Effect of the novel endothelin(A) receptor antagonist LU 208075 on contraction and relaxation of isolated rat basilar artery

Hartmut VATTER, Michael ZIMMERMANN, Carla JUNG, Edda WEYRAUCH, Josef LANG and Volker SEIFERT
Department of Neurosurgery, Johann Wolfgang Goethe-University, Schleusenweg 2–16, D-60528 Frankfurt am Main, Germany

ABSTRACT

Increased levels of endothelin (ET)-1 and bigET-1 may be responsible for enhanced cerebroarterial resistance under pathologic conditions. Therefore, the effect of LU 208075, a novel ET(A)-selective receptor antagonist was determined. The aim of the study was to investigate in vitro the inhibitory effect of LU 208075 on ET-1 and bigET-1 induced contraction and relaxation in rat basilar artery segments. Segments with (E +) and without (E −) endothelium were prepared for the measurement of isometric force. Concentration–effect curves (CECs) were constructed by cumulative application of ET-1 or bigET-1. The shift of the CECs in the presence of LU 208075 against the control curve was determined. Relaxation was investigated on precontracted segments, calculated in percentage decrease of precontraction and compared by the pD2 and Emax. ET-1 and bigET-1 induced contraction was dose dependently inhibited by LU 208075. Shifts of the CECs in the presence of LU 208075 (10−6 M and 10−5 M) were for ET-1 (1) in E+: 4.4 and 19.7; (2) in E−: 8.1 and 60.4 and for bigET-1 (3) in E+: 10.8 and (4) in E−: 26.0 respectively. LU 208075 (10−5 M) completely inhibited bigET-1-induced contraction. Relaxation by ET-1 or bigE-1 was only observed in the presence of LU 208075. CECs were shifted to the right by LU 208075 (10−5 M) by a factor of 24 (ET-1) and 4.5 (bigET-1). Emax values were 45±18% and 51±15% (ET-1; in the presence of 10−5 and 10−6 M LU 208075 respectively), and 56±20% and 49±17% (bigET-1; in the presence of 10−5 and 10−6 M LU 208075 respectively). The data suggests a competitive ET(A)-receptor inhibition by LU 208075. The enhanced inhibitory effect on bigET-1-induced contraction could indicate an additional inhibitory effect on endothelin-converting enzyme activity. The pronounced effect on E− vessels and the inhibition of relaxation may suggest an ET(B) receptor affinity.

INTRODUCTION

Endothelin (ET)-1 acts in cerebral vessels in vitro [1,2] and in vivo [3,4] as a potent and long-lasting vasoconstrictor. This contractile effect was enhanced after experimental trauma [5] and after experimentally induced subarachnoidal haemorrhage (SAH) [6]. Furthermore, an increase of ET-1 and its precursor, bigET-1, was observed in the cerebrospinal fluid of patients suffering from cerebral vasospasm following SAH [7,8] and in patients after cerebral ischaemia [9]. Therefore ET-1 and bigET-1 appear to be of major functional importance in the pathophysiology of enhanced cerebrovascular resistance under pathological conditions like severe head injury, ischaemia and most notably cerebral vasospasm following SAH. This enhanced resistance leads to reduced cerebral blood flow and may cause secondary ischaemic brain damage. Therefore interfering with the ET system...
may improve the reduced cerebral blood flow and the clinical outcome under the pathological conditions mentioned above.

The vasoactive effects of ET-1 are mediated by two specific receptors termed ET(A)- and ET(B) (for review see [10]). In the cerebral circulation, the ET(A) receptor located on smooth muscle cells mediates contraction [1], whereas relaxation results from activation of the ET(B) receptor on the endothelial cells [11]. The existence of an ET(B2) receptor subtype located on vascular muscle cells, which effects vasoconstriction on peripheral vessels [12], could not be proved in the cerebral circulation so far. However, some data suggest an ET(B) receptor-dependent vasospasm following experimental SAH [13]. In spite of these observations, most investigations suggest a simultaneous activation of both receptors resulting in vasoconstriction of cerebral vessels [1,2] caused by the predominant effect of the ET(A) receptor. Furthermore, an enhanced effect in reducing the cerebral vasospasm after experimental SAH in monkeys of an non-selective receptor antagonist for the treatment of cerebrovascular pathologically reduced cerebral blood flow.

The aim of the present study was to characterize the novel ET(A) receptor selective antagonist LU 208075 on constriction of isolated cerebral arteries by ET-1 and bigET-1. LU 208075 is a non-peptide compound, which is orally available and therefore suitable for prophylactic and therapeutic treatment. In binding experiments on membrane fractions expressing ET(A) and ET(B) receptors, a 77-fold selectivity for ET(A) receptors was observed (Muenter, K., personal communication). According to these results an additional aim of the present study was to determine if a relaxation of cerebral arteries could be induced by ET-1 or bigET-1 in the presence of LU 208075. A potent inhibitory effect of LU 208075 on the vasoconstriction by ET-1 and bigET-1 and the reversal of this contraction into relaxation in the presence of the receptor antagonist would strongly suggest the compound for clinical trials as a promising approach for the treatment of reduced cerebral blood flow under pathological conditions.

MATERIALS AND METHODS

The investigations were performed in basilar artery ring segments obtained from male Sprague–Dawley rats (250–400 g). The animals were sacrificed under carbon dioxide anaesthesia. Each basilar artery was dissected under microscopic control and cut into four ring segments. The ring segments were mounted on two L-shaped stainless steel wires (70 μm diameter) in organ baths of 2.5 ml volume (FMI, Seeheim, Germany) for measurement of isometric force as described previously [16]. Organ baths were filled with modified Krebs-Högestett solution of the following composition (mM): NaCl 119; KCl 3.0; NaH2PO4 1.2; CaCl2·H2O 1.5; MgCl2·6H2O 1.2; NaHCO3 20; glucose 10. They were continuously bubbled with humidified gas mixture (95% O2, 5% CO2) resulting in a pH around 7.35 at 37 °C. In a part of the segments the endothelium was removed mechanically by rubbing the intimal surface with a human hair. After mounting, an accommodation period of 60 min was kept. The segments were repeatedly stretched during this period until a stable level of resting tension of 2–3.5 mN was reached.

After accommodation a reference contraction was elicited by exchanging the bath solution with 124 mM K+-Krebs solution (NaCl replaced by KCl). Segments developing less than 2.5 mN force were discharged. The endothelial function or the complete removal of the endothelium was tested by relaxation induced by acetylcholine (10−5 M) after precontraction with 5-hydroxytryptamine (10−3 M). A relaxation of a segment of more than 30% of the precontraction was interpreted as functionally intact endothelium (E+). The removal of the endothelium (E−) was assessed successfully when the decrease of contraction induced by 5-hydroxytryptamine was less than 15% during the application of acetylcholine.

ET-1 (10−12–3 × 10−7 M) or bigET-1 (10−10–3 × 10−9 M) was applied cumulatively on segments under resting tension for the construction of concentration–effect curves (CECs). Contraction was measured in mN force and expressed as a percentage of the reference contraction. To compare the CECs, the observed maximum contraction (Emax) and the concentration at which 50% of the reference contraction occurred (EC50%K+) was calculated by linear regression analysis of the CEC after logarithmic transformation of the concentrations applied. The shift of the CECs on the level of the 50% reference contraction was calculated and used for the characterization of the inhibitory effect of the receptor antagonist LU 208075.

For the investigations concerning the relaxation segments were precontracted with prostaglandine F2α (10−6 M) before the application of ET-1 or bigET-1. Relaxation was calculated as the percentage decrease of precontraction, and determined according to the area under the curve method during an observation time of 20 min, as described previously [17]. To compare the CECs of the relaxation, the pD2 and the Emax values were calculated.

The receptor antagonist LU 208075 was used in concentrations of 10−6 M and 10−5 M. A preincubation
period of at least 30 min was used before application of ET-1 or bigET-1.

Human bigET-1 (1–38) and ET-1 was purchased from Calbiochem-Novabiochem, (Bad Soden, Germany), acetylcholine, 5-hydroxytryptamine and prostaglandine F2α from Sigma (Deisenhofen, Germany). LU 208075 was kindly provided by Dr K. Muenter (Knoll AG, Ludwigshafen, Germany).

Statistical analysis was performed using one way ANOVA followed by Tukey’s test for post-hoc comparison of mean values. A P-value < 0.05 was considered significant. All values in the text and in the Figures are given as means ± S.D.

RESULTS

Cumulative application of ET-1 and bigET-1 on segments under resting tension resulted in a dose-dependent contraction in vessels with and without endothelial function (Figures 1 to 4). Preincubation with LU 208075 resulted in a significant and dose-dependent rightwards shift of the CECs for the ET-1-induced vasocontraction in endothelium intact and denuded segments, as shown in Figures 1 and 2. The maximum contraction was not significantly reduced in the presence of both concentrations of the antagonist, which can be seen by the $E_{\text{max}}$ values in Table 1. The rightwards shift of the CECs for ET-1 on the EC$_{50\%K^+}$ level was increased in vessels without endothelial function (Table 1).
Effect of LU 208075 on cerebral arteries

Figure 4  CECs for contraction by bigET-1 in the absence and presence of LU 208075 for segments without endothelial function

The CECs are shifted rightwards and the maximum contraction is significantly reduced. Data are means ± S.D. * P < 0.05 versus bigET-1.

The CEC for the contraction by bigET-1 was shifted to the right in the presence of LU 208075 in the lower concentration (10⁻⁶ M) and the maximum contraction was significantly reduced in both segments with and without endothelial function (Table 1). The shift of the CEC for bigET-1 was similar to those of ET-1 in Epronounced in vessels without endothelium (Table 1). The presence of the higher concentration of LU 208075 (10⁻⁵ M) resulted in a complete inhibition of the contraction by bigET-1 (Figures 3 and 4). The inhibitory effect of LU 208075 was therefore markedly enhanced for the contraction by bigET-1 compared with ET-1, which is presented by the higher shifts on the 50% level of the reference contraction (Table 1).

In the absence of the receptor antagonist, neither ET-1 nor bigET-1 induced a significant relaxation of the precontracted segments. However, in the presence of LU 208075 a significant relaxation occurred for both compounds. The $E_{\text{max}}$ values for ET-1 were 45 ± 18% and 51 ± 15% in the presence of LU 208075 (10⁻⁶ M and 10⁻⁵ M) and 56 ± 20% and 49 ± 17% for bigET-1. The CEC for both ET-1 and bigET-1 was shifted to the right in the higher concentration of LU 208075 compared with the lower one. The pD₂ of the CEC for ET-1 in the presence of LU 208075 (10⁻⁵ M) was 9.23 whereas the corresponding value in the higher concentration of the antagonist (10⁻⁴ M) was 8.16, resulting in a shift on the level of the pD₂ of 24.0. The pD₂ values for the CECs for bigET-1 in the presence of LU 208075 were 7.46 and 6.94. The resulting shift of 4.5 was markedly reduced compared with ET-1.

**DISCUSSION**

LU 208075 was introduced as a novel, non-peptide and competitive ET receptor antagonist with a 77-fold selectivity for the ET(A) receptor (Muenter, K., personal communication). The parallel and dose-dependent shifting of the CEC for the contraction by ET-1 in the presence of LU 208075, without effecting the maximum contraction, are in good agreement with this observation. However, the enhanced inhibitory effect on the contraction by ET-1 on segments without endothelial function may indicate a co-inhibition of the ET(B)

| Table 1  | Comparison of the maximum contractions in percentage of reference contraction ($E_{\text{max}}$) and $EC_{50\%K^+}$ values for ET-1 and bigET-1 in the presence and absence of LU 208075 10⁻⁴ M and 10⁻⁵ M for segments with or without a functional intact endothelium |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | E+               | E-               | E+               | E-               | E+               | E-               |
|                | LU 208075        | LU 208075        | LU 208075        | LU 208075        | LU 208075        | LU 208075        |
| ET-1            |                  |                  |                  |                  |                  |                  |
| $E_{\text{max}}$ (%) | 110 ± 14        | 94 ± 20          | 92 ± 10          | 126 ± 17         | 118 ± 18         | 74 ± 33          |
| $EC_{50\%K^+}$ (M) | 2.32 × 10⁻⁸      | 1.01 × 10⁻⁷      | 4.57 × 10⁻⁷      | 6.41 × 10⁻⁸      | 5.19 × 10⁻⁸      | 3.87 × 10⁻⁸      |
| Shift versus control | –                | 4.4              | 19.7             | –                | 8.1              | 60.4             |
| bigET-1         |                  |                  |                  |                  |                  |                  |
| $E_{\text{max}}$ (%) | 97 ± 14          | 58 ± 25          | –                | 121 ± 12         | 62 ± 14          | –                |
| $EC_{50\%K^+}$ (M) | 9.47 × 10⁻⁸      | 1.02 × 10⁻⁴      | –                | 5.88 × 10⁻⁸      | 1.55 × 10⁻⁴      | –                |
| Shift versus control | –                | 10.8             | –                | –                | 26.0             | –                |
receptor, which would not be expected from the binding studies on membrane preparations. As contraction of cerebral vessels is mediated by the ET(A) receptor [1] and relaxation by the ET(B) receptor [11], both receptors represent functional antagonists. Therefore the co-inhibition of the ET(B) receptor leads to a decrease of the inhibitory effect in vessels possessing functional intact endothelium, whereas it has no effect in segments without endothelial function. Additionally a similar enhanced rightwards shift was observed on the contraction by bigET-1 on vessels without endothelium compared with endothelium intact segments.

The enhanced inhibitory effect of LU 208075 on contraction by bigET-1 compared with ET-1 suggest a further mechanism of inhibition, which seems to not be competitive. As bigET-1 itself is vasoactive [18], it has to be transformed to mature ET-1. Therefore, vasomotor effects of bigET-1 assumes the existence of a functional ET-converting enzyme (ECE)-activity [16,17]. An obvious explanation of the increased inhibitory effect on the contraction by bigET-1 in a non-competitive way is an additional co-inhibition of the functional relevant ECE activity. In discrepancy to this explanation is the bigET-1-induced relaxation in the present study, which also occurs in the presence of LU 208075 (10⁻⁵ M) and the reduced shift of the CECs for the relaxation by bigET-1 compared with the shift for ET-1. However, isoforms of the ECE were characterized, which may differ in their regional cellular distribution [19]. While the ECE-1 subtype appears to be most widely distributed in endothelial cells [19,20], ECE-1b is thought to be most abundantly expressed in vascular smooth muscle cells [20]. In contrast, ECE-2, derived from a different gene locus to ECE-1, may not have been contributed to ET-1-induced relaxation in the presence of LU 208075 on the functional ECE activity of the smooth muscle cells, but not on that located in the endothelium.

As expected an ET(B) receptor-mediated relaxation was observed by ET-1 and bigET-1 on precontracted vessels in the presence of the ET(A) receptor antagonist LU 208075. The rightward shift of the CEC for both compounds in the higher concentration of LU 208075 without effecting the maximum relaxation clearly indicates competitive inhibition of the ET(B) receptor in the presence of LU 208075 (10⁻⁵ M), which is in contrast to the observation in binding studies (Mueuter, K., personal communication).

In conclusion, the present results characterize LU 208075 as a competitive antagonist of the ET(A) receptor on cerebral arteries. Furthermore, LU 208075 seems to have a higher affinity to the ET(B) receptor than expected, according to receptor-binding assay and may possess an inhibitory effect on the functional relevant ECE. ET(A) selective and combined ET(A)/ET(B) receptor antagonists were successful in the treatment of experimental cerebral ischaemia [22,23] and cerebral vasospasm in different animal models [14,24]. Furthermore, a nearly complete reversal of a cerebral vasospasm in a rabbit model could be achieved by the ECE inhibitor CGS 26303 [25,26]. Therefore LU 208075, which seems to possess an ET(A) receptor antagonistic and an ECE-inhibitory component may present a promising approach for the treatment of reduced cerebral blood flow. Further investigations are necessary to confirm these promising results under pathological conditions such as SAH, stroke and brain injury.

ACKNOWLEDGMENTS

The authors thank Dr K. Muenter for providing LU 208075 and M. Heibel for expert technical assistance.

REFERENCES