The Vasorelaxant Properties of Novel Benzodiazepine-like Ligands on Isolated rat Thoracic Aorta

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Abstract

Background/Aim: In addition to well-established central effects, benzodiazepines, but also some other allosteric modulators of gamma-amino-butric acid (GABA) receptor exhibit significant vascular effects. However, there are currently no elucidated mechanisms for manifested vasodilatory properties and very little is known about GABA gamma-amino-butric acid function and GABA_A receptor expression within peripheral blood vessels.

Methods: In the present study, we demonstrated the vasorelaxant properties of diazepam, GABA and novel imidazobenzodiazepine amide ligands GL-II-73 and GL-II-74, which are characterized as positive allosteric modulators of α5-containing GABA_A receptor. Using isometric organ bath system, we examined the vascular responses to phenylephrine, in the presence and absence of various ligands, in the rat thoracic aorta.

Results: The observed significant and strong attenuation of the maximal contractile response of phenylephrine indicates a non-competitive antagonism of diazepam, GL-II-73 and GL-II-74 (p < 0.001), whereas GABA does not affect phenylephrine contraction.

Since the strongest inhibitory effect was observed with compound GL-II-74, that, compared to other tested ligands, exhibited a higher potentiation at α5 GABA_ARs, it could be assumed that the α5 subunit plays a significant role in the structure of putatively present “vascular” GABA_ARs.

Conclusion: This work emphasizes the importance of GABA_ARs research in the periphery and also points to the possibility of using α5-selective GABA_AR modulators as potential therapeutic targets for novel vasodilators.

Key words: GABA_A receptor, positive allosteric modulators, vasodilatation, rat thoracic aorta.

Introduction

In addition to being a major inhibitory neurotransmitter in the central nervous system (CNS), GABA has a functional importance in many peripheral tissues. Peripheral GABA regulation of cardiovascular function has long been known,1,2 but to date no distinct roles or exact mechanisms have been established.

The first studies with isolated cerebral blood vessels had suggested that GABA_A receptors (GABA_ARs) exist in vascular smooth muscle, where GABA or GABA-agonists produced a dilatation of cerebral arteries.3,4 Even though GABA_AR subunit mRNA expression has been demonstrated in various rat peripheral organs, such as kidneys, adrenal gland, ovary, testis, uterus and ileum5,6 very lit-
tle is known about GABA\textsubscript{R} expression and GABA function within the peripheral vascular smooth muscle.

GABA levels in the peripheral vessels and activity of GABA-related enzymes, especially glutamic acid decarboxylase (GAD) and gamma-aminobutyric acid-transaminase (GABA-T), have been found to be up to 1% of those in the brain\textsuperscript{2} and such a modest expression can be regarded as insufficient to directly elicit vasoactivity of GABA. However, the finding that cultured human aortic and umbilical vein endothelial cells synthesize GABA, which further exhibits direct effects on endothelial cell metabolism\textsuperscript{8} indicates the potential role of GABA as an autacoid for neighbouring smooth muscle cells.

Benzodiazepines (BZs) as positive modulators of GABA\textsubscript{R}s have a wide range of acute effects, such as anxiolytic, sedative, hypnotic, skeletal muscle relaxant, anticonvulsant, anterograde amnesic and ataxic action. In addition to well-established central role, BZ's also exhibit vasodilatory properties.\textsuperscript{9,10,11} However, there are currently no elucidated mechanisms of BZ's vasoactivity and propensity to reduce the intracellular influx of calcium into the smooth muscle cell.

Vascular effects similar to those of diazepam are also exhibited by other GABA\textsubscript{R} allosteric modulators, such as endogenous neurosteroids.\textsuperscript{12} Considering that the peripheral benzodiazepine receptor (officially known as translocator protein, TSPO) has no role in regulating smooth muscle contractility,\textsuperscript{10} the published results suggest that activation or positive modulation of GABA\textsubscript{R}s, such as that effected by diazepam, result in vascular dilation.\textsuperscript{12} However, the receptor subtype substrate of that action is totally unknown.

Herein, the vasorelaxant properties of novel ligands with imidazobenzodiazepine (IBZD) amide structure GL-II-73 and GL-II-74 were demonstrated, which are characterised as positive allosteric modulators (PAMs) of GABA\textsubscript{R} with preferential potentiation at \( \alpha_5 \) subunit-containing receptors.\textsuperscript{13} In order to examine their possible vasoactivity, isometric organ bath study of vascular responses to phenylephrine was conducted. Diazepam and GABA were used in the same protocols, and in this way the manifested effects were compared and thus the possible mechanisms of vasoactivity were assessed.

# Methods

## Vessel preparation

Wistar rats were obtained from the Military Medical Academy and housed in vivarium facilities of the Faculty of Pharmacy, University of Belgrade (Belgrade, Serbia) under normal housing conditions (temperature: 22 ± 1°C, relative humidity: 40-70%, 12/12 h light/dark period). As a part of a wider national project led by the senior author, the experiments were approved by the Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia. Male rats were anaesthetised with combination of ketamine hydrochloride (90 mg/kg, Ketamidor, Richter Pharma AG, Wels, Austria) and xylazine hydrochloride (10 mg/kg, Xylased, Biовeta, A. S., Ivanovice na Hane, Czech Republic). The descending thoracic aortas were dissected and cleared of surrounding adipose and connective tissue.

Aortic rings of approximately 3 mm length were obtained from isolated blood vessels bathed in Petri dish containing chilled (4°C) modified Krebs-bicarbonate solution (composition: 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 11 mM glucose).\textsuperscript{14} The aortic rings were rapidly placed for measurement of isometric contraction.

Experiments with isolated vascular rings

The aortic rings were suspended between two wire hooks in organ bath chambers filled with 15 mL modified Krebs-bicarbonate solution (composition: 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 11 mM glucose).\textsuperscript{14} The aortic rings were rapidly placed for measurement of isometric contraction.

The rings were placed under the optimal passive stretching tension of 4.0 g, defined previously.\textsuperscript{15} The equilibration period of the preparation lasted 60 min and during that time the bathing solution was changed every 10 min. Each aortic ring was subjected first to the initial challenging contraction with potassium chloride (6 x 10\textsuperscript{-2} M) to assess the viability of preparations. The rings
was then left to re-equilibrate for 40-50 min, before the appropriate protocol procedures were used.

**Experimental protocol:** experiments were aimed to investigate the effects of diazepam, GABA and novel imidazobenzodiazepine (IBZD) amide ligands (GL-II-73 and GL-II-74) on the contractile response induced by the α1 adrenoceptor agonist phenylephrine (PE), in the endothelium-intact aortic rings.

At the beginning of the protocol, to obtain a reference contraction, the contractile response induced by potassium (6 x 10^-2 M) was measured. After preparations were washed-out several times until tone returned to baseline, concentration-response curve of PE (control curve) was generated (10^-9-10^-4 M). Aortic ring had been washed-out again and test compound (each at concentration 10^-4 M and 10^-5 M, except for diazepam with the applied concentration of 10^-5 M) were added individually to the organ bath, 60 min before another PE-induced contraction was obtained. The effects of the test compound on the PE contraction were assessed by comparing the contractile response in the presence or absence of compound. Results were expressed in relation to the contraction achieved by the same ring previously contracted with isotonic potassium.

**Drugs and solutions**
Phenylephrine hydrochloride and GABA were purchased from Sigma-Aldrich (St. Louis, USA). Diazepam was generously supplied by Galenika (Belgrade, Serbia).

The ligands GL-II-73 ((R)-8-Ethynyl-6-(2-fluorophenyl)-N,N,4-trimethyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxamide) and GL-II-74 ((R)-N-Ethyl-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxamide) were synthesised at the Department of Chemistry and Biochemistry, University of Wisconsin, Milwaukee, USA.

All drugs were prepared as concentrated stock solutions 10^-1 M in 100 % ethyl alcohol, with exception for PE and GABA, the stocks of which were prepared in distilled water. The subsequent dilutions were carried out in mixture of solvent and distilled water, so that the final solvent concentration was never higher than 0.3 % in the 15 mL-organ bath.

**Statistical analysis**
Statistical analysis and graphs were prepared using LabChart 7 Pro software (AD Instruments) and SigmaPlot 11 (Systat Software Inc.) Results were summarised as the mean ± standard error of n replicates, where n is the number of aortic rings tested in one protocol, each obtained from a separate animal. The negative logarithm of the ligand concentration (pECs50) producing 50% of the maximum response was calculated in LabChart 7 Pro software. Statistical analyses were performed using Student’s paired t-test (p values less than 0.05 were considered statistically significant).

**Results**
Diazepam (10^-5 M) produced a significant attenuation (p < 0.001) of the maximal contractile response of PE (117.24 ± 5.30 % vs 66.62 ± 3.71 %), while it did not affect the pECs50 value of PE (Figure 1).

Although applied at a very high concentration (10^-4 M), GABA did not shift the PE concentration-response curve or affect the PE-induced maximal contraction (Figure 2A). GABA used at concentration of 10^-5 M also did not affect the PE contraction (data not shown).

The ligand GL-II-73 (10^-5 M) significantly decreased (p < 0.05) the maximal contractile re-
Discussion

The differential expression of total of nineteen GABA\_R subunits (\(\alpha1\)-6, \(\beta1\)-3, \(\gamma1\)-3, \(\delta\), \(\varepsilon\), \(\theta\), \(\pi\), \(\rho1\)-3) has been demonstrated in various peripheral organs, indicating that GABA\_R subunits are expressed in a tissue-specific manner.\(^{6,16}\) Immunohistochemical analyses, western blotting and real time reverse transcription polymerase chain
reaction (RT-PCR), had revealed the presence of functional GABA\_A\_Rs within the gastrointestinal tract,\textsuperscript{15} airway smooth muscle of trachea,\textsuperscript{16,17} pancreatic β cells.\textsuperscript{18} However, there is still no clear evidence for the expression of functional GABA\_A\_Rs on vascular smooth muscle cells. This study was based on the hypothesis that positive allosteric modulation of GABA\_A\_Rs that contain the α5 subunit contributes to vasodilating effects of BZs. The inhibiting influence of diazepam on the contractile activity of phenylephrine in isolated rat aorta was demonstrated, thus confirming the previous \textit{in vitro} studies, where diazepam inhibited PE-induced calcium oscillations,\textsuperscript{20} attenuated the PE-induced contractions in the rat aorta\textsuperscript{20} and produced vasodilation in the PE-precontracted rat aortic rings.\textsuperscript{9} The observed significant and strong attenuation of the maximal contractile response of PE indicates a non-competitive antagonism of diazepam, in terms of signalling mechanisms of contraction in vascular smooth muscle cells.

Concentration of GABA in the systemic circulation of humans was found to be between 0.5 to 3 µM.\textsuperscript{8} It has been suggested that apart from GABA produced by the pancreatic beta cells, adrenal gland and certain immune cells, an important source of GABA in circulation may be that related to endothelial cells of blood vessels.\textsuperscript{8} The examination of the effect of GABA on vascular response to PE in isolated rat aorta indicated that GABA did not affect PE contraction, even when applied in high concentration (100 µM). Findings of GABA indifference on contracted aortic rings found in this study may correlate with earlier data that no vasodilating effects on peripheral blood vessels have been reported for GABA.\textsuperscript{8,11,21} Nevertheless, the results from other studies with isolated blood vessels have shown that GABA has relaxatory effect on rat mesenteric bed.\textsuperscript{22,23} Diazepam, a standard non-selective PAM of GABA\_A\_Rs, was used as the reference ligand, in order to investigate the vasorelaxant properties of GL-II-73 and GL-II-74. Previously performed electrophysiological and binding studies showed that ligands GL-II-73 and GL-II-74 acted as PAMs with primary efficacy and affinity at α5-containing GABA\_A\_Rs,\textsuperscript{13} whereas diazepam modulates GABA\_A\_R activity as a non-selective PAM, with high affinity and efficacy at α1, α2, α3 or α5–containing GABA\_A\_Rs.\textsuperscript{24} Both ligands (GL-II-73 and GL-II-74) reduced the maximum contraction induced by PE, compared to the untreated rings, indicating similarity to the effects of diazepam in the same protocol.

It was also shown that the vascular responses to PE in the isolated aortic rings vary significantly, depending on concentrations of GL-II-73 and GL-II-74 used during incubation. When aortic rings were pre-treated with a higher concentration (10⁻⁴ M), the maximal contractile response was approximately 20-30 % of the corresponding control maximal contraction ie (without the presence of ligand), while at lower concentrations of tested ligand, the inhibitory effects were weaker (approximately 50 % reduction in contraction). This clearly indicates a concentration-dependent inhibitory effects of the tested IBZDs.

Concentrations of compounds used in this study were in accordance with those in studies of vascular effects of BZs on isolated blood vessels. Although these concentrations are too high to correspond with the clinical use of BZs, they can still be reached in cases of overdose or other abuse.\textsuperscript{11} In this regard, their vascular effects should not be neglected. Interestingly, a stronger inhibitory effect on the PE concentration-response curve was observed with compound GL-II-74 than with GL-II-73. This might be explained by the observed differences in their modulatory properties, taking into account that GL-II-74 exhibited a higher potentiation at α5 GABA\_A\_Rs than GL-II-73.\textsuperscript{23} Accordingly, it could be assumed that the presence of the α5 subunit in the structure of putatively present “vascular” GABA\_A\_Rs may play a substantial role in the overall observed vasoactivity.

**Conclusion**

The present work highlights the importance of GABA\_A\_Rs research in the periphery and also opens the possibility of using α5 selective GABA\_A\_R modulators as potential therapeutic targets for novel vasodilators.

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Conflict of interest

None.

References