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Participation of cyclooxygenase pathway in the vasoconstriction induced by 5-HT in the *in situ* autoperfused kidney of long-term diabetic rats

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ABSTRACT

We attempted to characterize the 5-hydroxytryptamine (5-HT) receptor type/subtype and mediator mechanisms involved in the contractile effects of 5-HT in the *in situ* autoperfused kidney of long-term diabetic rats. Diabetes was induced in male Wistar rats by a single subcutaneous injection of alloxan. Intra-arterial (i.a.) bolus injection of 5-HT (0.00000125 to 0.1 μg/kg) increased renal perfusion pressure in a dose dependent way but did not affect the systemic blood pressure in long-term diabetic rats. The selective 5-HT_2 receptor agonist, α-methyl-5-HT, caused a local vasoconstrictor effect in the *in situ* autoperfused rat kidney similar to 5-HT. However, BW723C86, a selective 5-HT_{2B} receptor agonist, m-CPP (1-(3-chlorophenyl)piperazine), a selective 5-$HT_{2B/2C}$ receptor agonist, the 5-HT_1 receptor agonist, 5-carboxamidotryptamine (5-CT), and the selective 5-HT_3 receptor agonist, 1-phenylbiguanide did not modify the renal perfusion pressure. In long-term diabetic rats, vasoconstriction elicited by 5-HT and α-methyl-5-HT was significantly decreased by ritanserin (a 5-HT_2 receptor antagonist), spiperone (a 5-HT_{2A} receptor antagonist), and the cyclooxygenase (COX) inhibitors, indomethacin (non-selective COX inhibitor), FR 122047 or nimesulide (selective COX-1 and COX-2 inhibitors, respectively), but was not modified by pretreatment with SB 206553 (3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2.b:4,5-b′] dipyrrole(1H)-carboxamide hydrochloride), a non-selective 5-HT_{2C} receptor antagonist, prazosin, propranolol, enalapril or losartan. The results of protein expression support these results: COX-1 and COX-2 are expressed in renal tissue. Inducible COX (COX-2) is overexpressed in long-term diabetes. Our data suggest that, in the *in-situ* autoperfused kidney of long-term diabetic rats, 5-HT vasoconstrictor action is mediated, through cyclooxygenase pathway, by local activation of 5-HT_{2A} receptors.

1. Introduction

Diabetes and hypertension are both associated with an increased risk of renal disease. 5-Hydroxytryptamine (5-HT) affects renal function (Shoji et al., 1989; Morán et al., 1997, 2008, 2009; Tian et al., 2002); however, the action of this amine in renal vasculature is controversial regarding both the effect (vasoconstriction/vasodilatation) and the magnitude of the response. Studies performed previously by us to evaluate 5-HT-induced hemodynamic changes in several autoperfused rat vascular beds confirmed the variability of these actions depending on the vascular bed analyzed (Fernández et al., 2000; Calama et al., 2002; Morán et al., 1997, 2008, 2009). Even, in the same vascular bed, 5-HT effects depend on several factors, such as the doses used or the existence of pathologies like hypertension or diabetes (Calama et al., 2003, 2004, 2005; García et al., 2005, 2006; Morán et al., 2008, 2009, 2010).

During diabetes, 5-HT vasoconstrictor effect is enhanced in renal, pulmonary or coronary arteries of rabbits and pigs (Miranda et al., 2002; El-Kashef, 1996; Miranda et al., 2000; Bagwell and Brophy, 2000), but variable results were found in aortas of diabetic rats (Orie et al., 1993; Sikorski et al., 1993; James et al., 1994; Hattori et al., 1995).

There is evidence that increases in 5-HT plasma levels may be related to the development of diabetic nephropathy through 5-HT_{2A} receptor activation in mesangial cells (Eto et al., 1997; Kasho et al., 1998); moreover, serum concentrations of serotonin are elevated in rabbit renal artery during diabetes (Miranda et al., 2002).

It seems likely that, as previously shown by us in pithed rats (García et al., 2006), 5-HT vasoconstrictor action in renal vascular bed during diabetes is linked to changes in intracellular signaling pathways. Nevertheless, the exact mechanism has not yet been elucidated.

In Krebs-perfused diabetic rat kidneys, the activation of vascular TXA_2 receptors increases 5-HT vasoconstrictor effect to a greater

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extent than in control kidneys (Hodgson et al., 1990). Others, however, describe abnormalities in the cascade of COX in several pathologies, such as diabetic nephropathy or renal hypertension (James and Hodgson, 1995; Hao and Breyer, 2008).

In streptozotocin-induced type 1 diabetic rats, renal synthesis of prostanoids is increased (Craven et al., 1987; Hao and Breyer, 2008). COX-2 expression is also increased in the thick ascending limbs and macula densa in both type I streptozotocin diabetic and type II diabetic Zucker rats (Komers et al., 2001, 2005; Cheng et al., 2002; Dey et al., 2004). Selective COX-2 inhibition significantly reduces glomerular hyperfiltration in streptozotocin-induced diabetic rats, which is consistent with the fact that COX-2-derived prostanoids increase hyperfiltration in diabetic kidney (Komers et al., 2001, 2005; Hao and Breyer, 2008; Yar et al., 2010).

In light of the above, in this work we aimed to determine if long-term diabetic state induces changes in the 5-HT receptor type/subtype involved in the 5-HT local vasoconstrictor effect in the *in-situ* autoperfused rat kidney and analyze the possible involvement of direct/indirect mechanisms.

2. Materials and methods

2.1. Ethical approval of the study protocol

Housing conditions and experimental procedures were in accordance with regulations provided by the European Union on the use of animals for scientific purposes (86/609/EEC, Article 5; Appendix II). This was enacted by Spanish legislation on 14 March 1988 (R.D. 223) and 10 October 2005 (R.D.1201).

Male Wistar rats (250–350 g) were used in the present study. Rats were kept and supplied by the Animal House of the Faculty of Pharmacy of the University of Salamanca (PAE-SA001; Salamanca, Spain).

2.2. Diabetes induction and animal maintenance

The rats were divided into two groups: normoglycemic and diabetic rats. Diabetes was induced by a single injection of alloxan (150 mg/kg, s.c.) in 0.9% NaCl (physiological saline). Rats were then maintained on tap water and regular food *ad libitum* for 8 weeks. Normal rats served as controls, and both groups, control and alloxan-diabetic rats, were all aged-matched. Body weight, systolic blood pressure, heart rate and blood glucose levels were determined before and at 2, 7, 14, 21, 28, 35, 42, 49 and 56 days after alloxan administration. Only rats with elevated blood glucose levels (>11 mM) at all time points were considered diabetic. Blood glucose levels were determined by test strips (Accu-Chek®). Systolic blood pressure and heart rate were measured in awake rat periodical using the tail-cuff method with a photoelectric sensor (NIPREM 546, Cibertec S.A, Madrid, Spain) along the study. Several determinations were made in each session for each animal and values were considered valid if five consecutive measurements did not differ by 10 mm Hg.

2.3. Animal preparation

Diabetic and normoglycemic rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). After the induction of anesthesia, a tracheotomy was carried out and catheters placed in the right and left carotid arteries. The right carotid artery was cannulated for measurement of blood pressure using a Spectramed model P23xL pressure transducer and Grass model 7 Physiograph recorders. The penis vein was cannulated for drug administration. Rats were kept warm with a heating lamp.

Rats were prepared for *in-situ* perfusion of the left kidney according to the method of Fink and Brody (1978). Vascular beds were perfused using an extracorporeal circuit and a constant-flow Gilson peristaltic pump. The left carotid artery was cannulated with the inflow end of the extracorporeal flow line. The abdominal aorta was exposed by a midline laparotomy and deflection of the intestines to the right side of the rat. A loose tie was placed around the aorta between the left renal artery and the origin of the right renal and superior mesenteric arteries. Additional ties were placed around the aorta 1 cm below the left renal artery and 1 cm above the iliac bifurcation. Heparin sodium (5 mg/kg, i.v.) was given. An intravenous infusion of normal (0.9%) saline was initiated at a rate of 2 ml/h and continued throughout the experiment.

When the aortic tie above the left renal artery was tightened, blood immediately began to flow from the carotid artery to the left renal artery; the circuit was established without interruption of blood flow to the kidney. Blood was pumped from the right carotid artery to an aortic pouch from which the left renal artery was the only outlet (Fink and Brody, 1978; Dupont et al., 1986; Roquebert et al., 1992; Morán et al., 2008, 2009).

The distal portion of the external circuit was connected to a Spectramed model P23xL pressure transducer for measurement of perfusion pressure, which was recorded on a Grass model 7 Physiograph recorder.

At the beginning of each experiment, flow was adjusted to render the perfusion pressure equal to the systemic pressure. Flow was kept constant throughout the experiment and changes in perfusion pressure reflected changes in vascular resistance. The flow rate through the renal vascular bed ranged from 2 ml/min to 2.9 ml/min (Roquebert et al., 1992; Morán et al., 1997, 2008, 2009). In all experiments, atropine (1 mg/kg, i.v.) was administered before saline infusion was started to block the cholinergic effect.

2.4. Experimental protocols

Experiments were carried out after a 15 min period to allow blood pressure and perfusion pressure to stabilize. Five rats were used to evaluate each dose of agonist or antagonist, and each animal preparation to evaluate only one agonist or antagonist.

The first group of experiments was carried out to confirm results from our laboratory (Morán et al., 2008) in normoglycemic control rats. In this group (n = 25), 5-HT (n = 5), the selective 5-HT_1 receptor agonist, 5-carboxamidotryptamine maleate (5-CT) (n = 5), the selective 5-HT_2 receptor agonist, α-methyl-5-HT (n = 5), and the selective 5-HT_3 receptor agonist, 1-phenylbiguanide (n = 5), were administered locally at doses of 0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg via the distal cannula intra-arterially (i.a.) by bolus injection of a maximum volume of 10 μl using a microsyringe (Exmire microsyringe), with a gap of 5 min between administration of each drug dose. Saline solution (10 μl) was administered (i.a.) in the control group (n = 5) in the same way.

In the first alloxan-treated diabetic group (n = 35): 5-HT (n = 5), 5-CT (n = 5), α-methyl-5-HT (n = 5), the selective 5-HT_{2B} receptor agonist, α-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride (BW723C86) (n = 5), the non-selective 5-HT_{2C} receptor agonist, 1-(3-chlorophenyl) piperazine dihydrochloride (m-CPP) (n = 5), and 1-phenylbiguanide (n = 5) were administered locally at doses of 0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg by bolus injection (i.a.) of a maximum volume of 10 μl, with a gap of 5 min between administration of each drug dose. Saline solution (10 μl) was administered (i.a.) in the diabetic groups (n = 5) in an identical fashion.

The second alloxan-treated diabetic group (n = 30) was run in parallel with the group described above to investigate the 5-HT_2 receptor subtype involved in 5-HT renal vascular effects. Several 5-HT_2 antagonists were administered (i.v.) 10 min before the corresponding dose–response curve of the agonist was obtained. The dose of each antagonist was selected after our previous

experience (Fernández et al., 2000; Calama et al., 2002; Morán et al., 2008, 2009). 5-HT_2 receptor antagonist, ritanserin (1 mg/kg), the 5-HT_{2A} receptor antagonist, spiperone (0.125 mg/kg), and the 5-$HT_{2B/2C}$ antagonist, 3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2.b:4,5-b′] dipyrrole (1H)-carboxamide hydrochloride SB206553 (0.5 mg/kg), were administered before administration of α-methyl-5-HT (n = 15, five rats for each antagonist) or 5-HT (n = 15, five rats for each antagonist).

The third alloxan-treated diabetic group (n = 70) was performed to analyze the mechanisms involved in the serotoninergic effect by using various antagonists. The dose of each antagonist was selected after consideration of recommendations of authors and our previous experience (Morán et al., 1997, 2008; Fernández et al., 2000; Ochi and Goto, 2001, 2002). The antagonists were administered intravenously as follows: non-selective cyclooxygenase (COX) inhibitor, 1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1-H-indole (indomethacin; 2 mg/kg), the selective COX-1 inhibitor, 1-[4, 5-bis (4-methoxyphenyl)-2-thiazolyl]-4-methylpipirazine hydrochloride (FR 122047; 1.5 mg/kg), the selective COX-2 inhibitor, N-(4-Nitro-2phenoxyphenyl) methanesulfonamide (nimesulide; 1.5 mg/kg), the α_1 antagonist, prazosin (0.1 mg/kg), a non-selective β-blocker, propranolol (2 mg/kg), the angiotensin converting enzyme inhibitor, enalapril (5 mg/kg) and the AT_1 antagonist, losartan (1 mg/kg), were administered 10 min before administration of α-methyl-5-HT (n = 35, five rats for each antagonist) or 5-HT (n = 35 five rats for each antagonist).

2.5. Western blot analyses

Renal tissue (from both normoglycemic and diabetic rats) was lysed on ice-cold lysis buffer and solubilized protein concentrations were determined as previously described (Rodriguez-Barbero et al., 2001). Protein samples were separated by SDS-PAGE and membranes blocked before incubation with the primary antibodies: COX-1 (11) and COX-2 (C-20) (Santa Cruz Biotechnology). Anti-alpha-tubulin (I-19) (Santa Cruz Biotechnology) antibody was used to confirm loading of comparable amount of protein in each lane. After incubation with HRP-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Protein expression was analyzed by densitometry using Scion Image software (Scion).

2.6. Drugs used

The following drugs were used: alloxan, monohydrate and pentobarbital sodium (Sigma-Aldrich), heparin sodium (Roche), atropine sulfate (Scharlau), 5-hydroxytryptamine-creatinine sulfate (5-HT; Sigma-Aldrich), 5-carboxamidotryptamine maleate (5-CT; Sigma-Research Biochemicals International), α-methyl-5-hydroxytryptamine maleate (Sigma-Research Biochemicals International), α-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride (BW723C86; Sigma-Research Biochemicals International), 1-(3-chlorophenyl)piperazine dihydrochloride (m-CPP; Sigma-Research Biochemicals International), 1-phenylbiguanide (1-PBG; Sigma-Research Biochemicals International), ritanserin (Janssen Pharmaceutical), spiperone hydrochloride (Sigma-Aldrich) and 3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2.b:4,5-b′]dipyrrole (1H)-carboxamide hydrochloride, SB206553, (Sigma-Research Biochemicals International), 1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1-H-indole (indomethacin; Tocris Cookson Ltd), N-(4-Nitro-2-phenoxyphenyl) methanesulfonamide (nimesulide; Sigma-Aldrich), 1-[4, 5-bis (4-methoxyphenyl)-2-thiazolyl]-4-methylpipirazine hydrochloride (FR 122047 hydrochloride; Biogen Scientific, Madrid, Spain), D-L-propranolol hydrochloride (ICI Pharmaceuticals), enalapril maleate (Merck, Sharp and Dohme), prazosin (Pfizer), and losartan (Merck). All drugs used were dissolved in distilled water at the time of the experiments, with the exception of ritanserin, m-CPP and nimesulide, which were dissolved in 0.04 mol/l lactic acid, 0.01 M HCl, and ethanol 50% respectively.

2.7. Statistics

Data are means ± S.E.M. of five experiments. Changes in renal vascular resistance are reported as increases, in mm Hg, and in perfusion pressure in comparison with control values. Statistical significance was calculated with one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test. $P<0.05$ was considered significant.

3. Results

3.1. Systemic hemodynamic variables

Alloxan-induced diabetes elicited a marked increase in serum glucose and systolic blood pressure and failure of the rats to increase their body weight in comparison with control rats. Table 1 shows the mean values of body weight, systolic blood pressure, heart rate and glycemia before and 8 weeks after the induction of diabetes for rats in diabetic group and in the control group. These values did not change significantly during the experiments and remained stable after intravenous (1 ml/kg) or intra-arterial (10 μl) administration of the different vehicles used: saline solution, HCl 0.01 mol/l, lactic acid 0.04 mol/l or ethanol 50%.

3.2. Renal vascular effects of the 5-hydroxytryptamine receptor agonists: 5-HT, 5-CT, α-methyl-5-HT, and 1-phenylbiguanide in normoglycemic anesthetized rats

Mean resting blood pressure, perfusion pressure, and heart rate in normoglycemic anesthetized rats were 90.5 ± 5.4 mm Hg, 91.4 ± 3.2 mm Hg and 390.3 ± 12.1 beats/min (bpm), (n = 25) respectively. These values did not change significantly during the experiments, and remained stable after intravenous (1 ml/kg) or i.a. (10 μl) administration of the vehicle saline solution or HCl 0.01 mol/l.

Local i.a. injection of graded doses of either 5-HT or the selective 5-HT_2 agonist, α-methyl-5-HT (0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg, n = 5 for each compound), had no effect on systemic blood pressure, but increased the perfusion pressure in the *in-situ* autoperfused rat kidney in a dose-dependent way (Fig. 1A and B).

Neither systemic blood pressure nor perfusion pressure was modified by local administration of similar doses of the selective 5-HT_1 receptor agonist, 5-CT (n = 5) or the selective 5-HT_3 receptor agonist, 1-phenylbiguanide (n = 5) (data not shown).

Table 1
Values of body weight, systolic blood pressure, heart rate and glycemia in control and diabetic rats.

	Body weight (g)	Systolic blood pressure (mm Hg)	Heart rate (bpm)	Glycemia (mM)	n
Control rats					
Initial time	219 ± 9.0	88.5 ± 5.0	375 ± 10.0	5.6 ± 0.2	25
8 weeks after	424 ± 16.0	90.5 ± 4.0	390 ± 12.1	4.8 ± 0.1	25
Diabetic rats					
Initial time	192 ± 10.0	124 ± 5.0	310 ± 6.0	5.6 ± 0.1	135
8 weeks after	309 ± 11.8[a]	147 ± 1.7[a]	340 ± 10.0	20.1 ± 0.7[a]	135

Results are means ± S.E.M. for "n" rats.
[a] Significantly different from the corresponding value in control rats, $P<0.05$.

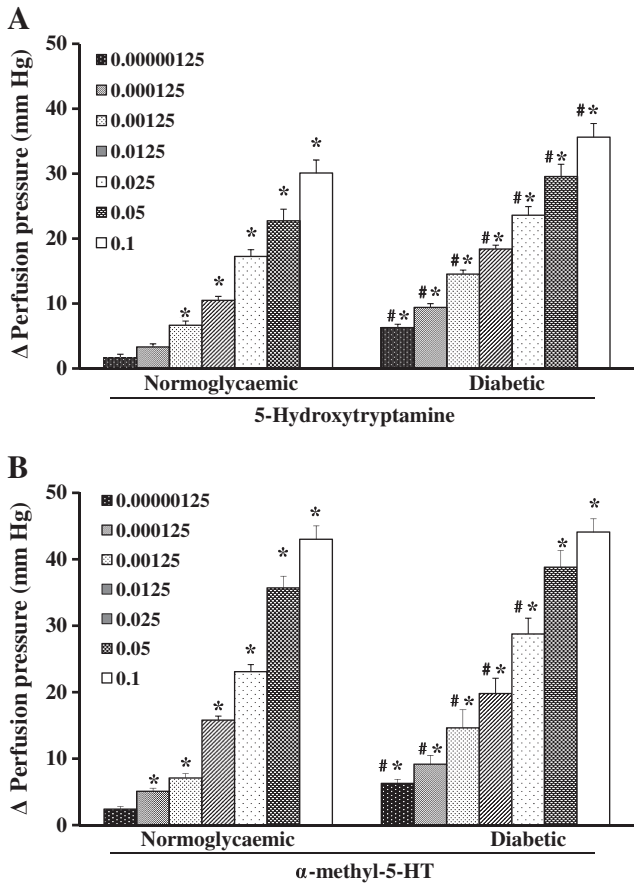

Fig. 1. Effect of intra-arterial renal administration in normoglycemic and alloxan-induced diabetic rats of different doses of: (A) 5-HT (0.00000125–0.1 μg/kg) (B) α-methyl-5-HT (0.00000125–0.1 μg/kg) on perfusion pressure in the *in-situ* autoperfused rat kidney. Data are means ± S.E.M. *P<0.05 with respect to basal perfusion pressure. #P<0.05 *vs* normoglycemic group.

3.3. Renal vascular effects of the 5-HT receptor agonists: 5-HT, 5-CT, α-methyl-5-HT, m-CPP, BW723C86 and 1-phenylbiguanide in long-term diabetic rats

Mean resting blood pressure, perfusion pressure, and heart rate in long-term diabetic rats were 147.6 ± 1.7 mm Hg, 146.2 ± 1.8 mm Hg and 345 ± 10.0 bpm (n = 135), respectively. These values did not change significantly during the experiments and remained stable after i.v. (1 ml/kg) or i.a. (10 μl) administration of the vehicle saline solution, HCl 0.01 mol/l, lactic acid 0.04 mol/l, or ethanol 50%.

Local i.a. injection of graded doses of 5-HT (0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg) (n = 5) had no effect on systemic blood pressure but increased perfusion pressure in the *in-situ* autoperfused rat kidney in a dose-dependent fashion (6.3 ± 0.5, 9.4 ± 0.6, 14.5 ± 0.6, 18.4 ± 0.6, 23.6 ± 1.4, 29.6 ± 1.9, and 35.6 ± 2.1 mm Hg, respectively) (Figs. 1A, 2). At doses of 0.00000125, 0.000125, 0.00125, 0.0125, 0.025, 0.05 and 0.1 μg/kg (n = 5), the selective 5-HT_2 receptor agonist, α-methyl-5-HT, increased perfusion pressure by 6.3 ± 0.6, 9.2 ± 1.3, 14.7 ± 2.7, 19.8 ± 2.3, 24.7 ± 2.3, 38.1 ± 2.5, and 44.1 ± 2.0 mm Hg, respectively (Figs. 1B, 2), without modifying systemic blood pressure.

Neither systemic blood pressure nor perfusion pressure was modified by local i.a. administration of similar doses of either the selective 5-HT_1 receptor agonist, 5-CT (n = 5), or the selective 5-HT_3 receptor agonist, 1-phenylbiguanide (n = 5) (Fig. 2), or the selective 5-HT_{2C} receptor agonist, m-CPP (n = 5) or the selective 5-HT_{2B} receptor agonist, BW723C86 (n = 5) (Fig. 2).

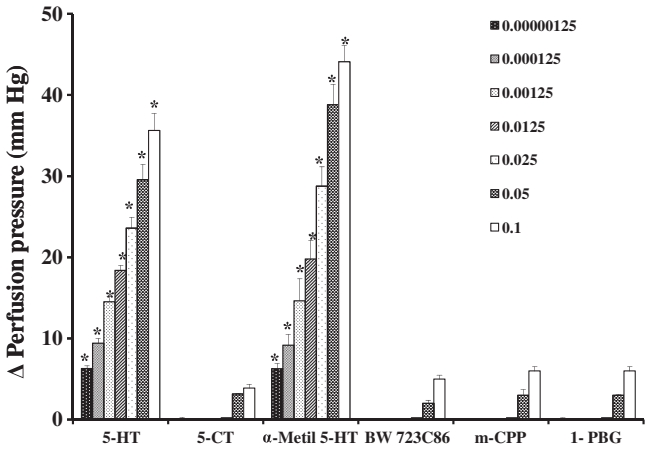

Fig. 2. Effect of intra-arterial renal administration of different doses of 5-HT receptor agonists (0.00000125–0.1 μg/kg) on perfusion pressure in the *in-situ* autoperfused rat kidney. 5-HT (5-hydroxytryptamine), 5-CT (5-Carboxamidotryptamine), α-methyl-5-HT, BW723C86 (α-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine hydrochloride), m-CPP (1-(3-chlorophenyl)piperazine dihydrochloride) and 1-phenylbiguanide (1-PBG). Data are means ± S.E.M. *P<0.05 with respect to basal perfusion pressure.

3.4. Effect of the selective 5-HT receptor antagonists (ritanserin, spiperone or SB206553) on the 5-HT or α-methyl-5-HT renal vasoconstrictor effect in long-term diabetic rats

Intravenous administration of the different antagonists tested (ritanserin (1 mg/kg); spiperone (0.125 mg/kg) or SB206553 (0.5 mg/kg)) did not induce changes in mean blood pressure or perfusion pressure (data not shown).

However, pretreatment with 1 mg/kg of the 5-HT_2 receptor antagonist, ritanserin abolished the local vasoconstrictor responses induced both by 5-HT (n = 5) (Fig. 3) and α-methyl-5-HT (n = 5) (Fig. 4) in renal vasculature.

This blockage was reproduced by the selective 5-HT_{2A} receptor antagonist, spiperone (0.125 mg/kg, n = 5 for each agonist), which significantly blocked the renal vasoconstrictor responses induced by i.a. administration of either 5-HT (Fig. 3) or α-methyl-5-HT (Fig. 4).

The effects of 5-HT and α-methyl-5-HT were also tested after treatment with SB206553, a 5-$HT_{2B/2C}$ antagonist (0.5 mg/kg, n = 5 for each agonist). The local renal vasoconstriction induced by 5-HT

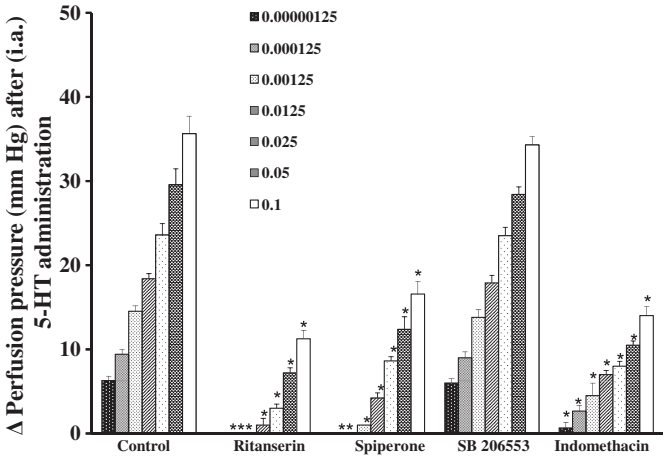

Fig. 3. Effect of pretreatment with ritanserin (1 mg/kg), spiperone (0.125 mg/kg), SB206553 (0.5 mg/kg) or indomethacin (2 mg/kg) on the renal vasoconstrictor effect induced by intra-arterial administration of 5-HT (0.00000125–0.1 μg/kg) in the *in-situ* autoperfused rat kidney. Data are means ± S.E.M. *P<0.05 *vs* the saline group (control), which received identical 5-HT doses without antagonist pretreatment.

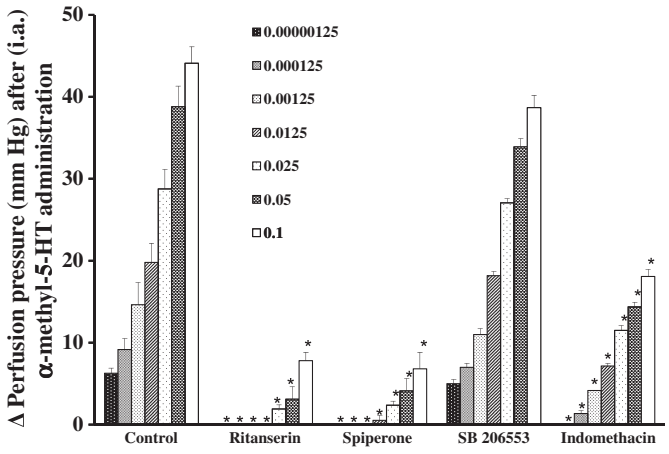

Fig. 4. Effect of pretreatment with ritanserin (1 mg/kg), spiperone (0.125 mg/kg), SB206553 (0.5 mg/kg) or indomethacin (2 mg/kg) on the renal vasoconstrictor effect induced by intra-arterial administration of α-methyl-5-HT (0.00000125–0.1 μg/kg) in the *in-situ* autoperfused rat kidney. Data are means ± S.E.M. *P<0.05 *vs* the saline group (control), which received identical α-methyl-5-HT doses without antagonist pretreatment.

and α-methyl-5-HT was not modified 10 min after i.v. pretreatment with the 5-$HT_{2B/2C}$ receptor antagonist, SB206553 (Figs. 3, 4).

3.5. Effect of other antagonists (indomethacin, FR122047, nimesulide, prazosin, propranolol, enalapril or losartan) on the 5-HT or α-methyl-5-HT renal vasoconstrictor action in long-term diabetic rats

Pretreatment with either 2 mg/kg of the non-selective cyclooxygenase (COX) inhibitor (COX-1 and COX-2) indomethacin, or the selective COX-1 inhibitor, FR 122047 hydrochloride (1.5 mg/kg), or the selective COX-2 inhibitor, nimesulide (1.5 mg/kg) abolished the renal vasoconstrictor responses induced by i.a. local administration of either 5-HT (n = 5 for each antagonist) (Fig. 4) or α-methyl-5-HT (n = 5 for each antagonist) (Fig. 5).

The effects of 5-HT and α-methyl-5-HT were also tested after pretreatment with either the α_1 antagonist, prazosin (0.1 mg/kg), or the non-selective β-blocker, propranolol (2 mg/kg), or the angiotensin converting enzyme inhibitor, enalapril (5 mg/kg) or antagonist AT_1, losartan (1 mg/kg) (data not shown).

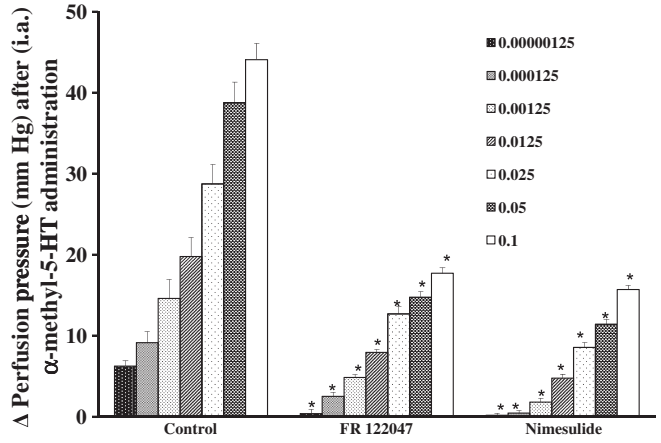

Fig. 5. Effect of pretreatment with FR122047 (1.5 mg/kg) or nimesulide (1.5 mg/kg), on the renal vasoconstrictor effect induced by intra-arterial administration of α-methyl-5-HT (0.00000125–0.1 μg/kg) in the *in-situ* autoperfused rat kidney. Data are means ± S.E.M. *P<0.05 *vs* the saline group (control), which received the same α-methyl-5-HT doses without antagonist pretreatment.

No changes in mean blood pressure or perfusion pressure were observed after administration of any of these antagonists (data not shown).

3.6. Study of the expression of COX in the kidney tissue in control and long-term diabetic rats

We examined the expression of COX-1 and COX-2 in kidneys of control and diabetic rats by Western blot analysis (n = 3 for each group of animals). No difference in COX-1 protein expression was observed between the Control, 4 weeks and 8 weeks diabetic kidneys (Fig. 6A). However, expression of COX-2 protein was higher in kidneys from 8 weeks diabetic rats compared with control and 4 weeks diabetic rats (Fig. 6B).

4. Discussion

This study aimed to characterize the 5-HT receptor type/subtype and the mechanisms involved in the serotonergic vascular responses in the *in situ* autoperfused kidney of long-term diabetic rats.

Alloxan, used as diabetogenic agent, induces a syndrome resembling type-1 diabetes mellitus (Agrawal et al., 1987) and is commonly used as an experimental model of diabetes (Miranda et al., 2002; García et al., 2006; Morán et al., 2010).

The technique used in our experiments, following the method of Fink and Brody (1978), allowed continuous measurement of renal blood flow in rat, permitting therefore the evaluation of rapid changes in renal blood flow induced by direct i.a. administration of drugs. Direct local renal action of 5-HT and possible indirect actions induced by the release of humoral vasoactive agents can be evaluated (Morán et al., 2009).

Cardiovascular reactivity to 5-HT during experimental diabetes showed marked variations in different vascular beds (Miranda et al.,

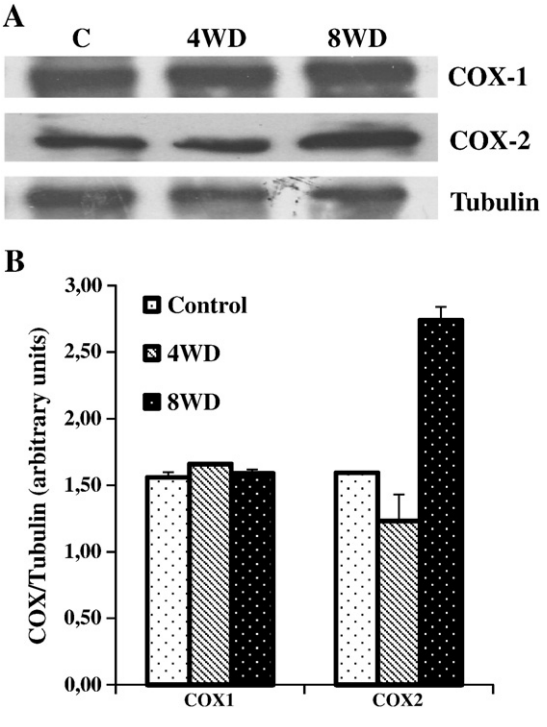

Fig. 6. COX expression in kidney diabetics rats: (A) Total protein extracts from control (C) and four (4WD) or 8 (8WD) weeks diabetic rats were analyzed by Western blot to detect COX-1 and COX-2 protein expression. Loading control included anti-tubulin antibody. A representative blot from three independent experiments is shown. Blots were analyzed by densitometric analysis. (B) The ratio of COX *versus* tubulin is depicted in the graph.

2000; García et al., 2005, 2006; Morán et al., 2010). Constrictor responses to 5-HT in kidneys of diabetic rats were significantly increased (Hodgson et al., 1990), whereas responses to 5-HT were reduced in rat hindquarters (Sikorski et al., 1991) and isolated aorta from diabetic rats (James et al., 1994; James and Hodgson, 1995). We and other authors have described that these variations also occur in experimental models of hypertension, where reactivity to 5-HT is increased in isolated kidneys and in the *in situ* autoperfused rat kidney (Tuncer and Vanhoutte, 1991; Boston and Hodgson, 1997; Morán et al., 2009); however, hypertension does not induce any changes in pithed rats (Haeusler and Finch, 1972; Cavaliere et al., 1980).

In this study, local i.a. administration of 5-HT in intact diabetic rats significantly augmented the renal perfusion pressure in a dose-dependent manner, which was significantly increased compared to normoglycemic rats. Our data are consistent, on one hand, with findings in rabbit renal artery (Miranda et al., 2002), in the *in situ* autoperfused rat kidney (Shoji et al., 1989; Endlich et al., 1993), in short and long-term diabetic pithed rats (García et al., 2006; Morán et al., 2010) and, on the other hand, with Van Buren et al. (1998) data, who reported an increase in the sensitivity of the basilar artery to 5-HT in short-term diabetic rats.

In long-term diabetic rats, 5-HT vasoconstrictor action in the *in situ* autoperfused rat kidney is due to 5-HT_2 receptor activation, since this effect was reproduced by similar doses of 5-HT_2 receptor agonist, α-methyl-5-HT (Baxter et al., 1995) and completely prevented by pretreatment with the selective 5-HT_2 receptor antagonist, ritanserin (Awouters et al., 1988). Neither the selective 5-HT_1 receptor agonist, 5-CT (Nowak et al., 1993; Wool et al., 2000) nor the selective 5-HT_3 receptor agonist, 1-phenylbiguanide (Cheng et al., 2002), reproduced the serotonergic vasoconstrictor action. Similar results have also been described by us in normoglycemic and hypertensive rats (Morán et al., 1997, 2008, 2009); and are in agreement with data from other authors using conscious, normoglycemic, hypertensive and diabetic rats (Zink et al., 1990; Ramage and Villalón, 2008; Kobayashi et al., 2008).

In this experimental model, 5-HT administration did not induce any vasodilator effect at any of the used doses, as already occurred in normoglycemic and hypertensive rats (Morán et al., 2008, 2009). However, our group has described both vasodilator and vasoconstrictor actions for 5-HT in autoperfused hindquarters of anesthetized rat depending on the doses used (Calama et al., 2002). Also, other authors have described a dual effect for serotonin (vasodilator and vasoconstrictor actions) at renal vascular level (Shoji et al., 1989; Endlich et al., 1993).

Accordingly, we focused on the determination of the 5-HT receptor subtypes involved in the serotoninergic vasoconstrictor effect in long-term diabetic rats. Both 5-HT and α-methyl-5-HT vasoconstriction was more marked in diabetic rats than in normoglycemic rats. In contrast with our previous observations in normoglycemic rats (Morán et al., 2008), i.a. administration of the selective 5-HT_{2C} receptor agonist, m-CPP, did not reproduce the vasoconstrictor effect observed with 5-HT or α-methyl-5-HT. Neither did the administration of the selective 5-HT_{2B} receptor agonist, BW723C86. Therefore, 5-$HT_{2B/2C}$ receptor subtypes are devoid of any serotonergic vasoconstrictor action in the *in situ* autoperfused kidney of long-term diabetic rats.

This fact was confirmed using selective antagonists: the selective 5-HT_2 receptor antagonist, ritanserin (Awouters et al., 1988) and the selective 5-HT_{2A} receptor antagonist, spiperone, (Hoyer et al., 1994) prevented the vasoconstrictor effect induced by either 5-HT or α-methyl-5-HT. The selective 5-$HT_{2B/2C}$ receptor antagonist, SB206553 (Kennett et al., 1996) did not inhibit either α-methyl-5-HT or 5-HT vasoconstrictor action.

Therefore, we surmise that 5-HT_{2A} receptor subtype is mainly involved in the 5-HT-induced renal vasoconstriction in long-term diabetic animals; whereas in the normoglycemic rat model the main receptor subtype involved was 5-HT_{2C} (Morán et al., 1997, 2008). Vanhoutte et al. showed that responses to serotonin are modulated by the endothelium (Vanhoutte and Houston, 1985; Houston and Vanhoutte, 1986). So, this change in receptor subtype could be due to endothelial changes, which are widely known to occur during diabetes, or to the augmented renal reactivity to 5-HT already described in diabetic SHR animals (Boston and Hodgson, 1997). Moreover, the long-term diabetic state in rats led to a hypertension similar to that induced by L-NAME where we have already determined similar results due to expression and activation of 5-HT_{2A} receptors in renal artery (Morán et al., 2009). In this line, Kobayashi et al. (2008) indicate that sarpogrelate, a 5-HT_{2A} receptor antagonist, improves glomerular endothelial function in rats with streptozotocin-induced diabetic nephropathy.

Our results are in agreement with those obtained by Zink et al. (1990) using conscious rats, by Tuncer and Vanhoutte (1991) in isolated perfused kidney, and with more recent studies carried out in isolated renal artery, where 5-HT_{2A} receptor subtype was reported to be the main responsible for the 5-HT-pressor responses (Watts and Thompson, 2004). However, our results differ from the data reported by Shoji et al. (1989), Endlich et al. (1993) and Tian et al. (2002), where 5-HT was found to have a dual effect (vasoconstrictor and vasodilator actions) at renal vascular level. Since alterations in endothelium and prostaglandins, or in their interaction with 5-HT have been long reported in different vascular beds of normoglycemic, hypertensive or diabetic models of mice, rats or dogs (Blackshear et al., 1991; Endlich et al., 1993; Tuncer and Vanhoutte, 1991; Guo et al., 2005; Morán et al., 2009), we analyzed if any indirect mechanisms were involved in this serotonergic vasoconstrictor effect. To do so, we tested the effect of different antagonists (the α_1 antagonist, prazosin, the non-selective β-blocker, propranolol, the angiotensin converting enzyme inhibitor, enalapril, the AT_1 antagonist, losartan or the inhibitors of the cyclooxygenase pathway (COX-1 and COX-2): indomethacin, FR 122047 and nimesulide, at doses commonly used to inhibit renal prostaglandin production) on the renal vasoconstrictor effect of 5-HT and α-methyl-5-HT. The vasoconstriction was completely blocked by inhibitors of cyclooxygenase pathway at all the doses tested, and was not modified by pretreatment with prazosin, propranolol, enalapril or losartan.

Therefore, we propose that, in the *in-situ* autoperfused kidney of long-term diabetic rats, 5-HT vasoconstrictor action is mediated by activation of the 5-HT_{2A} receptor that leads to the cyclooxygenase pathway activation.

To confirm our results, we carried out cyclooxygenase protein expression studies in kidney tissue. Two isoforms of COX are identified, COX-1 and COX-2. The first one is constitutively expressed in most tissues. In the normal adult kidney, COX-1 has been localized in arteries and arterioles, glomeruli and collecting ducts (Smith and Bell, 1978). In contrast, COX-2 operates as an inducible enzyme and its expression can be markedly increased by inflammation, diabetes or physical stimuli (Komers et al., 2001, 2005; Guo et al., 2005; Hao and Breyer, 2008). In mesangial cells, multiple stimuli can induce COX-2 expression, among them mediators that bind to G-protein-coupled-seven-transmembrane receptors such as 5-hydorxytriptamine (Stroebel and Goppelt-Struebe, 1994; Goppelt-Struebe and Stroebel, 1998; Goppelt-Struebe et al., 1999). Our studies revealed both COX-1 and COX-2 expression in the kidney tissue, as already reviewed by Harris and Breyer (2001). COX-2, inducible isoform, is overexpressed after eight weeks of diabetes induction in rats. Similar results have already been reported by Nacci et al. (2009) in aorta of eight-week streptozotocin diabetic mice and in other diabetic models, such as type I and type II Zucker diabetic rats (Komers et al., 2001, 2005; Cheng et al., 2002; Dey et al., 2004). Moreover, renal prostanoid synthesis has been shown to be increased in streptozotocin diabetic rats (Craven et al., 1987; Miranda et al., 2002). Also, in human diabetic kidneys, an increased expression of COX-2 has been demonstrated (Khan et al., 2001; Hao and Breyer, 2008).

Despite Tuncer and Vanhoutte (1991) findings, who reported that responses to 5-HT in kidneys of SHR rats are not affected by the

cyclooxygenase inhibitor indomethacin, our results, in a diabetic model, suggest a role for COX-2-derived prostaglandins in the pathogenesis of renal hemodynamic changes to 5-HT during diabetes.

5. Conclusion

In conclusion, our data suggest that, long-term diabetes induces changes in the 5-HT receptor subtype and in the indirect mechanisms involved in the serotonergic effect in the *in situ* autoperfused rat kidney. In long-term diabetic rats, 5-HT renal vasoconstrictor action is mediated by local 5-HT_{2A} receptor activation and by an increase in prostanoids formation.

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