Comparison of the vasoconstrictor effects of rizatriptan and sumatriptan in human isolated cranial arteries: immunohistological demonstration of the involvement of 5-HT_{1B}-receptors

J. Longmore,¹ Z. Razzaque,¹ D. Shaw,¹ A. P. Davenport,² J. Maguire,² J. D. Pickard,³ W. N. Schofield¹ & R. G. Hill¹

¹Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Eastwick Road, Harlow, Essex, UK, CM20 2QR, Departments of

²Clinical Pharmacology and ³Neurosurgery, Addenbrooke's Hospital, Hills Road, Cambridge, UK, CB2 2QQ

Aims We compared the vasoconstrictor effects of 5-HT with those of the selective 5-HT_{1B/1D}-receptor agonists sumatriptan and rizatriptan in human isolated cranial (middle meningeal) arteries. In addition selective 5-HT_{1B}- or 5-HT_{1D}-receptor antibodies were used in combination with semiquantitative immunohistochemical techniques to compare the levels of expression of these receptors in human middle meningeal and coronary arteries.

Methods Middle meningeal and coronary arteries were obtained (with consent) from either neurosurgical patients or donor hearts, respectively. Segments of middle meningeal artery were mounted in organ baths for isometric recording and cumulative concentration-effect curves to 5-HT, rizatriptan and sumatriptan were obtained. Frozen fresh sections of middle meningeal and coronary arteries were subjected to standard immunohistochemical techniques using specific 5-HT_{1B}- or 5-HT_{1D}-receptor primary antibodies and a radiolabelled secondary antibody. Data were subjected to analysis of variance (ANOVA) and nonlinear regression analysis.

Results 5-HT, rizatriptan and sumatriptan were potent vasoconstrictors in human isolated middle meningeal artery (EC₅₀ values = 32, 90 and 71 nM, respectively). A significantly higher level of 5-HT_{1B}-receptor immunoreactivity was detected in middle meningeal artery compared with coronary artery (ANOVA, $F=7.95$, $DF=1,4$, $P<0.05$).

Conclusions Rizatriptan and sumatriptan act selectively to cause vasoconstriction in human isolated middle meningeal artery and are 10-fold more potent than in human coronary artery. The higher level of expression of 5-HT_{1B}-receptors in middle meningeal compared with coronary artery provides a pharmacological basis for the craniovascular selectivity of both rizatriptan and sumatriptan.

Keywords: 5-HT_{1B/1D}-receptor agonists, human arteries, vasoconstriction

Introduction

It is generally accepted that migraine headache is accompanied by excessive vasodilation of extracerebral, intracranial blood vessels and that the therapeutic action of 5-HT_{1B/1D}-receptor agonists such as sumatriptan can result, at least in part, from cranial vasoconstriction [1–3] via activation of vascular 5-HT_{1B}-receptors [4–7]. (Note in this report we follow the recently adopted nomenclature of 5-HT_{1B}- and 5-HT_{1D}-receptors, however, these subtypes are also known in the literature as 5-HT_{1D β} and 5-HT_{1D α} receptors, respectively [8].) Sumatriptan-like drugs have been shown to cause vasoconstriction in human peripheral blood vessels both *in vitro* and *in vivo* [9–11] and their ability to cause unwanted coronary artery contraction is of particular

importance since this may lead to coronary adverse events [12, 13]. It has been reported that 5-HT_{1B}-receptors mediate vasoconstriction in cranial/cerebral arteries and also in coronary arteries [4–6]. However, sumatriptan acts selectively in cranial arteries compared with coronary arteries since the incidence of cardiac adverse-events related to sumatriptan administration in migraineurs is rare.

Previously we have reported two independent coronary artery studies (using different experimental protocols) where the maximum contraction evoked by the selective 5-HT_{1B/1D}-receptor agonist rizatriptan was found to be significantly smaller than that to sumatriptan [14] indicating that there are demonstrable differences between the effects of individual 5-HT_{1B/1D}-receptor agonists in human coronary artery. However, no previous studies have directly compared the effects of 5-HT_{1B/1D}-receptor agonists in human isolated cranial vessels.

The aims of the present experiments were two-fold: firstly to compare the vasoconstrictor effects of rizatriptan

Correspondence: Dr J. Longmore, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Eastwick Road, Harlow, Essex CM20 2QR.

*Present address: Department of Public Health and Community Medicine, Westmead Hospital, NSW, 2145 Australia.

and sumatriptan in human isolated cranial (i.e. middle meningeal) arteries. The second aim was to compare the levels of expression of 5-HT_{1B}- and 5-HT_{1D}-receptors in human meningeal and coronary arteries using semiquantitative immunocytochemical techniques and selective receptor subtype antibodies.

The collection of human material for these studies was approved by the local Ethics Committee. A preliminary communication of these experiments has been presented at the American Society for the Study of Headache [15].

Methods

Human tissue samples

Dural branches of the middle meningeal artery (2–4 mm in diameter) were obtained from discarded pieces of dura mater obtained from patients undergoing neurosurgery at Addenbrooke's Hospital, Cambridge, UK. After dissection, the arteries were placed in a modified Krebs's physiological salt solution maintained at 4 °C and transported to the Neuroscience Research Centre laboratory. Middle meningeal arteries which were macroscopically of normal appearance were obtained from nine patients (four females, five males, average age = 49 years, range 20–86 years) undergoing neurosurgery for subarachnoid haemorrhage ($n=3$), meningioma ($n=2$), middle cerebral artery aneurysm ($n=3$) or hypothalamic tumour ($n=1$). Most patients received calcium channel antagonists prior to neurosurgery and other preoperative medications included opiates, antibiotics, antiemetics, benzodiazepines, glucocorticoids, H₂-receptor antagonists, antiepileptics, diuretics, nitrates, NSAIDs, antidiuretic hormone, antipsychotics, neuromuscular blocker and thyroxine. For the immunohistochemical studies, normal coronary arteries were obtained from donor hearts which were unsuitable for cardiac transplantation.

Functional studies in human isolated middle meningeal artery

The vessels were cleaned of connective tissue, cut into ring segments (3–4 mm in length) with either 2 or 3 segments being obtained from the same artery. Segments were mounted for isometric tension recording in organ baths containing Krebs's physiological salt solution (37 °C, pH 7.4, aerated with 95% O₂, 5% CO₂). An initial resting load of 4 g was applied and tissues were allowed to relax to their own baseline tension (2 g). Following overnight washing to remove residual drugs (from patient medication at the time of surgery), the contractile response to 45 mM KCl (reference response) was obtained. Cumulative concentration-effect curves to the agonists (5-HT, rizatriptan and sumatriptan) were then obtained with each segment being exposed to more than one agonist. Any changes in contractile responsiveness over time or resulting from multiple exposures to an agonist, was tested for by measuring the response to 5-HT added as Curve 1, 2 or 3 ($n=5$, 6, and 5 segments for curves 1, 2 and 3, respectively). A washout period (>1 h) was used between individual agonist concentration-effect curves.

Immunostaining procedure in human middle meningeal and coronary arteries

Briefly, frozen sections (10 µm) of middle meningeal and coronary artery were prepared on a cryostat (Brights, UK) and brought to room temperature and immersed in 0.1 M phosphate buffered saline containing Tween 20 (0.1%, PBS-Tween 20 buffer) and 5% w/v nonfat milk (Marvel, 1 h, room temperature). The sections were transferred to the same buffer containing the primary 5-HT_{1B}- or 5-HT_{1D}-receptor antibodies (1:300 and 1:100 dilutions, respectively,^{5,16}) in a saturated humidity chamber (overnight, 4 °C). Following three washes in PBS/Tween 20 buffer, the sections were incubated in buffer containing 5% w/v nonfat milk and the secondary antibody (³⁵S-labelled goat antirabbit IgG, 0.1 mCi ml⁻¹, saturated humidity, 1 h, room temperature). Then, following washing in buffer, the sections were rinsed in water, air dried and exposed to film (Hyperfilm-Max) for 7–10 days. Measurements of optical density were made using an MCID M2 image analysis system (Imaging Res Inc, Canada).

Drugs and solutions

The composition of buffers were (in mM): Krebs's physiological salt used for transport of the vessels to the laboratory: NaCl 89; NaHCO₃ 29; KCl 5; MgSO₄ 0.5; CaCl₂ 2.25; Na₂HPO₄ 1; EDTA 0.04; Krebs's physiological salt solution used for the organ bath experiments: NaCl 118; NaHCO₃ 25.3; KCl 4.7; MgSO₄ 1.2; CaCl₂ 1.25; glucose 11.1; KH₂PO₄ 1.2; phosphate buffered saline (PBS, Sigma): phosphate buffer 10; KCl 2.7; NaCl 137.

Analysis of data

Functional studies in middle meningeal artery

Responses to the agonists were expressed as a percentage of the contraction evoked by 45 mM KCl. The data were subjected to a series of ANOVAS: (a) preliminary one-way ANOVAS to test for desensitization to multiple agonists by comparing order of agonist application (i.e. Curves 1, 2 or 3 with all other effects collapsed); differences in responsiveness between arteries obtained from different patients and between segments obtained from the same artery; (b) repeated measures ANOVA to test for differences in responses to the agonists. ANOVAS (using BMDP software, University of California Press, Los Angeles) were made for the maximum response measured, area under the curve (AOC), mean, median and slope and the results were consistent for each of these descriptive statistics.

In addition, concentration-effect curves for the mean responses for 5-HT, rizatriptan and sumatriptan were fitted to a model using weighted least squares nonlinear regression analysis and the equation:

$$E = E_{\max} / (1 + (EC_{50} / \text{agonist concentration})^{nH})$$

where $E_{\max}$ is the maximum contraction evoked by each agonist (relative to 45 mM KCl = 100%), EC_{50} is the half maximally effective concentration and nH is the Hill coefficient (Grafit Version 3.0, Erithacus Software Ltd,

Staines, UK). The weighting was the reciprocal of the error estimate for each mean value. Differences between the $E_{\max}$ and EC_{50} values obtained for the agonists were tested using asymptotic *t*-tests and error estimates from the fitting results.

Immunohistochemical studies

The levels of 5-HT_{1B}-immunoreactivity (i.r.) were expressed in terms of relative optical density. Statistical comparison of the values obtained in middle meningeal and coronary arteries was made using a mixed model analysis of variance, artery (2 levels)–segment (4 levels) with repeated measures on the second factor.

Results

Functional studies in middle meningeal artery

Some of the arterial samples were small and it was only possible to obtain two segments from these vessels, and therefore it was not possible to use a fully balanced crossover design for the order of agonist application. A total of 14 artery segments from 6 different arteries were used (4 segments were exposed to two agonists and 10 segments were exposed to 5-HT, rizatriptan and sumatriptan, giving a total of 40 agonist concentration–effect curves). The contractile responses to both rizatriptan and sumatriptan were studied in 12 segments (5-HT was also studied in some of these segments), with the remaining two segments being exposed to sumatriptan and 5-HT or to 5-HT only. ANOVA showed no significant desensitization resulting from exposure of a single segment to multiple agonists (maximum response measured, $F=0.58$, $DF=2,37$, $P=0.5671$; AOC, $F=1.69$, $DF=2,17$, $P=0.2149$).

The level of responsiveness between arteries from different patients was highly variable (for each dependent variable $P<0.001$) and this was controlled in the repeated measures ANOVAs used to make interagonist comparisons. The level of responsiveness between segments obtained from the same artery was also variable (AOC, $F=3.19$, $DF=2,17$, $P<0.07$; linear trend $F=3.35$, $DF=2,37$, $P=0.798$), however, a chi-square test comparing agonist by segment eliminated the possibility that intersegment responses may have confounded differences between agonists (chi-square = 4.31 $DF=4$, $P=0.37$). Repeated measures ANOVA showed no significant difference between the effects of 5-HT, rizatriptan or sumatriptan (for example see Table 1 for ANOVA summary relating to 'maximum response measured'), however, the power of these tests was low.

The overall mean concentration–effect curves obtained for 5-HT, rizatriptan and sumatriptan obtained in middle

meningeal artery are shown in Figure 1. All three agonists were potent vasoconstrictors (see Table 2). The $E_{\max}$ value for 5-HT was significantly higher than those for both rizatriptan and sumatriptan. In addition the $E_{\max}$ value for rizatriptan was significantly higher than that for sumatriptan (see Table 2). These results are consistent with those from the analyses of variance except that the fitting of the nonlinear function provides a better estimate of maximum value than the simple observed maximum of the data points.

Immunohistochemical studies

Three middle meningeal and three coronary arteries were used and four nonadjacent sections were obtained from each individual blood vessel. Measurements of relative optical density (ROD) showed that for all sections studied, 5-HT_{1D}-i.r. was negligible. In coronary artery, 5-HT_{1B}-i.r. was detected with similar levels being found in all 12 coronary artery sections studied (ranging from 0.0021 to 0.0032 ROD, mean = 0.0026 ROD [95% C.L. = 0.000176])

Figure 1 Concentration–effect curves to 5-HT (open squares), sumatriptan (closed circles) and rizatriptan (closed squares) obtained in isolated segments of middle meningeal artery. Points represent mean data expressed relative to the contraction evoked by 45 mM KCl. Vertical bars signify $\pm$ s.e. mean. Curves were fitted to the data using nonlinear regression analysis.

Table 1 Middle meningeal artery: analysis of variance summary for the observed maximum contractile response.

Source term	DF	Sum of squares	Mean square	F ratio	Probability level	Power (Alpha = 0.05)
Agonist	2	3 994.51	19 97.26	2.56	0.1386	13.9%
Patient	4	79 104.61	19 776.15	9.29	0.0002	
A $\times$ P	8	6 252.79	781.60	0.37	0.9259	
within	20	42 591.51	2 129.58			

	5-HT	Rizatriptan	Sumatriptan
Mean $E_{\max}$ [asymptotic error]	160.6 [8.8]	132.60 [6.2]**	105.3 [4.2]*
Mean EC_{50} [asymptotic error]	32 [8]	90 [2]*	71 [11]*

* Significantly different from 5-HT.

** Significantly different from both 5-HT and sumatriptan.

($P < 0.05$; asymptotic t -test, $n = 6-7$ arteries/group).

whereas in middle meningeal artery 5-HT_{1B}-i.r. was more variable (ranging from 0.00147 to 0.00546 ROD, mean = 0.0034 ROD [95% C.L. = 0.00097]). The level of 5-HT_{1B}-i.r. was significantly higher in middle meningeal artery than in coronary artery ($F = 7.95$, $DF = 1,4$, $P < 0.05$). See Figure 2 for representative autoradiographs.

Discussion

Both rizatriptan and sumatriptan caused vasoconstriction in human isolated middle meningeal artery. At low agonist concentrations (10 nM – 1 μ M) there was little difference between the drugs and the EC_{50} values were 90 nM and 71 nM for rizatriptan and sumatriptan, respectively. These values are similar to the clinically relevant plasma levels of 30–70 nM and 173 nM achieved following administration of rizatriptan (5 and 10 mg p.o.[17]) and sumatriptan (100 mg p.o.[17]) indicating that craniovascular vasoconstriction is likely to be achieved following clinical administration of these drugs. Note these concentrations were relatively

ineffective in producing vasoconstriction in human coronary artery [14, 18]. In middle meningeal artery, at high concentrations ($> 1 \mu$ M) a small difference between rizatriptan and sumatriptan was detected. The maximum contractile response to sumatriptan was not maintained and at concentrations beyond 1 μ M relaxation was seen. In contrast, the maximum contraction to rizatriptan was maintained and overall the maximum response to rizatriptan was significantly higher than that for sumatriptan. It is possible that at these high concentrations the selectivity of sumatriptan as a 5-HT_{1B/1D}-receptor agonist is lost and activation of another receptor type (e.g. histaminergic) causes vasorelaxation [19, 20]. Interestingly, a relaxant effect of sumatriptan (at concentrations $> 1 \mu$ M) has also been observed in human cerebral arteries [4].

Some of the samples of middle meningeal arteries used in this study were small and it was not always possible to obtain three segments which would have enabled a rigid crossover design for the order of agonist application. However, it is unlikely the small difference between

Figure 2 Representative autoradiographs obtained in sections of human middle meningeal artery (upper panel) and human coronary artery (lower panel). Sections were labelled with either 5-HT_{1B}-receptor (Panel A) or 5-HT_{1D}-receptor (Panel B) subtype specific primary antibodies and a radiolabelled secondary antibody. Levels of immunoreactivity were quantified using densitometry and an image analysis system. 5-HT_{1B}-immunoreactivity, but not no. 5-HT_{1D}-immunoreactivity, was detected in both middle meningeal and coronary arteries.

rizatriptan and sumatriptan is related to order of agonist application, since previous studies in our laboratory using vessels obtained from more than 70 patients have failed to detect any cross-over effects between agonists (unpublished observations). This is supported by the current analysis showing no desensitization and by previous studies showing no change in the sumatriptan concentration-effect curves following repeated application [21].

Vasoconstriction in cranial arteries may be important for the therapeutic effect of 5-HT_{1B/1D}-receptor agonists in relieving migraine headache whilst coronary artery contraction is relevant to the potential for cardiac adverse events associated with this class of drugs. Therefore, whilst there may be some potential issues concerning the comparison of vasoconstrictor effects in middle meningeal and coronary arteries obtained in separate experiments it is important that these comparisons are made. The present study shows that rizatriptan and sumatriptan are powerful vasoconstrictors in isolated middle meningeal artery with contractile responses being greater in magnitude (relative to 45 mM KCl = 100%) than those reported for coronary artery, for example for rizatriptan the E_{max} values were 132 and 22 [14], respectively relative to the reference agonist 45 mM KCl = 100%. In addition both rizatriptan and sumatriptan were 10-fold more potent in middle meningeal compared to coronary arteries (e.g. for rizatriptan EC_{50} values were 90 and 1020 nM [14], respectively). Finally, a three-way ANOVA (meta-analysis), comparing the overall concentration effect curves obtained in middle meningeal and coronary arteries [14], showed a significantly higher level of responsiveness to the agonists in middle meningeal artery (unpublished observations this laboratory summarized in Figure 3). This craniovascular selectivity may be achieved partly by differences in the distribution of 5-HT_{1B}- and 5-HT_{2A}-receptors [18, 19, 23], (both of which mediate vasoconstriction) in the different vascular beds. In human and primate cerebral arteries

Figure 3 Graphical summary of a three-way ANOVA (artery-agonist-concentration) to compare overall vasoconstrictor effects of 5-HT (circles), rizatriptan (triangles) and sumatriptan (squares) in middle meningeal artery and coronary artery. All three agonists were significantly more effective in middle meningeal compared to coronary artery (artery-agonist interaction $F = 19.4$, d.f. = 2 $P < 0.00009$). Post-analysis of variance multiple comparisons using Newman-Keuls showed significant differences between the effects of rizatriptan and sumatriptan in the two arteries ($P < 0.05$).

5-HT_{1B}-receptor activation contributes to more than 70% of the overall response to 5-HT [4, 7] and this agrees with the present data. However, in human coronary artery the relative contribution of 5-HT_{1B}-receptor activation to the overall 5-HT contractile response is generally lower between 21 and 71% [14, 18, 24]. Thus rizatriptan and sumatriptan (selective 5-HT_{1B/1D}-receptor agonists devoid of 5-HT₂-receptor affinity) will have a selective action in cranial vessels.

In the present study we used immunohistochemical techniques to compare the levels of 5-HT_{1B/1D}-receptor protein expression in middle meningeal and coronary arteries using 5-HT_{1B}- and 5-HT_{1D}-receptor antibodies. These antibodies were raised against unique peptide sequences located in the third intracellular loop of the receptors and have been fully characterized and shown to be specific for either 5-HT_{1B}- or 5-HT_{1D}-receptors with no cross-reactivity [16]. 5-HT_{1B}-receptor like protein, but not 5-HT_{1D}-receptor like protein, was detected within the smooth muscle wall of middle meningeal and coronary arteries indicating that vasoconstriction is 5-HT_{1B}-receptor mediated. This is consistent with published reports of RT-PCR studies showing selective expression of 5-HT_{1B}-mRNA in human blood vessels [4, 6] and functional studies showing a relationship between vasoconstrictor potency and 5-HT_{1B}-receptor affinity in human vessels including middle meningeal artery [18, 21, 22].

The current immunohistochemical techniques (using a radiolabelled secondary antibody) were sensitive and we were able to detect significantly higher levels of expression of 5-HT_{1B} receptor protein in middle meningeal artery compared to coronary artery. Due to the scarcity of fresh human coronary artery (normal) material it was only feasible to study a relatively small number of arteries in the immunohistochemical studies. The usual consequence of the lack of statistical power is the difficulty in finding a significant difference when one is truly there. However, on this occasion a significant difference was detected suggesting that statistical power was sufficient. Since agonist response is dependent on receptor number/density, the higher level of expression of 5-HT_{1B}-receptor protein in middle meningeal artery compared to coronary artery may also contribute to the differences in functional response in these two blood vessels and provide an additional pharmacological basis for the craniovascular over coronary artery selectivity of 5-HT_{1B/1D}-receptor agonists.

Conclusion

Rizatriptan and sumatriptan are powerful vasoconstrictors in human isolated middle meningeal arteries. Immunohistochemical studies showed that this response is 5-HT_{1B} receptor mediated and that there is a higher level of 5-HT_{1B}-receptor expression in middle meningeal arteries compared to coronary artery. This higher level of expression may contribute to the selective action of these drugs in the cranial vasculature.

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