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Involvement of 5-HT_{1B} receptors in collar-induced hypersensitivity to 5-hydroxytryptamine of the rabbit carotid artery

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1 In humans intimal thickening is a prerequisite of atherosclerosis. Application of a silicone collar around the rabbit carotid artery induces an intimal thickening but in addition it increases the sensitivity to the vasoconstrictor action of serotonin (5-hydroxytryptamine, 5-HT). The 5-HT receptors involved in collar-induced hypersensitivity to 5-HT were investigated using several agonists and antagonists.

2 One week after placement of collars around both carotid arteries of anaesthetized rabbits, rings (2 mm width) from inside (=collar) and outside (=sham) the collars were mounted in organ baths (10 ml) for isometric force measurements at 6 g loading tension.

3 Collared rings were more sensitive to the contractile effect of 5-HT (7.6 fold) and 5-carboxamidotryptamine (31 fold, 5-CT, 5-HT_1 agonist) in cumulative concentration response curves. Sumatriptan (5-$HT_{1B/1D}$ agonist) caused concentration-dependent constrictions in collared rings only.

4 Collar placement did not significantly alter pA_2 values (Schild regression) or apparent pK_b values (non-linear regression) of spiperone and methysergide (mixed 5-HT_{2A}/5-HT_1 antagonists) or ketanserin and ritanserin (5-HT_{2A} antagonists), indicating unchanged binding characteristics of the 5-HT_{2A} receptor. However, the reduced slope of the Schild regression pointed to a heterogeneous receptor population in collared rings.

5 In contrast, the apparent pK_b value of methiothepin (5-HT_{1B} antagonist) was significantly reduced by collar placement, and its antagonism shifted from non-surmountable in sham rings to surmountable in collared segments.

6 Taken together, this study demonstrates that the serotonergic receptor involved in the hypersensitivity to 5-HT of rabbit collared carotid artery is a 5-HT_{1B} receptor subtype.

Keywords: 5-HT_{1B} receptor; rabbit; carotid artery; collar; 5-HT

Abbreviations: CRC, concentration response curve; 5-CT, 5-carboxamidotryptamine; DMSO, dimethylsulphoxide; 5-HT, 5-hydroxytryptamine, serotonin; GR113808A, [1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate, maleate salt; L694,247, 2-[5-[3-(4methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethanamine; MDL72222, tropanyl 3,5-dichlorobenzoate; NAN-190, 1-(2-methoxyphenyl)-4-(4-phtalimidobutyl) piperazine; n_H, Hill coefficient; 8-OH-DPAT, (±)-8-hydroxy-2(di-n-propylamino)tetralin

Introduction

Patients with atherosclerosis are prone to the development of vasospasm to serotonin (5-hydroxytryptamine, 5-HT) (Hillis & Lange, 1991; Golino *et al*., 1991; Chester *et al*., 1993; McFadden *et al*., 1991) even when the atherosclerotic lesions are not angiographically detectable (Vrints *et al*., 1992). A similar hyperactivity to 5-HT develops when the rabbit carotid artery is surrounded by a flexible, silicone collar to induce intimal thickening (De Meyer *et al*., 1990; 1994; Sobey *et al*., 1991). In human arteries intimal thickening is an essential prerequisite for the development of atherosclerosis (Stary *et al*., 1995). Nevertheless three observations revealed that the hypersensitivity to 5-HT and intimal hyperplasia are separate responses to the damage of the media (De Meyer *et al*., 1997) evoked by the collar. The hypersensitivity to 5-HT preceded the development of intimal hyperplasia (Dusting *et al*., 1990; De Meyer *et al*., 1990); treatment with the glucocorticoid dexamethasone prevented intimal thickening, while raising the hypersensitivity to 5-HT even further (Van Put *et al*., 1995), whereas treatment with the calcium entry blocker verapamil had the opposite effect: it normalized the supersensitivity to 5-HT without affecting intimal hyperplasia (Üstünes *et al*., 1996).

The broad spectrum of cardiovascular effects of 5-HT is mediated through a variety of 5-HT receptor subtypes (Martin, 1994). In blood vessels 5-HT stimulates sympathetic nerves, the endothelium (Cocks & Angus, 1983) and vascular smooth muscle cells (Grandaw & Purdy, 1996). In normal rabbit carotid arteries vasoconstriction is a consequence of 5-HT_{2A} receptor stimulation, when α_1-adrenoceptors are not taken into consideration (Black *et al*., 1981; Yildiz & Tuncer, 1994). Activation of 5-$HT_{1B/1D}$ receptors evokes constrictions in endothelium-denuded human (Kaumann *et al*., 1994), pig (Kadokami *et al*., 1996), guinea-pig (Ellwood & Curtis, 1997a) and rabbit (Feletou *et al*., 1994; Ellwood & Curtis, 1997b) coronary arteries. Furthermore, a significant part of the contraction to 5-HT is mediated by 5-HT_1-like receptors when rabbit femoral (MacLennan & Martin, 1992; Randall *et al*., 1996), iliac and mesenteric (Yildiz & Tuncer, 1994) arteries are pre-contracted with a low concentration of angiotensin II, a thromboxane A_2 mimetic or prostaglandin $F_{2\alpha}$ before adding 5-HT. However, using the same strategy, Yildiz & Tuncer (1994) failed to find evidence for functional 5-HT_1-like receptors on vascular smooth muscle or endothelium in the normal rabbit carotid artery. The question arose whether

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receptor changes were responsible for the observed hypersensitivity in the collared carotid artery of the rabbit. Therefore the aim of our study was to dissect possible alterations of the vascular serotonergic receptor subtype(s) in this rabbit model using a pharmacological approach with different agonists and antagonists.

Methods

Experimental model

The experiments were approved by the ethical committee of the university. Male New Zealand White rabbits (2.2–3.5 kg) were fed on standard laboratory chow throughout the acclimatization, which lasted at least 1 week, and the experiment. After anaesthesia with sodium pentobarbitone (30 mg kg^{-1}, i.v., stock solution (60 mg ml^{-1}) diluted with 1 volume pyrogen-free sterile 0.9% NaCl) both carotid arteries were surgically exposed. Once the vessels had been dissected from the surrounding tissue, a flexible, non-occlusive, biological inert, silicone collar (inlet/outlet diameter 1.8 mm, 20 mm length; silicone MED-4211, Nusil Technology, U.S.A.) was placed around each carotid artery and closed with silicone glue (Dow Corning 732, Dow Corning Corp., U.S.A.) (Kockx *et al*., 1992; De Meyer *et al*., 1997). One week later the rabbits were anaesthetized again and both carotid arteries were excised. Thereafter the rabbits were sacrificed (overdose sodium pentobarbitone, 60 mg kg^{-1}, i.v.). The collar and loose connective tissue were removed carefully from the arteries which were immediately placed in cold gassed Krebs-Ringer solution.

Vascular reactivity

Segments (2 mm width) from the region inside (collar) and outside (sham) the collar were mounted horizontally on two parallel tungsten wire hooks in organ baths (10 ml) filled with Krebs-Ringer solution (37°C, continuously gassed with 95% O_2–5% CO_2) for force measurements at 6 g loading tension. As the segments outside the collar had been manipulated during collar implantation, they are designated sham rather than control throughout the text. After 45 min equilibration, during which the loading tension was readjusted when necessary, the study of the reactivity of the segments was started. Tension was measured isometrically with a Statham UC2 force transducer (Gould, Cleveland, U.S.A.) connected to a data acquisition system (Moise 3, EMKA Technologies, Paris, France). Between each concentration-response curve (CRC) the Krebs-Ringer solution was replaced three times to washout the agents. The rings were first contracted with a depolarizing potassium solution (50 mM). After washout this was followed by a cumulative CRC to the agonist in the absence of antagonists. Then, the arterial rings were incubated with the lowest concentration of the antagonist for 30 min, upon which a CRC to the agonist was performed. This was repeated twice, each time with an increasing concentration of the antagonist. In each experiment one sham and one collared segment did not receive any antagonist, to allow for correction of time effects. Each ring was exposed to one antagonist only and the number of carotid arteries reported (n) equals the number of rabbits used. For KCl responses and the first 5-HT CRC the average of the four rings from each rabbit was calculated and used for statistical analysis.

To exclude interference by metabolism and uptake of 5-HT, all experiments were carried out in the presence of inhibitors mono-amino-oxidase (pargyline, 0.15 mM) (Verbeuren *et al*., 1988), neuronal uptake (clomipramine, 30 nM) and extraneuronal uptake (cortisone-21-acetate, 10 μM) (Ellwood & Curtis, 1997b). To avoid the influence of adrenergic receptors, α_1- and α_2-adrenoceptor antagonists (respectively prazosin and idazoxan, both 1 μM) (Valentin *et al*., 1996) were added to the bath solution. The vasoactive prostanoids and the endothelial nitric oxide production was hindered by supplementing the salt solution with inhibitors of cyclo-oxygenase (indomethacin, 3 μM) (De Meyer *et al*., 1991) and nitric oxide synthase (nitro-L-arginine, 300 μM) (De Meyer *et al*., 1994).

At the end of the vascular reactivity tests the segments were fixed (formaldehyde 4% solution) and stained with hematoxylin/eosin. Intimal thickness (measured at 20 at random sites covering the whole ring and averaged per artery) was determined on one transverse section at 400× magnification using a computer assisted color image analysis (PC Image Color, Foster Findlay Associates, Newcastle-upon-Tyne, U.K.).

Calculation of pD_2, pA_2 and apparent pK_b-values

The KCl (50 mM) responses were used to normalize for the contractile capacity of each individual ring. Furthermore, a time dependent decrease in the maximum of the CRC to 5-HT was observed. Therefore all the results were corrected using the following equation:

$$E_a' = T(E_A/E_{KCl})100 \quad (1)$$

where E_a' is the corrected effect (% contraction), E_A the contraction induced by the agonist, E_{KCl} the contraction evoked by 50 mM KCl and T a correction factor. T was calculated for every CRC in sham and collared ring by determining the ratio E_{max_1}/E_{max_n} where E_{max_1} is the maximum response to the agonist in the first curve and E_{max_n} the maximum in first, second, third, fourth curve respectively of the simultaneous time control, i.e. rings not receiving antagonists. EC_{50} values ($pD_2 = -\log EC_{50}$) were obtained from individual concentration response curves by fitting the corrected data (E_a') to the four parameter logistic equation:

$$E_a' = E_{min} + (E_{max} - E_{min})/ (1 + 10^{\wedge}[(\log EC_{50} - \log A)n_H]) \quad (2)$$

where E_a' is the effect after correction, A the concentration of the agonist, E_{min} the minimum or initial contraction, E_{max} the maximum contraction, n_H the Hill slope and EC_{50} the concentration of the agonist giving half the maximal contraction. The curve fitting was carried out using Prism version 2.01 (GraphPad Software, San Diego, CA, U.S.A.).

To classify the 5-HT receptor types present in the vessel the pA_2 values of each antagonist were calculated using linear regression and the equation developed by Schild (Arunlakshana & Schild, 1959; Tallarida *et al*., 1979). Theoretically the pA_2 value of a given antagonist is expected to be independent of the effector tissue and the agonist used to stimulate a particular receptor. So within the same tissue (i.e. rabbit carotid artery) the pA_2 values were expected to be similar in both experimental conditions (sham or collar) unless the receptor characteristics had been changed by the collar placement.

Recently a non-linear regression method has been proposed to estimate apparent pK_b values (Peeters, 1998). The parameters describing the surmountable (pK_b) and non-surmountable (pD_2') portion of the inhibition provoked by an antagonist are estimated from the whole set of concentra-

tion response curves by fitting the normalized data to the following equation:

$$E_a' = ((E_{max} * 10^{\wedge} -pD_2')/(B + 10^{\wedge} -pD_2'))/$$

$$(1 + ((EC_{50}/A)(B + 10^{\wedge} -pK_b)/10^{\wedge} -pK_b)^{\wedge}n_H) \quad (3)$$

where E_a' is the normalized effect, A the concentration of the agonist, B the concentration of the antagonist, E_{max} the maximal contraction, n_H the Hill slope of the CRC without antagonist. The curve fitting was carried out using Table Curve 3D (SPSS ASC GmbH, Ekrath, Germany). In comparison to the Schild method (Arunlakshana & Schild, 1959) this model has the advantages that it incorporates non-surmountable antagonism, uses all available data instead of a single point (i.e. the EC_{50}) of the CRC, takes curves in which the dose-ratio is less than unity into consideration and does not attribute extra weight to the first CRC (Peeters, 1998).

Statistical analysis

The collared rings were compared to their proximal sham equivalents (same vessel) by paired Student's *t*-test. To determine the effect of an antagonist within one group (i.e. sham or collared rings) the normalized (%KCl) contractions were evaluated by means of a two-way analysis of variance (ANOVA) with the antagonist as column factor and the concentration of agonist as row factor. When the interaction between the two factors in the two-way ANOVA was statistically significant, an additional one-way ANOVA of the $-\log EC_{50}$ values and the E_{max} values, followed by Dunnett's Multiple Comparison *post-hoc* test, was performed. The pA_2 and apparent pK_b values obtained in sham and collared rings were compared by unpaired Student's *t*-test. The GraphPad Prism package was applied for these purposes. A probability of error less than 0.05 was selected as the criterion for statistical significance. All data are given as the mean ± standard error of the mean (s.e.mean).

Drugs

The following pharmacological agents were used: serotonin creatinine sulphate monohydrate (5-HT) (Acros organics, Geel, Belgium); clomipramine (Geigy, Brussels, Belgium); cortisone-21-acetate (Kremer-Louward, Braine-l'Alleud, Belgium); indomethacin (Merck Sharp and Dohme, Brussels, Belgium); idazoxan, N-$^{\omega}$-nitro-L-arginine, pargyline, prazosin, spiperone and (±)-8-hydroxy-2-(di-n-propylamino)tetralin ((±)-8-OH-DPAT) (all purchased from Sigma Chemical Company, Bornem, Belgium); 5-carboxamidotryptamine (5-CT), cyanopindolol, methiothepin, tropanyl 3,5-dichlorobenzoate (MDL 72222), 2-[5-[3-(4-methylsulphonyl amino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]-ethanamine (L694,247) and 1-(2-methoxyphenyl)-4-(4-phtalimidobutyl) piperazine (NAN-190) (all obtained from Tocris Cookson, Bristol, U.K.). Methysergide was kindly provided by Novartis (Brussels, Belgium), ketanserin and ritanserin by Janssen Research Foundation (Beerse, Belgium), sumatriptan and [1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3 carboxylate, maleate salt (GR 113808A) by Glaxo Wellcome Research and Development (Hertfordshire, U.K.).

MDL 72222, cyanopindolol and L694,247 were dissolved in dimethylsulphoxide (DMSO), methysergide in methanol, prazosin and spiperone in ethanol and ritanserin in tartaric acid (0.1 M). To prevent oxidation, 5-HT was dissolved in an aqueous solution of ascorbic acid (0.01%). All other products were dissolved in distilled water. The Krebs-Ringer salt solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025 and glucose 11.1. The 50 mM KCl solution was prepared by equimolar replacement of sodium by potassium in the physiological salt solution.

Results

Effect of KCl

Collar placement significantly raised intimal thickness (2.78 ± 0.06 μm sham; 8.87 ± 1.31 μm collar; $n = 31$,

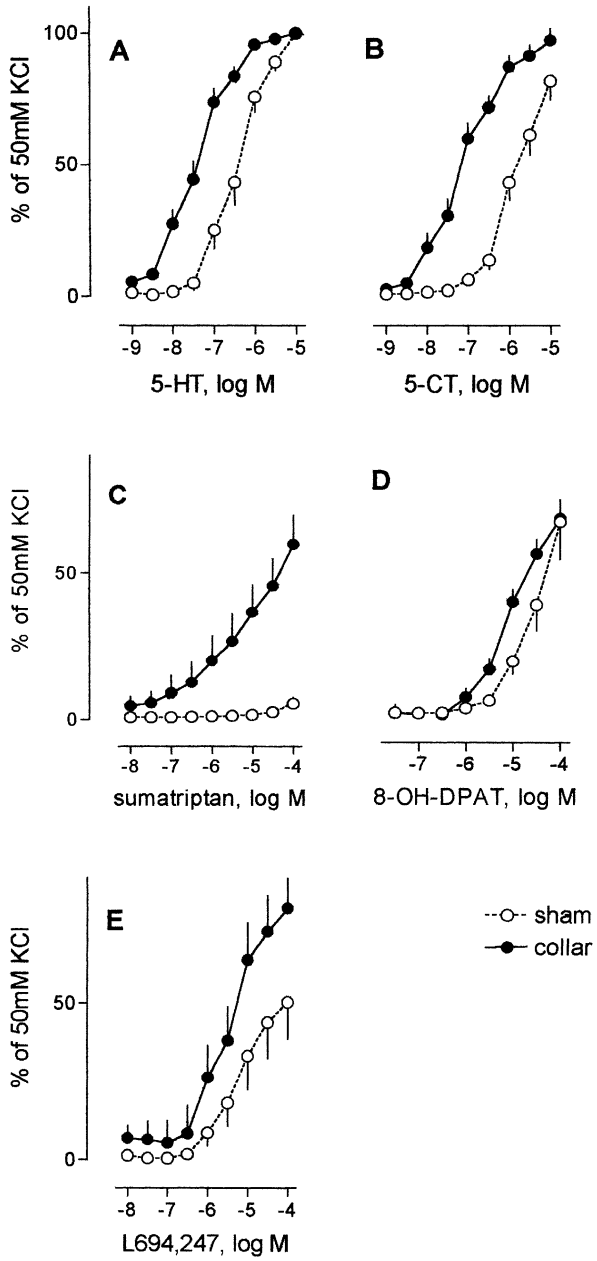

Figure 1 Contractile response curves to 5-HT (A, $n = 12$), 5-CT (B, $n = 8/7$), sumatriptan (C, $n = 4$), 8-OH-DPAT (D, $n = 6$) and L694,247 (E, $n = 7/8$) in sham and collared segments of the rabbit carotid artery. Responses are expressed as percentage of 50 mM KCl. Data are shown as mean ± s.e.mean.

$P<0.0001$ paired Student's t-test) and reduced the contractile response to the depolarization (50 mM KCl, 5.7 ± 0.2 g sham; 3.4 ± 0.2 g collar; $n=31$; $P<0.0001$ paired Student's t-test).

Effect of serotonergic agonists

5-HT (1 nM – 0.1 mM), 5-CT (0.1 nM – 10 μM), a preferential 5-HT$_1$ receptor agonist (Martin, 1994), L694,247, a selective 5-HT$_{1D}$ agonist (Beer *et al*., 1993), (1 nM – 0.1 mM) and 8-OH-DPAT, a selective 5-HT$_{1A}$ agonist (Glennon *et al*., 1988), (1 nM – 0.1 mM) induced concentration-dependent contractions in isolated sham and collared carotid arteries. In accordance with Yildiz & Tuncer (1994) sumatriptan, a 5-HT$_{1B/1D}$ agonist (Miller *et al*., 1992), (1 nM – 0.1 mM) did not elicit contractions in sham-operated rabbit carotid arteries, but concentration-dependent constrictions could be seen in collared rings, though the top concentration was insufficient to reach a plateau. The collared rings were significantly more sensitive to 5-HT (7.6 fold), 5-CT (31 fold), sumatriptan and L694,247 (2.6 fold) than sham segments (Figure 1 and Table 1). Repeated administration of the 5-HT$_1$ receptor agonists 5-CT, L694,247, 8-OH-DPAT and sumatriptan gave rise to the same maximum contraction and identical pD_2 values of the agonist (results not shown). However, a time dependent decrease in the maximum of the CRC to 5-HT was observed. Therefore all the results obtained with the agonist 5-HT have been corrected for this time effect. In sham arteries the decrease was 9 ± 6, 19 ± 5 and 30 ± 6% for respectively the second, third and fourth CRC. In collared segments the decrease of the maximum response was significantly less and respectively 5 ± 3, 4 ± 3 and 11 ± 3% for the second, third and fourth CRC. The CRC to 5-HT and 5-CT were less steep as shown by the significant reduction of the Hill coefficients in collared segments (Table 1).

Effects of antagonists

Ketanserin (1, 10 and 100 nM), a 5-HT$_{2A}$ receptor antagonist (Conolan *et al*., 1986; Van Nueten *et al*., 1986), induced parallel concentration-dependent rightward shifts of the 5-HT-induced contractions in sham and collared rings while the maximum responses were maintained. The inhibition was competitive, surmountable and statistically significant for all concentrations tested (Figure 2). At 10 and 100 nM ketanserin appeared to exert a similar competitive surmountable antagonism to 5-CT in sham and collared rings. A higher concentration (1 μM) of ketanserin resulted in a complete inhibition of 5-CT induced contraction in sham segments and a depression of the maximum response in collared rings (Figure 4A).

Ritanserin (1, 10 and 100 nM), a selective 5-HT$_{2A}$ receptor antagonist (Conolan *et al*., 1986; Van Nueten *et al*., 1986), reduced both the sensitivity and the maximal response to 5-HT in sham and collared segments pointing to a competitive but non-surmountable antagonism. However, collared segments seemed to be more resistant to the non-surmountable antagonism as 1 nM ritanserin did not reduce the maximum (Figure 3) and the depression of the E_{max} was less pronounced at 10 and 100 nM ritanserin. In sham segments the constrictions to 5-CT were progressively suppressed by 1, 10 and 100 nM ritanserin. In collar segments the antagonism of 5-CT contractions did not occur at 1 nM, and was still surmountable at 10 nM ritanserin. Only 100 nM ritanserin induced non-surmountable antagonism in collared segments (Figure 4B).

Spiperone (1, 10 and 100 nM) and methysergide (1, 10 and 100 nM), both predominantly 5-HT$_{2A}$ receptor antagonists (Feniuk *et al*., 1985; Vhora & Chiba, 1994), decreased dose-

Table 1 Effect of serotonergic agonists on isolated rabbit carotid artery

Agonist	n	$-log$ (EC_{50})	E_{max} (%)	n_H
5-HT				
sham	12	6.50 ± 0.06	93 ± 15	1.27 ± 0.08
collar	12	7.38 ± 0.07***	107 ± 15	0.90 ± 0.09**
5-CT				
sham	8	5.80 ± 0.14	82 ± 3	1.03 ± 0.03
collar	7	7.29 ± 0.08***	96 ± 5*	0.87 ± 0.04*
Sumatriptan				
sham	4	NC	5 ± 2	
collar	4	NC	60 ± 11**	
L694,247				
sham	7	5.06 ± 0.19	50 ± 13	1.24 ± 0.10
collar	8	5.48 ± 0.15*	80 ± 15*	1.15 ± 0.13
8-OH-DPAT				
sham	8	NC	64 ± 13	
collar	6	5.04 ± 0.11	64 ± 7	1.37 ± 0.16

NC: Dose-response curve did not reach a maximum at the highest concentration of the agonist, therefore pD_2-values could not be calculated. Values are shown as means $\pm$s.e.mean; n represents the number of rabbits, n_H the Hill slope, and E_{max} the maximum response expressed as percentage of contraction to 50 mM KCl. *$P<0.05$, **$P<0.01$, ***$P<0.001$ significance of difference between sham and collar in paired Student's t-test.

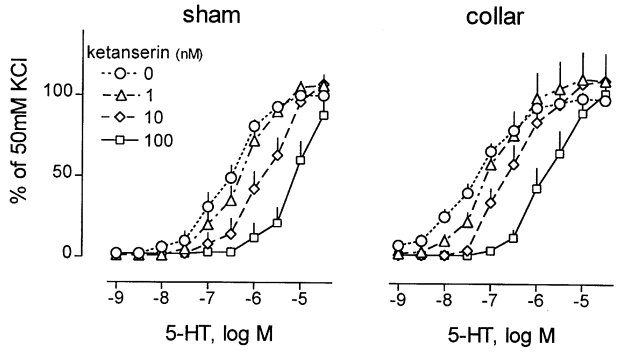

Figure 2 Effect of increasing concentrations of ketanserin on contractile responses to 5-HT in sham ($n=7$) and collared ($n=7$) rings. Responses are expressed as percentage of 50 mM KCl. Data are shown as mean$\pm$s.e.mean.

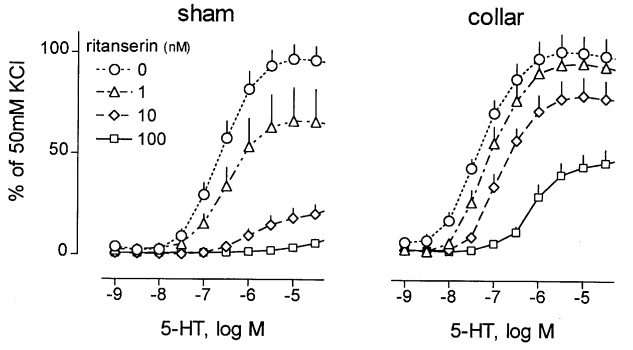

Figure 3 Effect of increasing concentrations of ritanserin on contractile responses to 5-HT in sham ($n=8$) and collared ($n=8$) rings. Responses are expressed as percentage of 50 mM KCl. Data are shown as mean$\pm$s.e.mean.

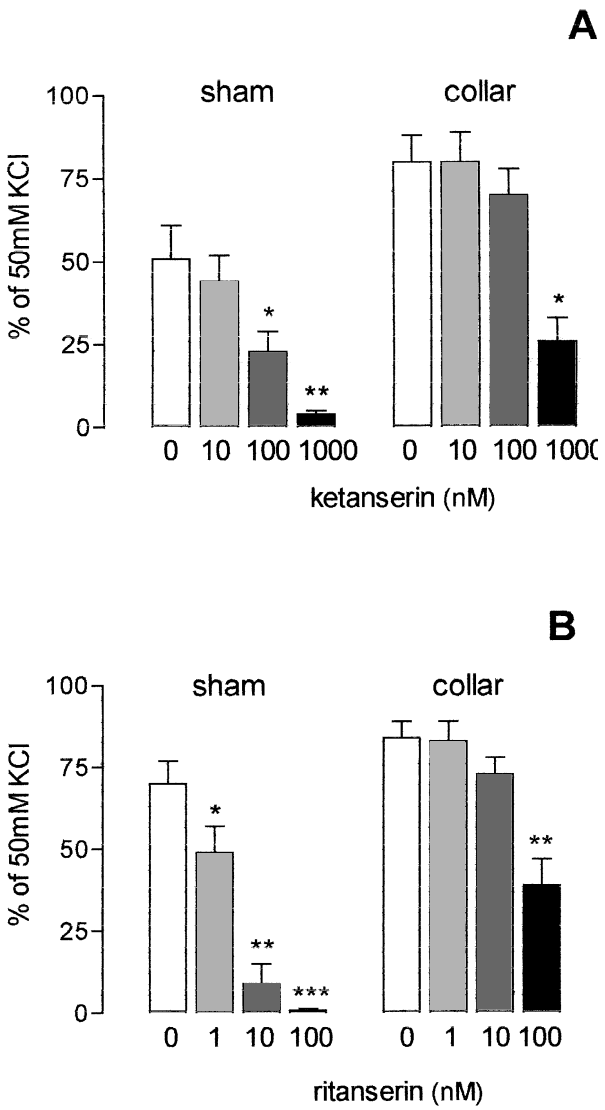

Figure 4 Effect of increasing concentrations of ketanserin (A) and ritanserin (B) on the contractile response to 5-CT (10 μM) in sham ($n=8$ for both antagonists) and collared ($n=7/8$ respectively) rings. Responses are expressed as percentage of 50 mM KCl. Data are shown as mean$\pm$s.e.mean. *$=P<0.05$, **$=P<0.01$, ***$=P<0.001$ significance of difference in one-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test.

dependently the sensitivity to serotonin, but did not influence the maximal response reached. They acted as competitive, surmountable antagonists in sham and collared rings (Table 2).

In sham rings methiothepin (0.1, 1 and 10 nM), a preferential rabbit 5-HT$_{1B}$ (Bard *et al*., 1996) and 5-HT$_{2A}$ receptor antagonist, caused concentration-dependent rightward displacements of the 5-HT curves and depression of the maximal response. In contrast, methiothepin produced a dose-dependent rightward shift of the 5-HT curves without depression of the maximum in collar segments (Figure 5). This indicates that collar placement had changed the type of antagonism for methiothepin from non-surmountable to competitive surmountable. A similar phenomenon was observed when 5-CT was used as agonist (Figure 5).

NAN-190 (0.01, 0.1 and 1 μM), a specific 5-HT$_{1A}$ receptor antagonist (Glennon *et al*., 1988), did not induce any statistically significant inhibition of the responses to 5-CT in sham and collared arteries (Table 3). In collared rings only, cyanopindolol (0.01, 0.1 and 1 μM), a 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonist (Giles *et al*., 1996), significantly shifted the 5-CT curves to the right, as indicated by the interaction between 5-CT and cyanopindolol concentrations ($P<0.05$). However in the subsequent one-way ANOVA the shift of the $-\log EC_{50}$ values just failed to atttain statistical significance ($P=0.051$). The constrictions evoked by 5-HT in sham and collared rings remained unchanged after incubation with MDL 72222 (1, 10 and 100 nM), a selective 5-HT$_3$ receptor antagonist (van Wijngaarden *et al*., 1990), or GR 113808A (1, 10 and 100 nM), a selective 5-HT$_4$ receptor antagonist (Kaumann, 1993; Gale *et al*., 1994) (Table 2).

Affinity values

According to the Schild analysis spiperone and methysergide were competitive surmountable antagonists in sham rings as the slopes did not differ from unity (Table 4). Their pA$_2$ values were not influenced by the collar ($P>0.05$, unpaired Student's *t*-test). In contrast, collar placement reduced the slope of spiperone and methysergide, which became significantly different from unity. The Schild slopes of ketanserin and ritanserin deviated slightly from unity in sham rings, whereas the collar led to a significant suppression. The suppression of the 5-HT responses by methiothepin was apparently not due to a simple, surmountable competitive antagonism, since the Schild slopes deviated significantly from unity. The Schild slope of methiothepin was strongly reduced by the collar.

Since the Schild analysis indicated that none of the antagonists behaved as a pure surmountable, competitive antagonist in collared rings, non-linear regression was used to estimate apparent pK_b values according to the model described by Peeters (1998). In sham operated rings the apparent pK_b values were not different from the pA$_2$ values for drugs (methysergide, spiperone) which displayed pure competitive surmountable antagonism and compounds (ketanserin, ritanserin) which deviated only slightly from the Schild regression. On the other hand for drugs which displayed an anomalous Schild regression, the apparent pK_b was significantly larger (methiothepin) than the (incorrect) estimate of the pA$_2$ (Table 4). In the collared rings the pA$_2$ and apparent pK_b values were not different for antagonists acting preferentially on 5-HT$_{2A}$ receptors (ketanserin, ritanserin and methysergide). The apparent pK_b values of methiothepin were smaller than the corresponding pA$_2$ values in collar rings. Finally, the apparent pK_b values of ketanserin and ritanserin were similar in sham and collared rings (Table 4). In contrast, collar placement significantly decreased the apparent pK_b value of methiothepin (Table 4, $P<0.0001$, unpaired Student's *t*-test).

Discussion

First of all our results confirmed the previous reports, i.e. decreased contractile responses to 50 mM KCl and increased sensitivity to 5-HT in rabbit carotid arteries surrounded by a flexible collar (Figure 1; Table 1) (Sobey *et al*., 1991; De Meyer *et al*., 1990) and extended those observations by demonstrating that the hypersensitivity occurred in the presence of inhibitors of serotonin uptake and metabolism, as well as α-adrenoceptor blockers. Therefore, it seems likely that the changes are to a large extent due to functional changes of 5-HT receptors. Previous studies reported the presence of 5-HT$_{2A}$ and 5-HT$_1$-like receptors in rabbit femoral arteries (Grandaw & Purdy, 1996; Randall *et al*., 1996; MacLennan & Martin, 1992), but functional evidence for 5-HT$_1$-like receptors was not found in the rabbit carotid artery (Yildiz & Tuncer, 1994). Moreover, the involvement of 5-HT$_3$ and 5-HT$_4$ receptors was ruled out

Table 2 Effect of antagonists on 5-HT-induced contractions in isolated rabbit carotid artery

		SHAM			*COLLAR*	
Antagonist	n	$-log$ (EC_{50})	E_{max} (%)	n	$-log$ (EC_{50})	E_{max} (%)
Spiperone						
0 nM	8	6.47±0.08	98±8	7	7.74±0.09	97±6
1 nM	8	6.14±0.11	89±10	7	7.29±0.14*	105±7
10 nM	8	5.60±0.10**	86±13	7	6.80±0.10**	105±6
100 nM	8	4.47±0.17**	78±11	7	5.89±0.12**	98±6
Methysergide						
0 μM	8	6.56±0.12	99±9	8	7.53±0.10	90±7
0.01 μM	8	6.19±0.08*	93±8	8	7.11±0.06**	102±10
0.1 μM	8	5.02±0.38**	85±9	8	6.46±0.06**	97±7
1 μM	8	4.52±0.08**	76±7	8	5.56±0.07**	89±7
MDL 72222						
0 nM	7	6.41±0.02	103±6	7	7.20±0.07	112±10
1 nM	7	6.24±0.02	92±5	7	6.94±0.08	119±19
10 nM	7	6.24±0.05	98±8	7	7.07±0.12	111±8
100 nM	7	6.02±0.04	90±14	7	6.81±0.12	132±24
GR 113808A						
0 nM	7	6.38±0.09	103±15	7	7.32±0.04	108±13
1 nM	7	6.27±0.06	104±18	7	7.13±0.03	117±22
10 nM	7	6.28±0.10	106±21	7	7.11±0.03	117±22
100 nM	7	6.08±0.07	109±22	7	6.69±0.05	122±27

Significance of concentrations in a two-way ANOVA of the CRCs

5-HT	$P<0.0001$	$P<0.0001$
Spiperone	$P<0.0001$	$P<0.0001$
Spiperone by 5-HT	$P<0.0001$	$P<0.0001$
5-HT	$P<0.0001$	$P<0.0001$
Methysergide	$P<0.0001$	$P<0.0001$
Methysergide by 5-HT	$P<0.0001$	$P<0.0001$
5-HT	$P<0.0001$	$P<0.0001$
MDL 72222	NS	NS
MDL 72222 by 5-HT	NS	NS
5-HT	$P<0.0001$	$P<0.0001$
GR 113808A	NS	NS
GR 113808A by 5-HT	NS	NS

Values are shown as means±s.e.mean; *n* represents the number of rabbits, P=significance of difference among concentrations in two-way analysis of variance (ANOVA) of all normalised contractions, NS: not significant, *=$P<0.05$, **$P<0.01$ in one-way ANOVA analysis of the $-\log(EC_{50})$ and E_{max} values followed by Dunnett's *post-hoc* test.

by the present experiments with MDL 72222, a selective 5-HT$_3$ receptor antagonist, and the 5-HT$_4$ receptor antagonist GR 113808A. Both antagonists failed to influence the 5-HT induced vasoconstriction in sham or collared segments (Table 2) indicating that 5-HT$_3$ and 5-HT$_4$ receptors do not contribute to the vasomotor responses to exogenous 5-HT in either condition. The presence of 5-HT$_{2A}$ receptors in normal rabbit carotid arteries (Yildiz & Tuncer, 1994) was confirmed by the response to the 5-HT$_{2A}$ receptor antagonists. As there were rightward displacements of the 5-HT CRCs by ketanserin (Figure 2), spiperone, methysergide and ritanserin (Figure 3), the 5-HT$_{2A}$ receptor appears to be functionally involved in the response to 5-HT. Since the affinity of ketanserin, ritanserin and methysergide was not changed by the collar, as shown by the apparent pK_b values (Table 4), it is assumed that the binding characteristics of this 5-HT$_{2A}$ receptor remained unchanged in collared arteries. This assumption was further confirmed by the unaltered pA_2 of spiperone and methysergide. Yet, these data do not exclude that the number of 5-HT$_{2A}$ receptors or the receptor-response coupling was changed by the collar.

Stimulation of the carotid artery with 5-CT, a preferential 5-HT$_1$ agonist, revealed that collared rings are 30 times more sensitive than the sham ones (Figure 1B). Since ketanserin and ritanserin are selective 5-HT$_{2A}$ antagonists at nanomolar concentrations (Van Nueten *et al*., 1986; Leff & Martin, 1986), the response observed in sham segments is presumably due to activation of 5-HT$_{2A}$ receptors by high concentrations of 5-CT as incubation with nanomolar concentrations of ketanserin (Figure 4A) and ritanserin (Figure 4B), significantly reduced those contractions. In contrast collared rings were more resistant to the 5-HT$_2$ blockers and at nanomolar concentrations ketanserin and ritanserin did not significantly alter the 5-CT induced contractions. This means that increased 5-HT$_1$ receptor activity is the basis for the hypersensitivity to 5-HT in collared rings. Three arguments strengthened this hypothesis. First, the significantly lower Hill coefficients of the CRCs to 5-HT and 5-CT in collared segments indicated that the curves were less steep. This is compatible with the idea that two receptor populations with different affinities for either agonist participated in the contractile responses of collared segments. Secondly, all antagonists displayed a reduced slope of the Schild regression in collared segments, which points to either the presence of heterogeneous receptors, or a non-equilibrium steady state in the tissue due to uptake of metabolism of the agonist (Kenakin, 1993). Since care had been taken to eliminate uptake and metabolism of 5-HT, the slope of the Schild regression for the four antagonists of less than unity could signify heterogeneous receptor populations in collared rings according to Kenakin (1985). Third, the time dependent decrease of the maximum response was only observed for 5-HT, which stimulates both 5-HT$_{2A}$ and 5-HT$_1$

receptors, but not with more selective 5-HT$_1$ receptor agonists (5-CT, sumatriptan). Since the reduction of the maximum response to 5-HT was less pronounced in collared rings, this suggests that the contribution of 5-HT$_1$ receptors to the 5-HT-induced constrictions became more pronounced in collared rings when compared to sham segments.

The 5-HT$_1$ receptor family is divided into five major subtypes: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-ht$_{1e}$ and 5-ht$_{1f}$. The physiological role of 5-ht$_{1e}$ and 5-ht$_{1f}$ receptors has not yet been identified (Martin, 1998) and there were no selective tools available to investigate the significance of the receptors in our study. Nevertheless, the presence of functional 5-ht$_{1e}$ and 5-ht$_{1f}$ receptors seems unlikely since 5-CT is far less potent than 5-HT as agonist for both 5-ht$_{1e}$ and 5-ht$_{1f}$ receptor expressed in recombinant mammalian cells systems (5-HT > RU24969 > 8-OH-DPAT > 5-CT > sumatriptan; N-dimethyl-5-HT > sumatriptan > 5-CT respectively) (Gerhardt & van Heerikhuizen, 1997; Martin, 1998) whereas pD$_2$ values of 5-HT and 5-CT were of a similar magnitude in sham as well as in collared rings. The involvement of 5-HT$_{1A}$ receptors is ruled out by two experimental results: 8-OH-DPAT, a selective 5-HT$_{1A}$ agonist, required extremely high concentrations (10 μM, Figure 1D and Table 1) to induce only a modest contraction of sham and collared rings. These responses are presumably the consequence of 5-HT$_{2A}$ receptor stimulation in view of the pK_i of 8-OH-DPAT (pK_i = 5.0) for 5-HT$_{2A}$ receptors (van Wijngaarden *et al*., 1990). Furthermore, NAN-190, a selective 5-HT$_{1A}$ receptor antagonist, did not influence the contractile response to 5-CT in either sham or collared segments (Table 3). In contrast, sumatriptan, a 5-HT$_{1B/1D}$ agonist, induced constrictions in collared vessels only (Figure 1C). Cyanopindolol, a 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonist, did not significantly alter the response to 5-CT in sham segments (Table 3). However, a significant rightward shift of the 5-CT curves was induced in collared rings (Table 3) pointing to 5-HT$_{1B}$ receptor antagonism in view of the results obtained with NAN-190 and 8-OH-DPAT. Both findings indicate that collar placement leads to up-regulation of 5-HT$_{1B}$ and/or 5-HT$_{1D}$ receptors which are scarcely present in normal rabbit carotid arteries (Black *et al*., 1981; Yildiz & Tuncer, 1994). This was further confirmed by the differences in antagonistic activity of the 5-HT$_{1B}$ antagonist methiothepin, which was non-surmountable in sham segments, but became surmountable in collared rings. An increased receptor reserve may explain the different profiles of methiothepin in sham and collared vessels (Figure 5) and the resistance of the collared rings to the inhibitory effect of ritanserin (Figure 4B) on contractions induced by 5-CT. When a large number of spare receptors exists, some drugs display competitive surmountable antagonism, whereas with a limited receptor reserve a non-surmountable antagonism occurs (Tallarida *et al*., 1979).

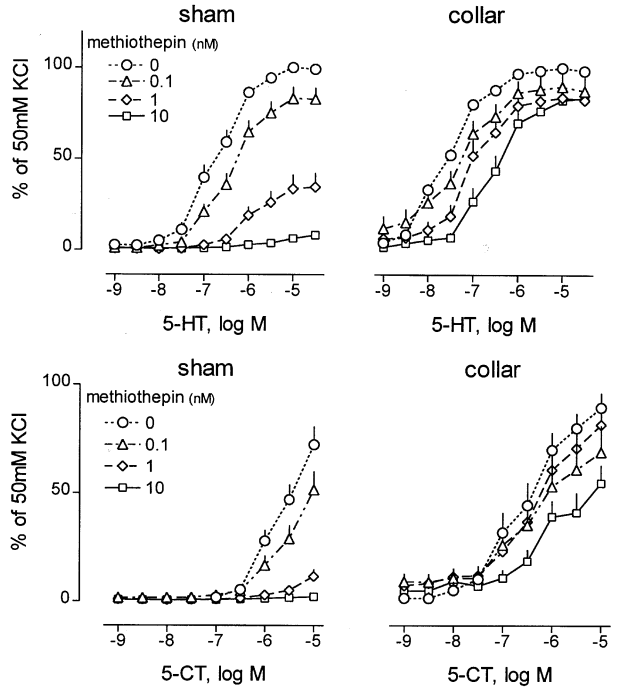

Figure 5 Effect of increasing concentrations of methiothepin on contractile responses to 5-HT and 5-CT in sham (n = 7/8 respectively) and collared (n = 8 for both agonists) rings. Responses are expressed as percentage of 50 mM KCl. Data are shown as mean ± s.e.mean.

Table 3 Effect of antagonists on 5-CT-induced contractions in isolated rabbit carotid artery

		SHAM			*COLLAR*	
Antagonist	n	$-log$ (EC$_{50}$)	E_{max} (%)	n	$-log$ (EC$_{50}$)	E_{max} (%)
NAN-190						
0 μM	7	5.84 ± 0.11	80 ± 4	7	6.78 ± 0.22	94 ± 4
0.01 μM	7	5.87 ± 0.11	73 ± 4	7	6.72 ± 0.23	83 ± 6
0.1 μM	7	5.92 ± 0.11	71 ± 4	7	6.66 ± 0.21	82 ± 6
1 μM	7	5.99 ± 0.11	68 ± 3	7	6.61 ± 0.24	72 ± 8
Cyanopindolol						
0 μM	8	5.56 ± 0.11	68 ± 6	7	6.91 ± 0.15	99 ± 3
0.01 μM	8	5.42 ± 0.09	57 ± 5	8	6.84 ± 0.07	97 ± 4
0.1 μM	7	5.50 ± 0.11	59 ± 6	8	6.74 ± 0.08	93 ± 3
1 μM	7	5.60 ± 0.10	62 ± 4	8	6.59 ± 0.08	91 ± 2

Significance of concentrations in a two-way ANOVA of the CRCs

5-HT	P < 0.0001	P < 0.0001
NAN-190	NS	NS
NAN-190 by 5-CT	NS	NS
5-CT	P < 0.0001	P < 0.0001
Cyanopindolol	NS	P < 0.0001
Cyanopindolol by 5-CT	NS	P < 0.05

Values are shown as means ± s.e.mean; n represents the number of rabbits; NS: differences among concentrations not significant in two-way ANOVA.

Table 4 Effect of collar on pA_2 and apparent pK_b values

	SHAM			*COLLAR*		
	pA_2	*slope*	*[95% CI]*	*pA_2*	*slope*	*[95% CI]*
Ketanserin	8.68±0.09	0.90±0.07	[0.83–0.97]	8.74±0.12	0.74±0.08	[0.66–0.82]
Ritanserin	8.54±0.09	1.12±0.11	[1.01–1.23]	8.47±0.09	0.78±0.07*	[0.71–0.85]
Spiperone	8.89±0.05	0.97±0.04	[0.93–1.01]	8.86±0.18	0.74±0.10*	[0.64–0.84]
Methysergide	7.99±0.08	0.99±0.06	[0.93–1.05]	7.79±0.10	0.88±0.08	[0.80–0.96]
Methiothepin	9.77±0.10	1.31±0.13	[1.18–1.43]	9.32±0.30	0.49±0.16***	[0.33–0.65]
	pK_b			*pK_b*		
Ketanserin	8.68±0.06			8.61±0.09		
Ritanserin	8.56±0.05			8.37±0.13		
Spiperone	8.83±0.05			8.53±0.06*#		
Methysergide	8.15±0.04			7.87±0.06***		
Methiothepin	10.11±0.07#			9.03±0.09***		

Values are represented as mean±s.e.mean, *$P<0.05$; **$P<0.01$; ***$P<0.001$, significance of difference between collar and sham; #$P<0.05$ apparent pK_b different from pA_2 estimate, unpaired Student's *t*-tests.

An attempt was made to discriminate between the involvement of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Ketanserin and ritanserin are, besides selective 5-HT$_{2A}$ antagonists at nanomolar concentrations (Van Nueten *et al*., 1986; Leff & Martin, 1986), 5-HT$_{1D}$ antagonists at micromolar concentrations (Kaumann *et al*., 1994; Peroutka, 1994; Pauwels & Colpaert, 1995; Kaumann *et al*., 1993). However other studies have shown that the ability of ketanserin and ritanserin to differentiate between 5-HT$_{1B}$ and 5-HT$_{1D}$ is doubtful. Ketanserin and ritanserin exhibit a 70 (Zgombick *et al*., 1995; Bard *et al*., 1996) and 20 (Zgombick *et al*., 1995) fold affinity differences between human cloned 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Therefore, as suggested by Ellwood & Curtis (1997b), methiothepin served as antagonist for rabbit 5-HT$_{1B}$ receptors (not human) (Bard *et al*., 1996; Zgombick *et al*., 1996). The collared rings displayed a decreased affinity for methiothepin as indicated by the apparent pK_b values (Table 4), again suggesting changes in the contribution of the 5-HT$_{1B}$ receptor population. Although, L694,247, a selective 5-HT$_{1D}$ agonist (Beer *et al*., 1993), induced constrictions in both sham and collared arteries, these responses were presumably due to 5-HT$_{2A}$ receptor stimulation because the $-\log(EC_{50})$ (5.5) was more than 1000 fold higher than the affinity value reported for cloned 5-HT$_{1D}$ receptors (10.0) (Walsh *et al*., 1995).

Taken together these results suggest that contractile responses to 5-HT are primarily mediated by 5-HT$_{2A}$ receptors in normal rabbit carotid arteries, and that an up-regulation of the 5-HT$_{1B}$ receptor activity explains the increased sensitivity to 5-HT in collared arteries. Nevertheless, these functional observations ought to be confirmed on a molecular level by determining the expression of rabbit 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors at mRNA and protein level. Moreover, besides an increase in number of 5-HT$_{1B}$ receptors, a more efficient 5-HT$_1$ receptor-induced signal transduction as well as an altered cellular calcium metabolism may be involved. As rabbit 5-HT$_{1B}$ receptor shows greater than 90% sequence homology to cloned human 5-HT$_{1B}$ receptor (Bard *et al*., 1996; Gerhardt & van Heerikhuizen, 1997; Wurch *et al*., 1996) and the activation of 5-HT$_{1B}$ is involved in 5-HT-induced constriction of human coronary artery (Kaumann *et al*., 1994), this model could provide new insights into the pathophysiology and the treatment of human coronary vasospasm.

In conclusion, this study indicates that the serotonergic receptor involved in the increased sensitivity to 5-HT of the rabbit collared carotid arteries is a 5-HT$_{1B}$ receptor subtype. Yet it has to be determined whether an increased number of receptors or an enhanced receptor-signal transduction causes the hypersensitivity to 5-HT.

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