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Endothelium-dependent Vasorelaxant and Anti-aggregatory Effect and Mechanism of Action of Some Antifibrinogen RGD (Arg-Gly-Asp-containing) Peptides

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Abstract

Vasorelaxation caused by some antifibrinogen RGD (Arg-Gly-Asp-containing) peptides and their basic mechanism of action was studied on rabbit isolated thoracic aortic rings preconstricted with 0·25 μM phenylephrine.

GRGDS (Gly-Arg-Gly-Asp-Ser-OH) and RGDV (Arg-Gly-Asp-Val-OH) caused dose-dependent relaxation. RGDS (Arg-Gly-Asp-Ser-OH) had a biphasic effect (a transient relaxation followed by a contraction) while GRGDS-[SE] (Gly-Arg-Gly-Asp-Ser(SO_3)-OH) did not change the isometric tone of precontracted aortic preparations. GRGDS and RGDV exerted no relaxing effect on endothelium-denuded blood vessels suggesting that the vascular action of these peptides was entirely dependent on the presence of functionally intact endothelium. L-N^G-Nitro-arginine (30 μM) attenuated the relaxation induced by GRGDS and abolished that induced by RGDV. All of the four RGD congeners inhibited ADP-induced aggregation of human platelets.

These findings indicate that the relaxant effect of RGDV is mediated exclusively by the nitric oxide pathway, but GRGDS could cause, besides nitric oxide release, the release of another substance which is different from nitric oxide. Because the rank order of the vasorelaxant potencies of RGD peptides differed from that found for their anti-aggregatory activities, a vascular effector mechanism mediated by an RGD-recognizing structure other than the known glycoprotein IIb/IIIa-like RGD-binding site is suggested.

The adhesion of platelets to the subendothelial extracellular matrix (Sixma et al 1987), especially to the freshly presented collagen content (Sakariassen et al 1986) of an injured vessel wall and the ensuing platelet aggregation are cardinal phases in the beginning of unrestrained platelet deposition on thrombogenic surfaces (Scharf & Harker 1987). Such damage of vessels caused, for example, by the rupture of an atherosclerotic plaque (Fuster et al 1990), may induce vascular occlusions resulting in myocardial infarction (Forrester et al 1991) or cerebral stroke (Carr et al 1996). For expansion of the thrombus to occur, the fibrinogen cross-linkings between platelets have been found to be essential (Hawinger 1987). This process is mediated via activated forms of the platelet fibrinogen receptor, the glycoprotein IIb/IIIa (gpIIb/IIIa) complex (Shattil et al 1985). To ward off the development of haemostatic plugs with the aim of blocking this step seemed to be useful (Ruggeri et al 1986). Monoclonal antibodies against gpIIb/IIIa (Coller & Scudder 1986) and an army of peptide (Samanen et al 1991) and non-peptide (Alig et al 1992) analogues mimicking the Arg-Gly-Asp-containing gpIIb/IIIa binding site of the fibrinogen α-chain (Doolittle et al 1979) were evolved.

GRGDS (Gly-Arg-Gly-Asp-Ser-OH), RGDV (Arg-Gly-Asp-Val-OH) and RGDS- (Arg-Gly-Asp-Ser-OH), are representatives of platelet aggregation inhibitor RGD peptides (Samanen et al 1991). RGDS, GRGDS and RGDV are not only antifibrinogen peptides, but they can also block other RGD-mediated receptor-ligand interactions, e.g. binding of fibronectin, vitronectin, von Willebrand factor, thrombospondin (Cox et al

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1994) and disintegrins (Gould et al 1990) to their own receptor sites. Although it was previously reported that systematically administered RGD peptides could cause mild hypotension (Roux et al 1993), it is not completely clear how RGD peptides could influence the tone of the vessel wall. It has recently been reported that some RGD congeners could act as an arteriolar vasodilator (Mogford et al 1996) utilizing a subendothelial smooth muscle $\alpha_v\beta_3$ integrin receptor. According to our previous findings (Torday et al 1993a,b, 1994), the aforementioned congeners could exert their effect not only on a smooth muscle, but also on an endothelial receptor. In the present work the vascular and antiaggregatory effect and the mechanism of action of some RGD peptides was studied.

Materials and Methods

Measurements of vascular tension

We followed the procedure described previously by Toth et al (1995). New Zealand rabbits, 1·5–2·5 kg, of either sex were used. After injecting 500 int. units kg^{-1} heparin into their marginal ear vein, rabbits were killed. After opening the chest, the heart was cut out immediately. Following complete exsanguination the thoracic aorta was excised and washed from remaining blood with Krebs–Hensleit solution of the following composition: 120 mM NaCl, 4·2 mM KCl, 1·5 mM $CaCl_2$, 20 mM $NaHCO_3$, 1·2 mM $MgCl_2$, 1·2 mM KH_2PO_4 and 11 mM glucose. Then the vessel was carefully cleaned from surrounding connective tissue and was sliced with sharp blades into rings 5 mm wide. To achieve steady-state equilibration, preparations were mounted in water-thermostated (at 37°C) recording chambers containing 2 mL freshly prepared Krebs–Hensleit solution which was continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The medium was changed every 15 min. The isometric tension was recorded using a force transducer (Experimetria, Hungary). Continuously readjusting the tension to 10 mN, rings reached stable equilibrium within 45 min. We tested each ring before experiments concerning the functional integrity of endothelium using 0·25 μM phenylephrine as a preconstricting agent. The rings were considered suitable if the amplitude of relaxation caused by 0·25 μM acetylcholine, which was administered 5 min after the beginning of the steady state of contraction, was at least 50% of the steady-state tone induced by the phenylephrine. After an appropriate washing period, peptides were cumulatively administered using the same preconstricting methodology. Each concentration was allowed to act for 10 min. By the end of this period a new equilibrium (plateau) was always attained.

Denudation process

Denuded aortic rings were prepared by gentle rubbing of the internal surface with a wet-cotton-covered glass rod. The endothelial layer was considered to be removed if the rings that had been preconstricted by 0·25 μM phenylephrine did not relax, but contracted after administration of 0·25 μM acetylcholine.

Inhibition of endothelial nitric oxide synthase

To block the ability of the endothelial cells to release nitric oxide we used 30 μM L-N^G-nitro-arginine, which is one of the most effective constitutive nitric oxide synthase inhibitors (Moore et al 1990). Thirty minutes was chosen as the preincubation period for aortic rings.

Preparation of platelet-rich plasma

Freshly drawn venous blood from cubital veins of drug-free volunteers was anticoagulated with 0·1 vol 3·8% (w/v) trisodium citrate and then centrifuged at 200 *g* for 15 min. Thus other cellular elements of the plasma were removed. Platelet counts of prepared platelet-rich plasma samples were adjusted to 280 000–330 000 platelets μL^{-1} by using autologous platelet-poor plasma (obtained by centrifuging the pellet of the remaining blood at 1000 *g* for 10 min).

ADP-induced platelet aggregation

We induced the aggregation of the platelets by adding 8·3 mM adenosine diphosphate (ADP) after a 2-min incubation period with different concentrations of peptides in a thermostated (at 37°C) 600-μL glass chamber. The changes of light transmittance was measured turbidimetrically (Born 1962) with a Born-aggregometer. The degree of inhibition of the platelet aggregation was measured as the percent of the effect of a single shot of 8·3 μM ADP.

Drugs and peptides

Components of the Krebs–Hensleit solution were purchased from Reanal Fine Chemicals Ltd (Budapest, Hungary). Phenylephrine was the product of Sigma Co. (St Louis, MO). Acetylcholine was procured from Fluka AG (Switzerland). The RGD peptides were synthesized on solid phase by the Department of Medical Chemistry (Albert Szent-Györgyi Medical University, Szeged, Hungary) using the synthesis cycle published by Penke et al (1984) and were purified by preparative high-performance liquid chromatography (HPLC). The

structure of the analogues was verified by amino-acid analysis and mass spectrometry, the purity was checked by thin-layer chromatography and analytical HPLC.

Statistical analysis

All values are expressed as mean ± s.e.m. Differences between means were compared by Student's *t*-test for paired data. When the probability that the difference arose by chance was lower than 0·05, the difference was considered statistically significant. n refers to the number of rings from different rabbit aortas as used or the number of different blood samples. For the calculation of IC50 values the $(a*x)/(x+b)$ logistic equation was fitted to the individual dose–response values.

Results

The effect of four RGD-analogue peptides was studied on phenylephrine-preconstricted endothelium-intact rabbit aortic rings. GRGDS exhibited a dose-dependent relaxant effect over a concentration range of 10 μM to 0·4 mM. The exerted maximum relaxation was 81·4 ± 3·4% (Figure 1, n = 13). The IC50 of this effect was found to be $2{\cdot}7 \pm 0{\cdot}5 \times 10^{-5}$ M. RGDV showed a similar action; its maximum relaxant effect amounted to 98·0 ± 2·9% (Figure 2, n = 19), and the IC50 was $1{\cdot}1 \pm 0{\cdot}1 \times 10^{-4}$ M. The sulphate ester congener of GRGDS (GRGDS-[SE]) had no effect on the tone of the preconstricted aortic rings (Table 1, n = 3). We administered RGDS from 0·5 up to 2·3 mM and its effect could be described as an initial mild relaxation not exceeding 10% followed by a strong contractile response up to about 30% above the phenylephrine-induced tone (n = 3).

We also investigated the effect of aortic-ring denudation on previously experienced peptide effects. We have found that denudation totally abolished the relaxation caused by either GRGDS (Figure 1) or RGDV (Figure 2), and decreased by 50% the initial relaxant phase of RGDS action (n = 5). Then we examined the changes resulting from pre-incubation of the aortic rings with 30 μM L-N^G-nitro-arginine. This procedure decreased the maximum vasorelaxant effect of GRGDS to 33·6 ± 7·4% (Figure 1, n = 5), and caused the vascular action of RGDV to practically disappear (Figure 2, n = 5).

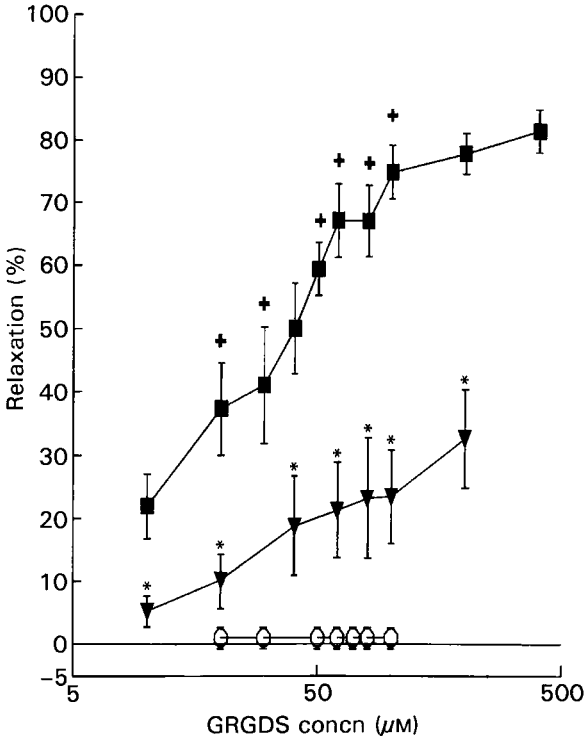

Figure 1. The relaxing effect of GRGDS on endothelium-intact (■), denuded (○) and 30 mM L-N^G-nitro-arginine-pretreated (▼) rabbit aortic rings preconstricted with 25 mM phenylephrine. Significant differences in corresponding values between denuded and endothelium-intact rings are marked by +, and by * between L-N^G-nitro-arginine-pretreated and endothelium-intact rings.

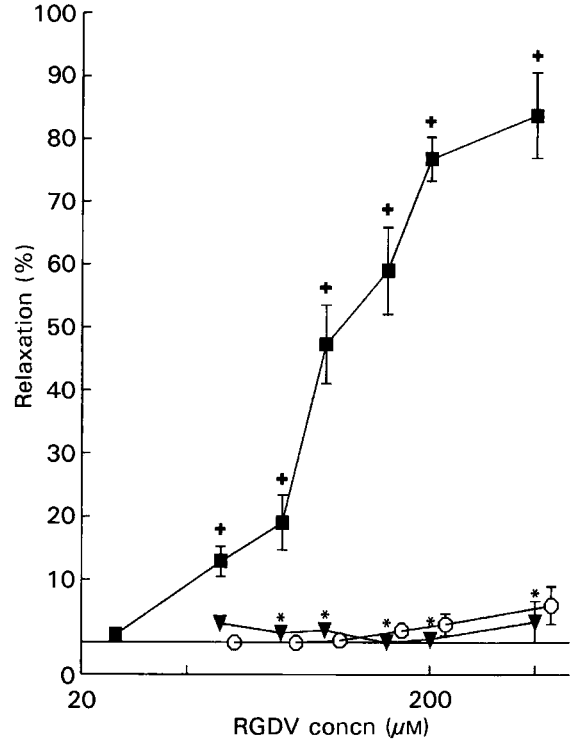

Figure 2. The relaxing effect of RGDV on endothelium-intact (■), denuded (○) and 30 mM L-N^G-nitro-arginine-pretreated (▼) rabbit aortic rings preconstricted with 25 mM phenyl ephrine. Significant differences in corresponding values between denuded and endothelium-intact rings are marked by +, and by * between L-N^G-nitro-arginine-pretreated and endothelium-intact rings.

Table 1. Effect of peptides on ADP-induced human platelet aggregation. IC50 values for vasorelaxing effect are also presented for comparison.

	IC50 of inhibition of ADP-induced human platelet aggregation	IC50 of vasodilator effect
Peptide sequence	($\times 10^{-5}$ M)	($\times 10^{-5}$ M)
RGDV	8·7 ± 1·5	11·3 ± 0·1*
GRGDS	11·6 ± 2·5	2·7 ± 0·5
GRGDS-[SE]	11·9 ± 3·8	< 100

*Significant difference from the corresponding value of GRGDS ($P < 0.05$). GRGDS: Gly-Arg-Gly-Asp-Ser-OH; RGDV: Arg-Gly-Asp-Val-OH; GRGDS-[SE]: Gly-Arg-Gly-Asp-Ser(SO_3)-OH.

IC50 values of three RGD congeners on ADP-induced human platelet aggregation were in the same 10^{-5} range. However IC50 values of vasodilator effects differed considerably. Moreover, the rank order of platelet inhibitory effect was also different from that obtained on endothelium-intact rabbit aorta. Results are shown in Table 1.

Discussion

The most important finding of the present study is that two of the investigated RGD peptides, GRGDS and RGDV, could act as powerful endothelium-dependent vasodilators. RGDS showed a biphasic action and the relaxation phase appeared partially endothelium dependent while GRGDS-[SE] had no vascular activity.

These results are in agreement with a very recent finding of Muller et al (1997) who used endothelium-intact pig coronary arterioles. In their experiments, RGD peptides, and also an intraluminally administered β_3 integrin function-blocking antibody (F11), successfully inhibited the shear-stress-induced vascular relaxation which is known to be completely endothelium dependent (Kuo et al 1990). Very interestingly, Muller's group also found that pronectin F, which is a large, synthetic 13 RGD-copy containing molecule, inhibited the spontaneous tone of arterioles. They suggested the existence of an endothelial RGD-receptorial site which could mediate the release of endothelial relaxing factor(s). Moreover, Mogford et al (1996) demonstrated that at least three of the RGD congeners with different receptor selectivity could induce vascular dilatation in denuded rat cremaster muscle arterioles. In their study, a difference was found between the response of denuded and that of endothelium-intact vessels, but it was not significant statistically. They were able to inhibit the peptide-induced vasorelaxation using monoclonal antibodies against $\alpha_v\beta_3$ integrin, suggesting that the vasoactive effect of their peptides was mediated mainly via this type of receptor located on arteriolar smooth muscle cells.

On the basis of our own results, we suggest the presence of RGD-recognizing receptors both on the endothelial and the smooth muscle cells. It is of interest that the sulphate ester congener of GRGDS was not effective, providing further evidence for the existence of a specific receptorial site.

Vasorelaxation by RGDV appeared to be mediated via the nitric oxide pathway, because L-N^G-nitro-arginine completely blocked the nitric-oxide mediated components of endothelium-dependent relaxations. It was not the case with GRGDS suggesting the involvement of other endothelial-dependent mechanism(s) too, such as the release of endothelium-derived hyperpolarizing factor (EDHF). Endothelial α_v integrins, recognizing the RGD sequence of fibronectin, could induce elevation of intracellular Ca^{2+} levels (Schwartz & Denninghoff 1994). This change of ionic milieu inside a vascular endothelial cell could trigger the release of nitric oxide (Busse et al 1991) or EDHF, or both (Chen & Suzuki 1990). The effector role of prostaglandins can be excluded because prostaglandins play negligible roles in the regulation of rabbit aortic tone (Förstermann et al 1984).

It is well known that most of the RGD oligopeptides are platelet-aggregation inhibitors (Alig et al 1992) including the peptides investigated in the current experiments (Torday et al 1994). Comparing the order of potencies regarding endothelium-dependent relaxing effects and platelet aggregation inhibitory activities, we could not find any association. This means that the relaxing effect of our RGD peptides may not be mediated via a platelet-type RGD receptor, such as gpIIb/IIIa. In conclusion, the results of the present investigation provides pharmacological evidence to suggest that an RGD-recognizing endothelial receptor exists in rabbit aorta which mediates its vasorelaxant action via a nitric-oxide signalling pathway.

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