renal electrical stimuli (2, 4, 6 Hz; 12.5 ± 2.5 V; 1 ms; 10 s) and the NA administration (0.05, 0.1 and 0.4 μg/kg, i.a.) were fixed in sequential 3–5 min intervals. The responses obtained showed increases in renal-PP (without alteration of SBP) and renal-PP came back to basal values immediately after the stimulation ended or the NA administration.

2.4.1. Periarterial renal nerves electrical stimulation

Increases in renal-PP (Δ renal-PP) were performed by electrical stimulation of the renal artery at increasing frequencies (by placing an electrode in the left renal artery coupled to a Cibertec Stimulator C9, using the conditions previously described) at 5 min intervals between each frequency. The control S-R curve (E0) was achieved in 15 min.

The first DM-S group (S-R curve, Fig. 1) was performed to study the effect of 5-HT receptor selective agonists on the renal sympathetic vasoconstrictor responses induced by electrical stimulation. This group (group I, total n=70; n=5 for each agonist and dose) received the intraarterial bolus injection in a maximum volume of 10 μL using a micro-syringe of: (i) saline, ethanol 5 % or HCl 0.01 M (vehicles), (ii) 5-HT (0.00000125, 0.0125 and 0.1 μg/kg), (iii) the selective 5-HT receptor agonists, at 0.0125 μg/kg: 5-CT (5-$HT_{1/5/7}$), 1- PBG (5-HT_3), cisapride (5-HT_4), AS-19 (5-HT_7), 8-OH-DPAT (5-HT_{1A}), CGS 12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}), LY 344864 (5-HT_{1F}). After 5 min of the i.a. administration, a new S-R curve (E1) was executed as described for the E0 (control curve).

In the second one (group II, n=15), the effect of the selective 5-$HT_{1/5/7}$ agonist (5-CT, 0.0125 μg/kg) was studied in presence of the different 5-HT receptor antagonists: SB 699551 (5-HT_{5A}; 1 mg/kg; i.v.) a mixture of GR 127935 (5-$HT_{1B/1D/1F}$; 310 μg/kg; i.v.) plus SB 258719 (5-HT_7, 1 mg/kg; i.v.) or their vehicle (saline,1 mL/kg, i.v.). This set of experiments was used to confirm the receptor type/subtype(s) involved in the modulation of the renal sympathetic nerve vascular activity. Firstly, after 10 min of each antagonist (or their vehicle) administration the $E0_{saline}$ (control), $E0_{SB\ 699551}$ or $E0_{GR\ 127935+SB\ 258719}$ were completed as previously described. Then, in the presence of each antagonist, the animals received 0.0125 μg/kg of 5-CT (i.a) and a new S-R curve (E1) was performed.

2.4.2. Vasopressor responses by exogenous NA

In the third group of DM-S animals (group III, n= 20), the Δ renal-PP were induced by increasing doses of exogenous NA administered i.a. (as previously reported). The D-R curve was performed before (E0') and after 5 min of i.a. administration of saline (10 μL), L-694,247, LY 344864 or AS-19 (0.0125 μg/kg, each) to obtain a new D-R curve (E1').

2.5. Blood parameters and organs hypertrophy determination

Complementary studies were performed in three groups of animals: NG, DM and DM-S (n=10 each). After the anesthetic administration (pentobarbital, 60 mg/kg, i.p.), blood samples were collected from the carotid artery, and centrifuged at 3000 rpm for 10 min. Serum samples were kept at -80 ºC until analysis.

To determine renal function, total proteins, blood urea nitrogen (BUN) and creatinine were measured in each group. To do so, serum samples were placed in multiparametric SpotChem EZ® analytic strips and analyzed using the biochemistry autoanalyzer SpotChem EZ® (ARKRAY, Japan). Creatinine clearance was calculated from every animal blood creatinine and weight value by using ACLARA, a web-based, openly available estimated creatinine clearance (eClCr) calculator (http://idal.uv.es/acalara) [22].

The right kidneys and left ventricles were collected and prepared as previously stated [21]. Then, left ventricle hypertrophy (LVH) index was calculated using left ventricle weight/tibia length ratio (mg/mm), whereas the renal hypertrophy (RH) index was calculated considering the kidney weight/tibia length ratio (mg/mm).

2.6. Statistical evaluation and data presentation

All data in the text, figures, and tables are presented as mean ± S.E.M. of five experiments, unless otherwise stated. Changes in the renal vascular resistance from the baseline values, both induced by electrical stimulation or i.a. NA administration are presented as Δ renal-PP (mm Hg). Comparison of the results from the experimental groups and their corresponding control group was carried out with one-way ANOVA followed by the Student-Newman-Keul's post hoc test. Statistical significance was accepted at $p < 0.05$.

Note that: Δ renal-PP after saline i.a. administration did not differ from those obtained in the E0 curve (nothing), hence to simplify statistical analysis is only carried out *vs* saline.

3. Results

3.1. Metabolic and hemodynamic parameters in DM-S rats

In DM-S, the glycemic values augmented from 112 ± 3 mg/dL (day 0; prior alloxan administration) to 537 ± 7 mg/dL (day 2; post-alloxan administration), ($p < 0.05$ *vs day 0)*. This hyperglycemic state was maintained until the day 28 (548 ± 10 mg/dL), and it did not differ from the DM control group (554 ± 6 mg/dL).

However, body weight significantly decreased two days after alloxan administration (279 ± 2 g *vs* 300 ± 2 g prior administration; $p < 0.05$ *vs day 0*), and this body weight was maintained until day 28.

After anesthesia, SBP, HR and renal-PP were 91 ± 3 mm Hg, 295 ± 6 beats per min (bpm) and 109 ± 4 mm Hg, respectively in the DM-S animals. These basal values were not modified by the i.a. administration of selective 5-HT receptor agonists, vehicles (saline, ethanol 0.5 % and HCl 0.01 M) or the i.v. 5-HT antagonist administrations. In contrast, only i.a. administration of higher doses of 5-HT (0.0125 and 0.1 μg/kg) evoked a transient but significant Δ renal-PP (13.6 ± 3.5 and 16.2 ± 3.0, respectively; $p < 0.05$ *vs* basal values).

3.2. Renal sympathetic-induced vasopressor responses in diabetic rats treated with sarpogrelate

The electrical stimulation of renal periarterial nerves induced endogenous NA release, and consequently immediate and frequency-dependent Δ renal-PP, that were 23.9 ± 2.1, 59.5 ± 1.9 and 80.5 ± 3.5 for 2, 4 and 6 Hz, respectively (S-R curve; Fig. 2). Similarly, exogenous NA i.a. administration in ascending doses induced dose-dependent renal vasopressor responses (Δ Renal-PP), that were 26.5 ± 3.5, 42.2 ± 3.9 and 72.5 ±8.5 mm Hg for 0.05, 0.1 and 0.4 μg/kg i.a. NA

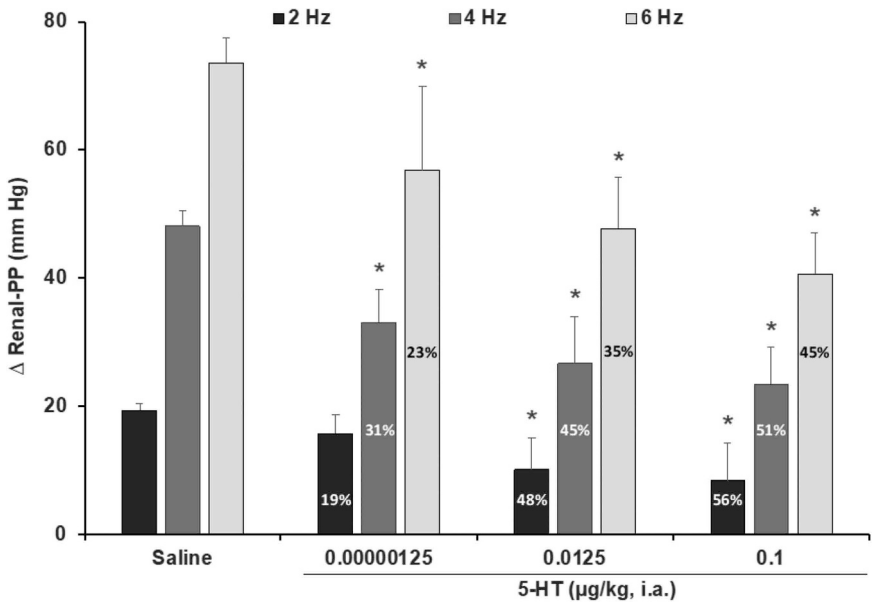

Fig. 2. Effect of intraarterial bolus of 10 μL of saline (control; n=5) and increasing doses of 5-HT (0.00000125–0.1 μg/kg; n=5 each dose) on the increases in renal perfusion pressure (Δ Renal-PP) evoked by the selective stimulation of renal sympathetic nerves in the in situ autoperfused kidney of sarpogrelate-treated diabetic rats. Percentual values shown inside the bars indicates the percentages of inhibition vs saline. *p < 0.05 vs saline using one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

doses, respectively (D-R curve, control). These vasoconstrictor responses (electrical or exogenous NA) were selectively induced in the renal vascular bed, so neither SBP nor HR were altered (data not shown).

3.3. *Effect of vehicles, 5-HT or selective 5-HT receptor type agonists on the renal vasopressor responses induced by electrical stimulation in DM-S rats*

The Δ renal-PP due to electrical stimulation at increasing frequencies (2, 4 and 6 Hz; Fig. 2) were not modified by the intraarterial administration of 10 μL of saline (Fig. 2), ethanol 5 % or HCl 0.01 M (data not shown). In contrast, i.a. 5-HT administration (0.00000125, 0.0125 and 0.1 μg/kg) induced a dose and frequency-dependent inhibition of the sympathetic-induced **Δ** renal-PP (Fig. 2) in DM-S rats.

As shown in Fig. 3, the electrically evoked Δ renal-PP were not altered by the intraarterial administration of the 5-HT_3 or 5-HT_4 receptor agonists (1-PBG or cisapride, respectively at 0.0125 μg/kg). In contrast, i.a. 5-CT (5-$HT_{1/5/7}$ agonist; 0,0125 μg/kg) mimicked the 5-HT inhibitory effect on the vasopressor responses (Fig. 3). To the same extent, AS-19 (5-HT_7 selective agonist; 0,0125 μg/kg, i.a.) significantly reduced the electrical-induced renal vasoconstrictor responses (Fig. 3).

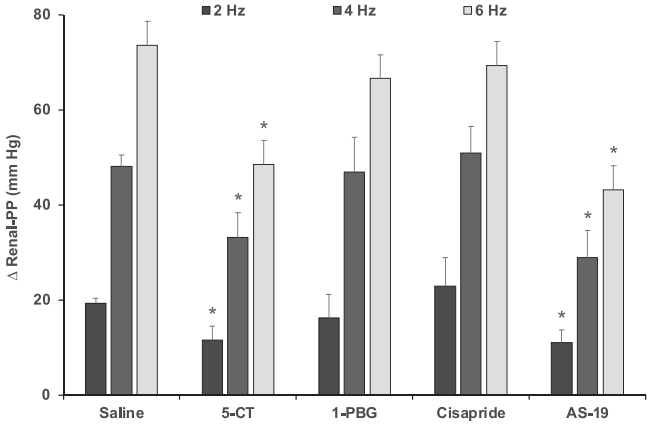

Fig. 3. Effect of the i.a. bolus of saline (10 μL; n=5) and 0.0125 μg/kg of either 5-CT (5-$HT_{1/5/7}$ receptor agonist), or 1-PBG (5-HT_3 receptor agonist),or cisapride (5-HT_4 receptor agonist) or AS-19 (5-HT_7 receptor agonist) (n=5 each agonist) on the vasoconstrictor responses (Δ Renal-PP) elicited by electrical stimulation of renal sympathetic nerves in the in situ autoperfused left kidney of sarpogrelate-treated diabetic rats. *p < 0.05 vs saline, using one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

3.4. *Effect of the different 5-HT_1 receptor subtype agonists on the increases in renal perfusion pressure evoked by electrical stimulation in DM-S rats*

To further analyze the 5-HT receptor subtype/s involved in the renal serotonergic sympathoinhibitory action, different 5-HT_1 subtype agonists were intraarterially administered. 8-OH-DPAT (5-HT_{1A}) and CGS 12066B (5-HT_{1B}) failed to modify the sympathetic-induced Δ renal-PP (Fig. 4). On the other hand, both L-694,247 (5-HT_{1D}) and LY 344864 (5-HT_{1F}) significantly inhibited the electrically induced renal vasoconstrictions (Fig. 4).

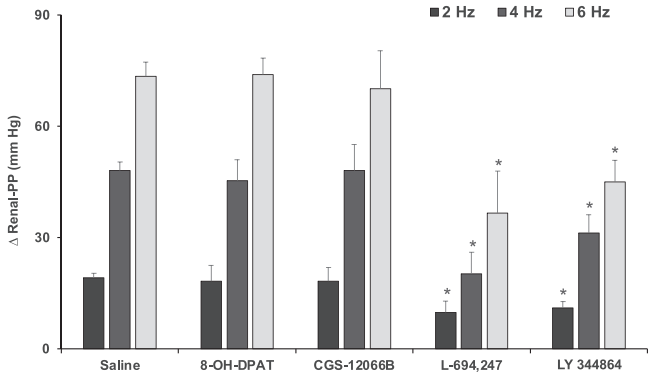

Fig. 4. Effect of i.a. bolus of saline (10 μL; n=5), 8-OH-DPAT (5-HT_{1A}), CGS-12066B (5-HT_{1B}), L-694,247 (5-HT_{1D}) or LY 344864 (5-HT_{1F}) (0.0125 μg/kg and n=5 for each agonist) on the vasopressor responses (Δ Renal-PP) elicited by electrical stimulation of the periarterial renal nerves in sarpogrelate-treated diabetic rats. *p < 0.05 vs saline, with one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

3.5. Impact of saline or different 5-HT receptor type/subtype antagonist on the 5-CT renal sympatho-inhibitory effect in DM-S rats

The i.v. administration of the 5-HT_{5A} selective antagonist (SB 69951, 1 mg/kg), or a mixture of GR 127935 (5-$HT_{1B/1D/1F}$ selective antagonist, 310 μg/kg) plus SB 258719 (5-HT_7 selective antagonist, 1 mg/kg) did not induce *per se* any effect in the renal vasopressor responses induce by electrical stimulation of periarterial renal nerves (data not shown). Furthermore, antagonist's vehicle (saline, 1 mL/kg, i.v.) did not modify the 5-CT-induced renal sympathetic inhibition (Fig. 5).

The i.v. administration of 1 mg/kg of SB 699551 (5-HT_{5A} antagonist) was not able to abolish the renal sympatho-inhibitory effect of 5-CT (0.0125 μg/kg, i.a.). Whereas, i.v. administration of a cocktail of GR 127935 (5-$HT_{1B/1D/1F}$ antagonist, 310 μg/kg) and SB 258719 (5-HT_7 antagonist, 1 mg/kg) completely blocked the inhibitory effect of 5-CT on the Δ renal-PP (Fig. 5).

3.6. Effect of selective 5-HT agonists on the Δ renal-PP induced by the intraarterial administration of exogenous NA

The i.a. administration of exogenous NA evoked (Δ renal-PP) in a dose dependent manner, as described in 3.2. Neither saline (10 μL) nor any of the 5-HT selective receptor agonists tested: L-694,247 (5-HT_{1D}), LY 344864 (5-HT_{1F}) and AS-19 (5-HT_7) (at a dose 0.0125 μg/kg each agonist) of modified these vasopressor responses (Table 1).

3.7. Organ hypertrophy and blood parameters

The LVH index did not show differences among NG, DM or DM-S rats (data not shown); however, RH index (mg/mm) of the NG, DM and DM-S animals were 29.3 ± 1.1, 40.4 ± 1.4* and 35.1 ± 1.1*#, respectively; (*$p < 0.05$ *vs* NG; #$p < 0.05$ *vs* DM; n=10, each group).

As described in Table 2, the diabetic state (DM group) decreased the total proteins concentration ($p < 0.05$) in the serum samples compared with the NG group. Although sarpogrelate-treatment (DM-S group) seems to increase total protein concentration, the values obtained were not significantly different from either DM or NG groups.

In relation to other renal function markers, BUN and creatinine were significantly increased in DM animals compared with NG group ($p < 0.05$) (Table 2). The chronic blockade of 5-HT_2 was able to reduce BUN in diabetic animals ($p < 0.05$ *vs* diabetic), although DM-S BUN values did not reach NG group BUN values ($p < 0.05$ *vs* normoglycemic). Besides, the DM-S rats showed restored normoglycemic creatinine values ($p < 0.05$ *vs* diabetic).

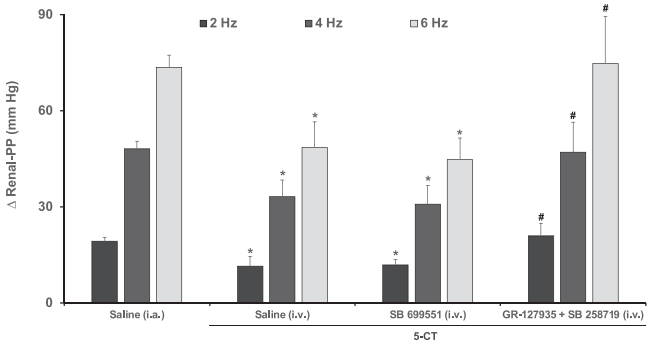

Fig. 5. Effect of the i.a. administration of 5-CT (0.0125 μg/kg) in the presence of saline (1 mL/kg, i.v.), SB 699551 (1 mg/kg, i.v.) or a cocktail of GR 127935 (310 μg/kg, i.v.) + SB 258719 (1 mg/kg, i.v.) on the vasopressor responses (Δ Renal-PP) elicited by electrical stimulation of renal sympathetic nerves in sarpogrelate-treated diabetic rats. n=5 for each treatment. *$p < 0.05$ vs i.a. saline; #$p < 0.05$ vs 5-CT, using one-way ANOVA followed by the Student-Newman-Keuls post hoc test.

Table 1
Effect of i.a. bolus of saline (10 μL), L-694,247, LY 344864 or AS-19 (0.0125 μg/kg, each agonist) on the vasopressor responses elicited by the i.a. administration of noradrenaline (0.05, 0.1 and 0.4 μg/kg) in diabetic rats treated with sarpogrelate.

	i.a. NA doses (μg/kg)		
Drugs	*0.05*	*0.1*	*0.4*
Saline	29.9 ± 2.3	45 ± 3.2	78.9 ± 5.5
L-694,247	29.9 ± 6.9	40.5 ± 7.0	77.5 ± 9.7
LY 344864	26.2 ± 4.0	40.1 ± 7.2	70.6 ± 8.9
AS-19	29.8 ± 4.3	41.5 ± 5.7	71.3 ± 8.1
	Δ Renal-PP (mm Hg)		

Note that responses induced by the i.a. administration of exogenous NA administration were not modified by any of the selective 5-HT receptor type agonists ($p > 0.05$ *vs* saline)

Table 2
Renal parameters comparison among the different study groups from serum blood samples (normoglycemic, diabetic and diabetic treated with sarpogrelate).

	NG	DM	DM-S
Total proteins (g/dL)	4.87 ± 0.13	4.34 ± 0.16*	4.64 ± 0.47
Blood Urea Nitrogen (mg/dL)	22.44 ± 0.91	35.58 ± 1.45*	28.38 ± 4.48*#
Creatinine (mg/dL)	0.64 ± 0.02	0.81 ± 0.05*	0.64 ± 0.06#
†eClCr (mL/min)	0.94 ± 0.03	0.66 ± 0.06*	0.81 ± 0.04*#

†Estimated creatinine clearance (eClCr) was calculated by online ACLARA calculator from Intelligent Data Analysis Laboratory (IDAL) from University of Valencia, from a single value of blood creatinine (mg/dL) and body weight (g). NG: normoglycaemic group; DM: non-treated (control) diabetic group; DM-S: sarpogrelate-treated diabetic group. Values shown as means ± S.E.M. of n=10. *$p < 0.05$ vs normoglycemic; #$p < 0.05$ vs diabetic.

4. Discussion

4.1. General: metabolic and haemodynamic parameters

Due to the great prevalence of diabetes nowadays, chronic derived complications have become a worldwide public health. The most common associated pathologies are microvascular alterations, neuropathy (characterized by sympathetic hyperactivity) and renal function alterations [3,4]. The combination of these three may lead to final renal failure and death [23]. Hence, understanding this intricate interplay, and finding new pharmacological strategies to containing it, has emerged as a must nowadays and in the coming years. In this line, modulating the serotonergic system (due to its great variety of receptors and peripheral actions) seems a promising procedure for the development of novel therapeutic interventions.

In this spirit, we studied the effect of the 5-HT_2 chronic blockade on the renal sympathetic neurotransmission and its modulation by the serotonergic system in experimental diabetic animals. Moreover, we determined the changes that this treatment may induce in renal function during diabetes. To do so, we utilized sarpogrelate as a 5-HT_2 antagonist because it has already been postulated as a pharmacological tool in diabetic microvascular complications and in renal function damage induced by diabetes [17,18,24,25].

Alloxan is a commonly used diabetogenic compound in experimental rodent models [19,20,26]. Chronic hyperglycemia appears on the second day post-subcutaneous administration (due to β-cell destruction) and is maintained during the 28 days in which the animals remain penned in the animal facilities [7,20]. The chronic hyperglycemia leads to a variety of complications, including renal damage and neuropathy [19,27].

14-day oral sarpogrelate treatment, at a dose of 30 mg/kg.day [19,21] does not modify glycemia in diabetic animals as stated by us and other authors [19,28].

The *in vivo* experimental model (*in situ* autoperfused rat kidney) utilized in our experiments allows to characterize the direct action of a drug in the kidney having into account all physiopathological compensatory responses in the entire organism [10]. In this work, we continuously recorded SBP, HR and renal blood pressure, assessing changes in the renal blood flow (without modifying SBP and HR) induced either by selective stimulation of sympathetic periarterial renal nerves or i.a. administration of exogenous NA in sarpogrelate-treated diabetic rats, as previously described in other experimental models [9, 19].

The renal system is stated as the leading long-term regulator of blood pressure; thus, renal alterations together with hypertension (one of common diabetes comorbidities) are directly involved in the renal damage [29] and final kidney failure. Our experimental technique allows us to stablish the effect of chronic blockade of 5-HT_2 receptors on hemodynamic parameters (HR, SBP and RPP). On this basis, elevated renal-PP and SBP was already reported before in a long-term diabetic animal model using the same experimental technique [30], which indicates the acute development of diabetic hypertension. Recently, our group has demonstrated that renal-PP is also augmented in a short-term model of diabetes, whereas there are no changes in SBP compared to normoglycemic animals [7,9,12], probably due to the timing of diabetes. Although sarpogrelate treatment has been demonstrated to reduce high blood pressure in awake diabetic animals [21,31], our current data, in anaesthetized animals, show that 14-day oral sarpogrelate treatment does not alter SBP or the augmented perfusion pressure in the kidney during diabetes. Similarly, the significant decrease observed in HR in diabetic rats [7] *versus* normoglycemic is not modified by the chronic blockade of 5-HT_2 receptors.

It is well established that diabetes induces changes in the sympathetic nervous system [32,33]. The hyperactivation of adrenergic input in diabetes is showed in different manners: higher adrenergic responses [7] and increases in systemic and/or renal/adrenal catecholamines concentration [34,35], among others. Moreover, adrenergic innervation is extensive in the kidney [36]. Thus, renal sympathetic hyperactivity, previously described in several diabetic animal models, [7,37] may contribute to the development of hypertension (one of common diabetes comorbidities), and progressively, generate a cross-talk that contributes to renal damage [29]. Therefore, controlling the renal sympathetic (hyper)activity may be a target in diabetes in order to reduce the associated complications [37,38]. In this sense, we demonstrate that vasoconstrictor responses induced by either electrical stimulation of the renal sympathetic nerves (that induces endogenous NA release) or exogenous NA administration are significantly lower in the DM-S rats than in the DM ones [7]. These results in the renal bed are in agreement which those recently reported by us in total vascular noradrenergic innervation [19], where the vasopressor responses were attenuated in sarpogrelate-treated in comparison to non-treated diabetic rats. This fact proves that sarpogrelate treatment regulates the sympathetic hyperactivity originated by diabetes, and consequently, decreases the magnitude of the increased vasopressor responses due to diabetic state [19, current data]. Hence, chronic treatment with a 5-HT_2 blocker becomes a potential target in the regulation of sympathetic hyperactivity, which highly contributes to cardiovascular and renal complications during diabetes.

4.2. Inhibitory profile of renal sympathetic neurotransmission of different 5-HT agonists in DM-S animals

The serotonergic system is stablished as one of the modulators of the blood pressure control. Anyhow, none of the administered 5-HT agonists (i.a.) or antagonists (i.v.) altered basal parameters (SBP, HR or renal-PP). Only the higher doses of 5-HT (0.0125 and 0.1 μg/kg, i.a.) increased renal-PP that came back to basal values within 2 min. It is important to remark that this fact has been previously shown by us both in normoglycemic and diabetic rats [7,12] and it may be due to the vasoconstrictor role of serotonin at renal level mainly mediated by 5-HT_2 receptor activation [10,13,30]. In fact, chronic sarpogrelate treatment halves this acute vasoconstrictor effect of 5-HT (0.1 μg/kg, i.a.; current results) compared to diabetic rats [7].

In relation to serotonergic modulatory role of sympathetic activity, the inhibitory effect of 5-HT on the sympathetic neurotransmission has been reported before in different experimental model and vascular territories [9,19,25,39,40].

As 5-HT_2 receptors are (1) functional and widely expressed in renal tissues and (2) mediate deleterious effects such as vasoconstriction [10, 13], inflammation, and fibrosis, which are key processes in the development and progression of diabetic nephropathy [8,14], our hypothesis remained that chronic blockade of 5-HT_2 receptor might unmask or potentiate other 5-HT receptor activation to control sympathetic hyperactivity during diabetes. Our study confirms that 5-HT inhibitory effect is revealed at lower doses in diabetes (sarpogrelate-treated or not) than in normoglycemic animals. As stated, the 0.0125 μg/kg dose of 5-HT inhibited the sympathetic responses in the DM and in DM-S animals [7; current data], but not in the normoglycemic animals [12]. Of particular interest is that the 5-HT_2 blockade induces, in DM-S group, a higher percentage of 5-HT inhibition than in DM animals, which, in fact, would indicate that, in diabetic animals chronically treated with sarpogrelate, lower doses of 5-HT may induce a greater inhibitory action.

In DM-S rats, the inhibitory serotonergic profile is mainly mediated by the 5-$HT_{1/7}$ receptor type, since the selective receptor agonists 1-PBG (5-HT_3) and cisapride (5-HT_4) did not modify the vasopressor responses in the renal bed of DM-S rats. Only the non-selective 5-$HT_{1/5/7}$ agonist, 5-CT, and the selective 5-HT_7 agonist, AS-19, reproduced 5-HT renal sympatho-inhibitory action, and 5-HT_{5A} receptor was devoid of this inhibitory action since the selective 5-HT_{5A} blockade (SB 699551) did not abolish 5-CT action on renal noradrenergic input. In pithed rats, 5-HT_1 receptor type has longly been described as sympathoinhibitory at vascular level in normoglycemic and diabetic state [20,41]. Furthermore, in this experimental model (normoglycemic pithed rat) treated with sarpogrelate, as occurs in our current data in the kidney, 5-HT_7 is unmasked as sympatholytic [25]. Anyhow, in diabetic pithed rats, the 5-HT_{5A} receptor was involved in the serotonergic sympathoinhibitory action at cardiac level [39].

To further analyse the 5-HT receptor subtype involved in the renal sympatholytic effect of 5-HT, we utilized selective agonists. Neither 8-OH-DPAT nor CGS-12066B (5-HT_{1A} and 5-HT_{1B} receptor agonists, respectively) were able to reproduce 5-CT inhibitory action; however, L-694,247 (5-HT_{1D}) and LY 344864 (5-HT_{1F}) mimicked serotonin inhibitory effect.

To confirm the 5-$HT_{1D/1F}$ and 5-HT_7 involvement in the serotonergic sympatholytic action, we used a mixture of GR 127935 (5-$HT_{1B/1D/1F}$) plus SB 258719 (5-HT_7), that completely abolished 5-CT inhibition of the vasoconstrictor responses (see results, 3.5 section).

All these data evidence that chronic blockade of 5-HT_2 receptors in experimental diabetes show up the 5-HT_{1D}, 5-HT_{1F} and 5-HT_7 receptors as responsible of the renal vasculature sympathoinhibition by the serotonergic system, which is partly in agreement with results in DM animals [7], where 5-HT_{1D} receptors were the sole responsible of the sympatho-inhibitory action. Thus, sarpogrelate treatment in diabetic rats unmasks the 5-HT_{1F} and 5-HT_7 receptors involved in the inhibition of the renal sympathetic vascular tone. The 5-HT_{1D} receptor has been entailed in the renal noradrenergic-neurotransmission inhibition also in normoglycemic animals [9]. Moreover, the inhibitory role of this receptor subtype has already been described in the sympathetic vascular and cardiac innervation [41,42] and, also, in the cardiac vagal input [43, 44] in different experimental models. Also, the 5-HT_7 activation has been reported as a sympathoinhibitor in sarpogrelate-treated normoglycemic [25] and diabetic [19] pithed animals. In addition, this inhibitory effect by 5-HT_7 could be explained by the activation of G_s protein, and consequently, the K^+ channel activation followed of the membrane hyperpolarization. Of particular interest is the participation

of 5-HT_{1F} in the sympatho-inhibitory effect, that has never been described before at renal level; however, our results tally with those obtained at cardiac level in saprogrelate-treated normoglycemic pithed animals [45]. The unmasking of 5-HT_{1F} receptor as sympatho-modulator at renal level in DM-S rats may become of great interest, since there are some studies that showed up how 5-HT_{1F} receptor activation play a significant role in improving renal damage [46,47]. Both 5-$HT_{1D/1F}$ receptors acts as neurotransmitter inhibitor by the inhibition of $G_{i/o}$ protein and adenylyl cyclase inhibition [6,48].

The implication of these three receptor subtypes (5-$HT_{1D/1F}$ and 5-HT_7) could, at least in part, explain the inhibitory effect of 5-HT in DM-S rats at lower doses than in DM rats. Furthemore, sarpogrelate treatment enhances the endothelial function in the diabetic rats thought reducing oxidative stress or inflammatory factors [18,21].

The administration of increasing doses of NA induced dose-dependent vasoconstrictor responses on the renal vasculature. The intraarterial administration of the L-694,247, LY 344864 and AS-19 (5-HT_{1D}, 5-HT_{1F} and 5-HT_7 receptor agonist, respectively at a dose of 0.0125 μg/kg each) did not modify the NA-induced the Δ renal-PP. This leads us to confirm the prejunctional nature of this inhibition. On this basis, the prejunctional locus of serotonergic receptors as inhibitors of noradrenergic input was previously demonstrated at renal, cardiac and vascular levels in normoglycemic [9,12,41,42] and diabetic animals [7,20,39].

4.3. Renal parameters and organs hypertrophy

Diabetic renal disease clinical manifestations appear to be heterogenous. Anyhow, kidney hypertrophy, total proteins, creatinine levels (and/or creatinine clearance) and blood urea nitrogen are common markers to evaluate the functionality of the kidney [8,49]. In this sense, we tried to determine whether our experimental model of diabetes originates alteration in these parameters; the present results confirm increased levels of serum creatinine and BUN, and decreased levels of serum total proteins and creatinine clearance (estimated by ACLARA, a web-based, openly available estimated creatinine clearence (eClCr) calculator [22]) which confirm that 28-day alloxan-induced hyperglycemia induces renal disturbances, as previously described [50,51]. 14-day sarpogrelate-treatment in diabetic animal is able to reduce the elevation of both serum BUN and creatinine values in diabetic animals; and also, 5-HT_2 receptor blockade significantly reversed eClCr, although it did not come back to control values (NG group). Anyhow, we may affirm that changes induced in the renal function by diabetes tend to improve with 5-HT_2 blockade.

This fact is also consistent with the decrease observed of RH rate in the diabetic rats treated with sarpogrelate from the diabetic control ($p < 0.05$) [21, current data]. Although some studies, using other models of sarpogrelate treatment, did not find significant effect of the 5-HT receptor blockade on the renal parameters or organ hypertrophy in diabetic rats [24], our results agree with those from other authors who reported that blocking 5-HT_2 receptor ameliorates altered renal parameters in chronic kidney disease in rats [52] or mice [49].

4.4. Final considerations

The present work has some curbs to be taken into account: (1) our experimental model in vivo allows to determine renal sympathetic activity as an indirect assessment by measuring Δ renal-PP (which is not a direct measurement of NA release), (2) we selectively measure renal-PP/sympathetic innervation in the left kidney, and not in the right kidney, but it is important to remark that perform the *in situ* autoperfusion of the right kidney might lead to the blockage of the mesenteric arterial irrigation (by surgical necessities), which could induce a loss in the hemodynamic control and several systemic changes and (3) our results are based in males; thus, we may further analyze these results in female rats to avoid sex or gender bias; anyhow, it must be considered that in a pithed rat model, the sympathetic input at vascular level shows no differences between male and female rats [53].

Despite all these constraints, current data bring to light new possibilities in the treatment of diabetic complications. We have demonstrated that chronic sarpogrelate treatment attenuates the augmented diabetic renal vascular responses due to sympathetic hyperactivity [7]. Moreover, sarpogrelate chronic therapy reduce renal hypertrophy and ameliorates markers of renal dysfunction (BUN or creatinine), which can trigger damage in the kidney during diabetes. Besides, 5-HT_2 blockade shows up the inhibitory effect of prejunctional 5-$HT_{1D/1F}$ and 5-HT_7 on the renal sympathetic ouflow. Thus, modulating serotonergic system may contribute to ameliorate renal alterations (reduce hypertrophy and improve markers) and reduce the microvascular disturbances due to noradrenergic hyperactivation during diabetes.

5. Conclusions

Chronic 5-HT_2 receptor blockade (by sarpogrelate) reduces renal hypertrophy, ameliorates renal dysfunction markers (BUN and creatinine), decreases the renal sympathetic hyperactivity and shows up a renal sympatholytic effect by prejuncional 5-HT_{1D}, 5-HT_{1F} and 5-HT_7 receptor activation in experimental diabetes. So, these results open new ways to explore in the current treatment of diabetic complications, postulating the 5-HT_2 antagonism as a pharmacological tool to mitigate kidney dysfunction in diabetes.

CRediT authorship contribution statement

Anais-Clara Terol-Úbeda: Methodology, Investigation, Data curation. **José-Ángel García-Pedraza:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Juan-Francisco Fernández-González:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Asunción Morán:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **María-Luisa Martín:** Data curation, Conceptualization. **Mónica García-Domingo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Authors' statement

Anaïs Clara Teról-Úbeda: Performed *in vivo* experiments, performed study of blood parameters. Asunción Morán: Designed the *in vivo* experiments, Analyzed the data, and Writing-Reviewing and Editing. José Ángel García-Pedraza: Performed *in vivo* experiments, designed blood parameters and organ hypertrophy study, Analyzed the data, and Writing-Reviewing and Editing. Juan Francisco Fernández-González: Performed *in vivo* experiments, study blood parameters and organ hypertrophy, Analyzed the data, and Writing-Reviewing and Editing. María Luisa Martín: Designed the *in vivo* experiments. Mónica García-Domingo: Designed the *in vivo* experiments and blood parameter study, Analyzed the data, and Writing-Reviewing and Editing. All authors read and approved the final manuscript.

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.

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