RGDN Peptide Interaction with Endothelial $\alpha_5\beta_1$ Integrin Causes Sustained Endothelin-dependent Vasoconstriction of Rat Skeletal Muscle Arterioles

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Abstract

The ability of an integrin-binding Arg-Gly-Asp-Asn (RGDN)-containing peptide to influence vascular tone by interacting with the $\alpha_5\beta_1$ integrin was studied using rat skeletal muscle arterioles. After blockade of β_3 integrin function, isolated arterioles with spontaneous tone showed concentration-dependent vasoconstrictions to topical application of GRGDNP, a peptide that shows a greater ability to interact with $\alpha_5\beta_1$ than with $\alpha_v\beta_3$. The constriction to GRGDNP (2.1 mM) was inhibited by blocking α_5 integrin function, and was intensified by blocking β_3 integrin function. In contrast, GRGDSP, a peptide that interacts better with $\alpha_v\beta_3$, was unable to induce sustained constrictions. Removal of the endothelium abolished the vasoconstriction in response to GRGDNP, suggesting that the response was due to release of an endothelium-dependent factor. Indeed, blockade of ET_A endothelin receptors with BQ-610 (1 μM), similar to removal of the endothelium and α_5 integrin blockade, inhibited the vasoconstriction. These data indicate that interaction of RGD peptides, and in particular the RGDN sequence with endothelial cell $\alpha_5\beta_1$, causes endothelin-mediated arteriolar vasoconstriction. These results indicate that integrins are novel signaling receptors within the vascular wall that affect vasomotor tone, and may play an important role in vascular control. (*J. Clin. Invest.* 1997. 100:1647–1653.) Key words: vasoconstriction • arginine-glycine-aspartic acid (RGD) • $\alpha_v\beta_3$ integrin • vascular smooth muscle • microcirculation

Introduction

Integrins are a family of heterodimeric transmembrane glycoproteins composed of α and β subunits (1, 2). At this time, there are 15 α and 8 β subunits described that combine to form over 20 heterodimers (1–3). A majority of the integrins mediate cellular attachment to extracellular matrix (ECM)[1] components including collagens, laminin, and fibronectin, with each heterodimer displaying unique binding properties (1–3). In addition to mediating cell attachment, integrin-dependent cell binding to ECM components has been shown to initiate a number of cellular responses, including changes in gene expression, intracellular ion concentrations, and the activation/generation of traditional second messengers such as tyrosine kinases, phospholipase C, and protein kinase C (4, 5). Although the majority of studies have focused on the ability of insoluble ECM to activate signaling pathways through integrins, it is evident that soluble integrin ligands also have this ability (6, 7).

A significant advance in the understanding of integrin function was the identification of the minimal integrin-binding sequences within various ligands. The first such sequence discovered was the arg-gly-asp (RGD) sequence found in fibronectin (8). RGD has since been found in both ECM (e.g., collagen, osteopontin, and vitronectin) and non-ECM proteins (e.g., disintegrins) (9–12), and is recognized by several integrins, including $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ (13–17). Synthetic peptides containing the RGD sequence have primarily been used to investigate the RGD dependence of cell adhesion to RGD-containing proteins. The integrin affinity for these peptides is influenced in part by the amino acid at the RGDX position (18, 19). For example, cell adhesion studies have shown that the synthetic RGD peptides GRGDNP and GRGDSP target both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (18). GRGDNP, however, appears to interfere with $\alpha_5\beta_1$-dependent functions more effectively than $\alpha_v\beta_3$-dependent functions when compared with GRGDSP (18). Thus, RGD-containing peptides in which the surrounding amino acids are substituted may provide useful tools for more selective investigation of the functional role of different integrins.

We recently demonstrated that soluble synthetic RGD peptides cause vasodilation of isolated rat skeletal muscle arterioles by interacting with the vascular smooth muscle (VSMC) integrin $\alpha_v\beta_3$ (20). On the basis of these studies, we proposed that other VSMC or endothelial cell (EC) integrins capable of interacting with RGD could also be involved in vascular control. A likely candidate integrin is $\alpha_5\beta_1$, the classic fibronectin receptor (14). $\alpha_5\beta_1$-dependent cell adhesion to fibronectin is associated with various cellular responses including migration, cytokine production, alterations in gene expression, and growth responses (21–24). The goal of these studies was to investigate the hypothesis that ligand interaction with $\alpha_5\beta_1$ integrin can alter vascular tone. To test this hypothesis, the differential binding properties of RGDN vs. RGD peptides for $\alpha_5\beta_1$ and function-blocking antibodies were used as tools to investigate a role for $\alpha_5\beta_1$.

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Received for publication 18 December 1996 and accepted in revised form 17 June 1997.

J. Clin. Invest.

0021-9738/97/09/1647/07 $2.00
Volume 100, Number 6, September 1997, 1647–1653
http://www.jci.org

1. *Abbreviations used in this paper:* ECM, extracellular matrix; RGD, arginine-glycine-aspartic acid; VSMC, vascular smooth muscle cell; EC, endothelial cell.

Methods

Vessel preparation. The preparation of isolated arterioles for study has been previously described in detail (20). In brief, male Sprague-Dawley rats (180–300 g) were anesthetized with pentobarbital sodium (100 mg/kg i.p.). The right cremaster muscle was excised and pinned flat in a refrigerated chamber containing cold (4°C) physiological saline solution (PSS; in mM): 145 NaCl, 4.7 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.2 NaH_2PO_4, 5 dextrose, 3 MOPS (3-[*N*-Morpholino]-propanesulfonic acid) buffer, 2 pyruvate, 0.02 EDTA, 0.15 albumin; pH 7.4±0.1. A segment of first-order (1A) arteriole (150–211 μm passive internal diameter) was surgically isolated and transferred to a chamber with a transparent glass bottom that fits into a microscope stage plate. The chamber was filled with PSS without albumin. The proximal end of the vessel was cannulated and tied to an open-ended, heat-polished glass pipette (80–100 μm tip diameter) filled with PSS with albumin. After flushing out red blood cells by applying slight positive pressure through the pipette, the distal end of the vessel was cannulated with a closed-end pipette and the vessel was set to in situ length. The cannulated vessel was then placed on an inverted microscope (Axiovert 100 TV; Carl Zeiss Inc., Germany) for observation. The vessel was checked for leaks, warmed to 34.5±0.5°C, and superfused with PSS (290–310 mosm/liter). After a 1-h equilibration period, intraluminal pressure was increased to the vessel's in vivo value of 90 cm H_2O (∼ 68 mmHg). Only vessels that developed spontaneous tone, resulting in a decrease in internal diameter during the equilibration period, were studied. Internal diameter was recorded by closed-circuit video microscopy system with an online calibrated video caliper (25). At the completion of each experiment, vessel viability was verified by addition of the α_1 adrenergic receptor agonist phenylephrine (1 μM), and passive diameter was determined by exchanging the vessel bath with Ca^{++}-free PBS containing adenosine (1 mM), an endothelium-independent vasodilator. All animal handling procedures followed institutional guidelines. Average internal diameter of all vessels studied (n = 49) after development of spontaneous tone was 104±2 μm (61±1% of passive diameter). Average passive diameter was 171±2 μm.

Peptide addition. GRGDNP and GRGDSP (Gibco BRL, Gaithersburg, MD) were solubilized in fresh PSS. Cumulative doses (0.21 μM–2.1 mM) of the peptides were carefully added to the vessel bath (abluminal addition) to determine the concentration-dependent responses of the arterioles to the peptides. Maximal diameter changes and minute interval diameter values were recorded and quantified as a percentage of arteriolar diameter with spontaneous tone at 90 cm H_2O, denoted as % control.

Deendothelialization of isolated arterioles. Removal of a functional endothelium was required to determine the role of the endothelium in the RGD-induced responses. Deendothelialization was accomplished as previously described by passing a rough-edged glass pipette through the vessel lumen several times before cannulation and pressurization (26). The absence of a functional endothelium was confirmed by a lack of response to the endothelium-dependent vasodilator acetylcholine (1 mM).

Integrin function blockade. Isolated arterioles were pretreated for 15 min with function-blocking monoclonal antibodies directed against either β_3 integrin (clone F11; 100 μg), α_5 integrin (clone Hmα5-1; 200 μg) or α_1 integrin (clone Ha31/8; 200 μg). All three antibodies were purchased from PharMingen, San Diego, CA. GRGDNP was then added to the vessel bath to assess the effects of the antibodies.

Endothelin receptor blockade. The involvement of endothelin in the RGD-induced vasoconstriction was determined by pretreating the arterioles for 30 min with 1 μM of the ET_A endothelin receptor antagonist BQ-610 (Peninsula Laboratories, Inc., Belmont, CA). The ET_A receptor is reported to be the predominant endothelin receptor expressed by arterial vascular smooth muscle in humans and rats (27, 28). The vessels were then treated with GRGDNP (2.1 mM), followed by endothelin-1 (10 nM), to test the effectiveness of the ET_A blockade. The α_1 adrenergic agonist phenylephrine (1 μM) was added to demonstrate the ability of the vessel to respond to another receptor-mediated contractile agonist. The vessels were then washed extensively to remove the BQ-610, and treatment with GRGDNP was repeated.

Luminal application of fibronectin. Fibronectin, a known ligand for $\alpha_5\beta_1$ integrin, is normally found as an insoluble component of the ECM and as a soluble plasma protein. Determination of the effect of soluble fibronectin on spontaneous tone and on the GRGDNP-induced vasoconstriction was made by introducing rat plasma fibronectin (1 μM; Gibco BRL) into the arteriolar lumen. After a 25-min incubation period, the level of spontaneous tone was assessed followed by treatment with 2.1 mM GRGDNP.

Data analysis. All data are expressed as mean±SEM. Analysis of time-dependent responses was performed by repeated measures ANOVA combined with Fischer's Least Squares Difference when appropriate. In all analyses, $P < 0.05$ represents the level of significance.

Results

RGD peptides induce vasoconstriction after β_3 integrin function blockade. In the isolated arteriole preparation, GRGDNP, GRGDSP, and a cyclic RGD peptide (GPenGRGDSPCA where Pen = penicillamine; Gibco BRL) induce concentration-dependent arteriolar dilation with the cyclic peptide showing greater potency consistent with its predominant $\alpha_v\beta_3$-binding properties (20). The purpose of these experiments was to determine if the role of $\alpha_5\beta_1$ would be made evident after blockade of β_3 integrin function. This purpose was accomplished by pretreating arterioles with the β_3 function–blocking monoclonal antibody F11 (100 μg; 50 μg/ml) before determining the concentration-dependent responses to either GRGDNP and GRGDSP. Arterioles pretreated with F11 showed concentration-dependent vasoconstriction to GRGDNP (Fig. 1) and GRGDSP (data not shown) from 70 μM and 700 μM.

In the absence of β_3 blockade, sustained vasoconstriction (30±4% of control diameter) could be induced by treating arterioles with GRGDNP in excess of 700 μM (e.g., 2.1 mM) (Fig. 2). The average starting diameter before addition of the peptide was 105±5 μm whereas after peptide addition average diameter decreased over the first 2 min to 39±2 μm. After 2 min, the arterioles relaxed slightly but continued to maintain an ∼ 69% constriction (72±5 μm) throughout the observation period. In several experiments (n = 5), the constrictions were monitored for 25 min, and were found to persist.

Compared to GRGDNP, addition of 2.1 mM GRGDSP in the absence of β_3 integrin blockade produced an initial transient vasoconstriction that was not maintained (Fig. 2). After the initial vasoconstriction, arterioles rapidly dilated to 148±2% of control diameter, and maintained a dilated state. At the same concentration, the inactive control peptide GRGESP had no vasoactive effects (data not shown).

We postulated that the partial recovery after the initial vasoconstriction in response to GRGDNP was the result of peptide interaction with VSMC $\alpha_v\beta_3$ integrin. To examine this possibility, arterioles were pretreated with the β_3 function blocking antibody as described above. After β_3 blockade, the vasoconstrictor response to GRGDNP was intensified, and the partial recovery after the initial vasoconstriction was eliminated (Fig. 2). Average diameter for F11-treated arterioles over the 10-min period was 42±1.3 μm (42±1% of starting diameter).

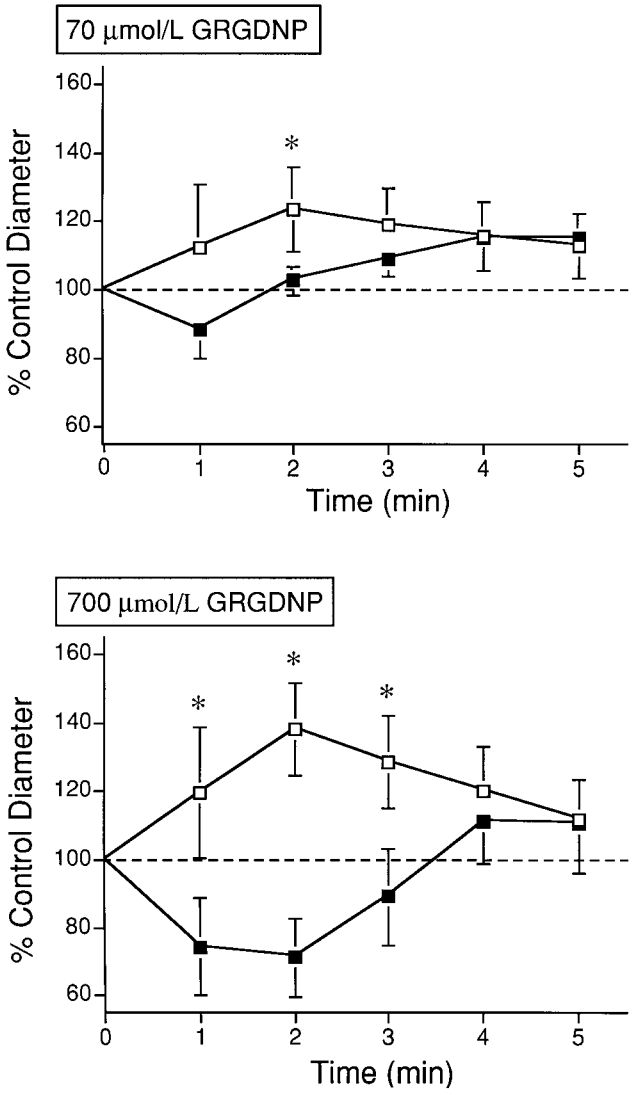

Figure 1. Arteriolar responses to 70 and 700 μM GRGDNP with (*open squares*, β_3 function intact, $n = 5$), or without (*closed squares*, β_3 function blocked, $n = 8$) β_3 integrin function blockade. Pretreatment of isolated arterioles with the β_3 integrin function–blocking monoclonal antibody F11 (100 μg) for 15 min unveiled a vasoconstrictor response to the higher concentrations of GRGDNP. *Significant difference between groups at a particular time point ($P < 0.05$) determined by multiple comparison ANOVA. Data are represented as mean±SEM.

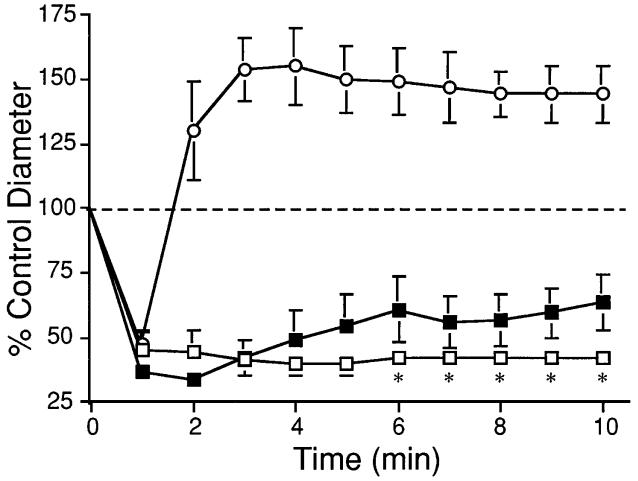

Figure 2. Arteriolar responses to 2.1 mM GRGDNP. In response to 2.1 mM GRGDNP, arterioles constricted maximally to 30±4% of control diameter (31±4 μm). The 10-min time course demonstrates maintenance of the vasoconstrictor response to GRGDNP. Blockade of β_3 function with the monoclonal antibody F11 enhanced the ability of the arterioles to maintain the constriction, while 2.1 mM GRGDSP did not induce a maintained constrictor response. *Significant difference between GRGDNP and GRGDNP + F11 at a particular time point ($P < 0.05$) determined by multiple comparison ANOVA. Data are represented as mean±SEM. *Closed squares*, GRGDNP ($n = 12$); *open squares*, GRGDNP + β_3 function blockade ($n = 4$); *open circles*, GRGDSP ($n = 3$).

$\alpha_5\beta_1$ integrin function blockade inhibits the maintained vasoconstriction in response to GRGDNP. To address whether the vasoconstriction involved the $\alpha_5\beta_1$ integrin, arterioles were pretreated with an α_5 integrin function–blocking monoclonal antibody (clone HMα5-1; 200 μg). Addition of 2.1 mM GRGDNP after α_5 integrin blockade resulted in an initial transient constriction followed by rapid dilation (within 30 sec) to 143±2% of the starting diameter, corresponding to 134±6 μm or 83±1% of passive diameter achieved with 1 mM adenosine in Ca^{++}-free PBS (Fig. 3). Thus, after α_5 blockade the response appeared similar to the arteriolar response to GRGDSP. Arteriolar vasoconstriction to 2.1 mM GRGDNP was not altered significantly by pretreatment with the IgG and integrin-binding control anti-α_1 function-blocking antibody (clone Ha31/8; 200 μg; Fig. 3). Control arteriolar diameter was not affected by addition of either the anti-α_5 or anti-α_1 antibody.

Removal of the endothelium inhibits the maintained vasoconstriction in response to 2.1 mM GRGDNP. To determine if the vasoconstrictor response was being mediated by peptide interaction with VSMC or EC, the ECs were mechanically removed from arterioles before addition of 2.1 mM GRGDNP. Successful removal of the endothelium was confirmed by lack of dilation in response to acetylcholine (1 mM), an endothelium-dependent vasodilator. In the absence of a functional endothelium, addition of GRGDNP produced an initial transient vasoconstriction, but vessels rapidly dilated to an average of 111±3% control diameter (132±3 μm or 82±1% of passive diameter) (Fig. 4), similar to the response observed after α_5 blockade. These data suggest that the sustained constrictor response was mediated by peptide interaction with $\alpha_5\beta_1$ expressed by EC. Immunocytochemical labeling of isolated arterioles confirmed the presence of the α_5 subunit on arteriolar EC (data not shown).

Inhibition of the maintained vasoconstriction by blockade of ET_A endothelin receptors. To investigate whether the RGD peptide–induced vasoconstrictions were due to endothelial release of the potent vasoconstrictor endothelin (29–31), isolated arterioles with a functional endothelium were incubated for 30 min with BQ-610 (1 μM). BQ-610 is a highly selective blocker of the endothelin-A (ET_A) receptor (32), the primary endothelin receptor expressed by VSMCs in humans and rats (27, 28). Pretreatment with BQ-610 blocked the maintained constrictor

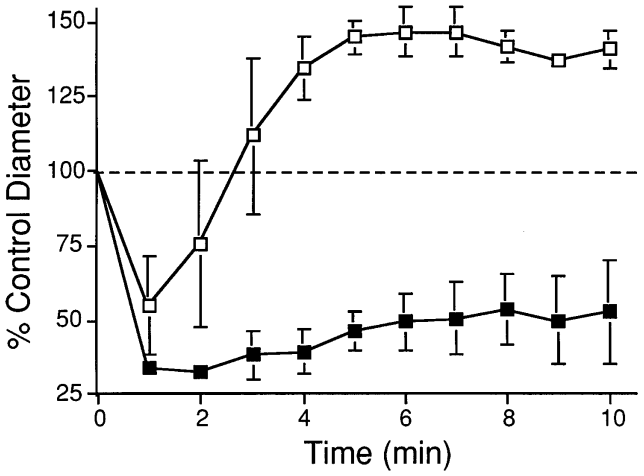

Figure 3. α_5 integrin function blockade effect on the arteriolar response to 2.1 mM GRGDNP. Pretreatment of arterioles with an α_5 integrin function blocking antibody (100 μg/ml; *open squares*, $n = 3$) prevented the maintenance of the 2.1-mM GRGDNP-induced vasoconstriction. Arterioles dilated to an average sustained maximal diameter 83±1% of passive diameter (143±2% control diameter) within 4–5 min. An IgG and integrin-binding control antibody directed against α_1 integrin subunit did not significantly alter responses to 2.1-mM GRGDNP when compared with untreated arterioles (shown in Fig. 2). Data are represented as mean±SEM. *Closed squares*, α_1 function blockade, $n = 3$.

response of the arterioles to 2.1 mM GRGDNP (Fig. 5). The effects of BQ-610 on the constrictor response to the peptide were similar to those observed after removal of the endothelium or blockade of α_5 function. Arterioles treated with BQ-610 did not respond to the abluminal application of endothelin-1 (10 nM), confirming the effectiveness of the blockade. BQ-610 also did not alter control arteriolar diameter nor did it affect the receptor-mediated response to the α_1 adrenergic agonist phenylephrine (1 μM). ET_A receptor blockade was reversible as the constrictor response to GRGDNP was fully restored after several bath exchanges to remove BQ-610 (Fig. 5).

Presence of luminal fibronectin does not alter arteriolar responses to GRGDNP. $\alpha_5\beta_1$ integrin is reported to be expressed on the luminal as well as basolateral surfaces of endothelial cells (33, 34). The basolateral receptors are exposed to insoluble cellular fibronectin found in the basement membrane, while the luminal receptors are exposed to soluble plasma fibronectin. Rat plasma fibronectin (1 μM) was added to the arteriolar lumen to more accurately reproduce the in vivo integrin–ligand environment. The soluble rat fibronectin was added to the lumen of arterioles with tone. After a 25-min incubation with the luminal fibronectin, the arterioles showed no difference in basal or spontaneous tone, and no alteration in the response to 2.1 mM GRGDNP (Fig. 6). Intraluminal administration of 2.1 mM GRGDNP peptide did not induce the vasoconstrictor response, suggesting that $\alpha_5\beta_1$ integrin expressed on the luminal surface of the EC is not involved in mediating the vasoconstriction.

Discussion

The arginine-glycine-aspartic acid tripeptide sequence, commonly known as RGD, is found in numerous proteins, including both insoluble components of extracellular matrices and soluble proteins such as the disintegrins found in snake venoms. While RGD is the minimal sequence within these proteins required for integrin binding, the specificity of the binding is

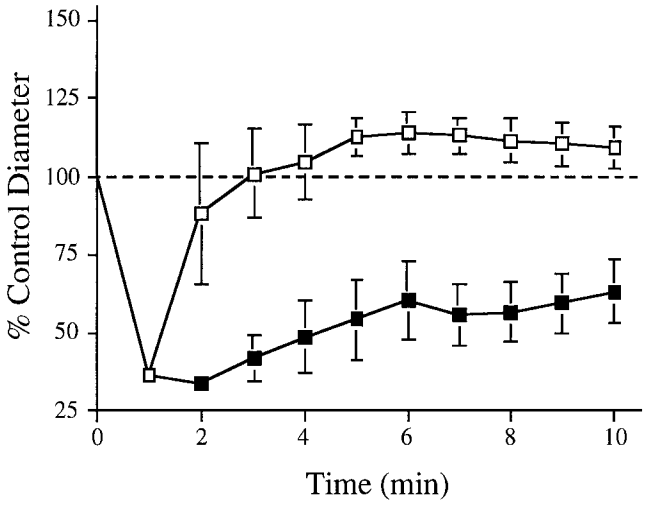

Figure 4. Effect of the removal of a functional endothelium on the arteriolar response to 2.1 mM GRGDNP. Arterioles were denuded of the endothelium as described in the Methods. Complete removal of a functional endothelium was verified by a lack of response to acetylcholine (1 mM), an endothelium-dependent vasodilator. Successfully denuded arterioles were unable to maintain the vasoconstriction induced by 2.1 mM GRGDNP dilating to 82±1% passive diameter (111±3% control diameter) by 4–5 min. Data are represented as mean±SEM. *Closed squares*, endothelium intact ($n = 12$), *open squares*, endothelium denuded ($n = 4$).

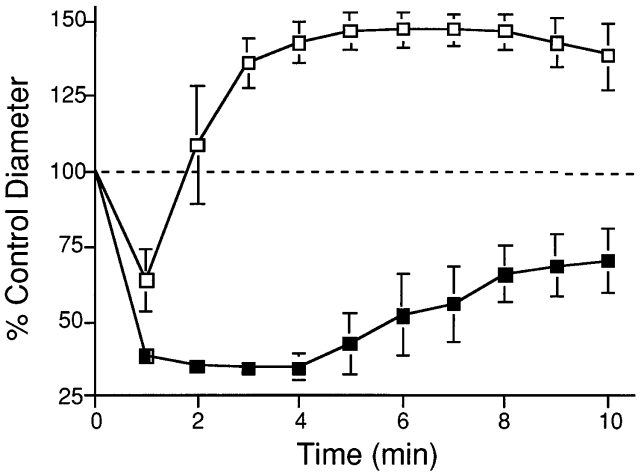

Figure 5. Effect of endothelin-A receptor blockade on the arteriolar response to 2.1 mM GRGDNP. Arterioles were pretreated for 30 min with BQ-610, a specific blocker of the endothelin-A receptor to determine the involvement of endothelin, a potent endothelial-derived vasoconstrictor, in the 2.1 mM GRGDNP-induced vasoconstriction. BQ-610 (1 μM) prevented maintenance of the vasoconstriction induced by 2.1 mM GRGDNP. *Open squares*, (+)BQ-610 ($n = 4$). The arterioles showed identical constrictor responses to the peptide as untreated vessels after removal of BQ-610 (*closed squares*, $n = 4$). Data are represented as mean±SEM.

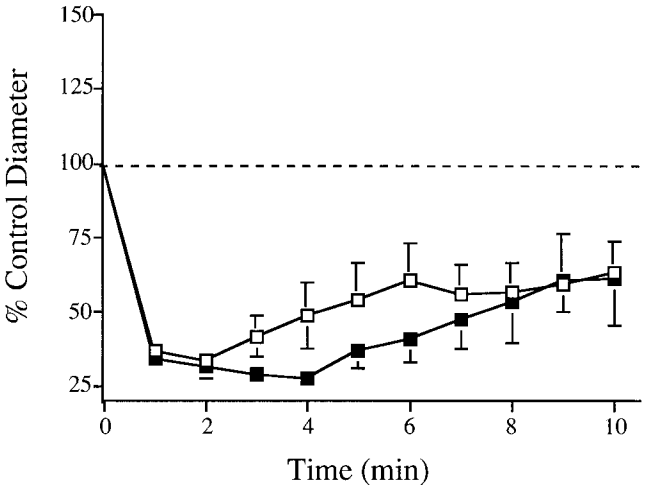

Figure 6. Effect of luminal application of soluble fibronectin and GRGDNP peptide. The effect of plasma fibronectin on the arteriolar response to GRGDNP was assessed by adding rat plasma fibronectin (1 μM) to the lumenal compartment of arterioles with spontaneous tone. Lumenal presence of fibronectin did not alter the level of spontaneous tone, nor did it affect the vasoconstrictor response to abluminal application of GRGDNP. Lumenal application of 2.1 mM GRGDNP alone did not induce any change in arteriolar diameter, illustrated by no deviation from control diameter. Data are represented as mean±SEM. *Open squares*, GRGDNP (n = 12); *closed squares*, GRGDNP + luminal Fn (n = 3).

greatly influenced by the conformation of RGD-containing peptide (18). For example, constraint of the RGD conformation by peptide cyclization produces a peptide that is essentially specific for blocking $\alpha_v\beta_3$-dependent adhesion to vitronectin, while having little effect on $\alpha_5\beta_1$-dependent binding to fibronectin (18). This same cyclic peptide was found to be a more potent vasodilator than the linear peptides GRGDSP and GRGDNP in an isolated rat skeletal muscle arteriole preparation, a response shown to be significantly blocked by a β_3 integrin function–blocking antibody (20).

In addition to the effects of conformation, the integrin affinity for the linear synthetic RGD peptides is significantly affected by the particular amino acid in the RGDX position (18, 19). Both GRGDNP (N, asparagine) and GRGDSP (S, serine) block cell adhesion to fibronectin and vitronectin, interpreted as demonstrating an ability of the peptides to interact with both the $\alpha_5\beta_1$ and $\alpha_v\beta_3$, respectively. More quantitative comparisons indicate that the presence of the asparagine at the RGDX position produces a peptide with increased ability to interact with $\alpha_5\beta_1$ over $\alpha_v\beta_3$, while serine in this position has the opposite effect (18). In this regard, our previous study has shown that GRGDSP was a slightly more potent vasodilator than GRGDNP, suggesting that it interacted more effectively with $\alpha_v\beta_3$ (20). Based on these observations, we reasoned that functional blockade of the β_3 integrin would enhance detection of any vasoactive responses due to interaction of GRGDSP or GRGDNP with $\alpha_5\beta_1$.

The results of this study are consistent with this reasoning, and support a vasoregulatory role for $\alpha_5\beta_1$. Our evidence for this is that β_3 blockade unveiled transient arteriolar constriction to higher concentrations (70–700 μM) of both GRGDNP and GRGDSP. Vasodilation of the arterioles to both peptides was blunted by pretreatment of the vessels with F11, further demonstrating a role for β_3 integrin in the vasodilatory response to the peptides. These data suggested that GRGDNP and GRGDSP were mediating vasoconstriction through an integrin other than $\alpha_v\beta_3$.

A higher concentration of GRGDNP was tested in an effort to produce a sustained vasoconstrictor response without the use of the β_3 function-blocking antibody. Addition of 2.1 mM of the peptide to the vessel bath consistently induced powerful and long-lived vasoconstrictions. The RGD-dependence of the response was confirmed by a lack of arteriolar response to the control peptide GRGESP (data not shown). Pretreatment of vessels with F11 before addition of 2.1 mM GRGDNP enhanced the vasoconstrictor response, suggesting that GRGDNP may continue to activate a vasodilatory pathway through $\alpha_v\beta_3$, but that the constrictor response predominates at this peptide concentration.

The higher concentration of GRGDNP required to induce a sustained vasoconstrictor response may reflect a higher affinity of small RGD peptides (< 1 kD) for the $\alpha_v\beta_3$ integrin, resulting in the predominance of the $\alpha_v\beta_3$-dependent vasodilations. This possibility is supported by the finding that the 120-kD fragment of fibronectin supports $\alpha_5\beta_1$ binding, while smaller fragments (< 5 kD) of the protein display a greater affinity for $\alpha_v\beta_3$ (35). Also, we have previously determined that GRGDNP and GRGDSP are more potent on a molar basis at blocking rat aortic VSMC binding to vitronectin than to fibronectin (unpublished observations). More potent $\alpha_5\beta_1$-specific RGD proteins may exist naturally, similar to certain disintegrins that are capable of blocking $\alpha_{IIb}\beta_3$-dependent platelet adhesion to fibrinogen in the nanomolar range compared to micromolar concentrations required for synthetic RGD peptides (36). In this regard, the RGDN sequence is found in the active site of disintegrins from the venom of at least 10 species of snakes from the Viperidae family (19), and these disintegrins were more potent antagonists of $\alpha_5\beta_1$-fibronectin binding compared to $\alpha_v\beta_3$-vitronectin binding. Other natural sources include matrix and plasma proteins containing the RGDN sequence in an insoluble or cryptic form that may become active upon injury-induced proteolysis (37). Alternatively, the higher concentration of GRGDNP may be necessary to overcome sequestration of the peptide, or diffusion barriers associated with reaching the EC, since the peptide was added abluminally in our study (38). It is interesting, however, that luminal application of GRGDNP did not cause vasoconstriction. Further studies will be required to resolve these issues.

A specific role for $\alpha_5\beta_1$ integrin in the constrictor response was further addressed by treating the vessel with an α_5 integrin function–blocking antibody before addition of GRGDNP. The α_5 subunit is known to only associate with the β_1 subunit, making this antibody specific for the $\alpha_5\beta_1$ heterodimer (1). Arterioles pretreated with this antibody were unable to maintain the peptide-induced constriction, dilating to a maximum of 143% of starting diameter within 5 min. A function-blocking antibody against the α_1 integrin subunit, chosen as an isotype and integrin-binding control for the α_5 antibody, had no effect on the arteriolar response to 2.1 mM GRGDNP. The α_1 subunit combines with β_1 to form the $\alpha_1\beta_1$ heterodimer reported to bind collagen and laminin (1). These results strongly indicate the involvement of $\alpha_5\beta_1$ integrin in the sustained GRGDNP-induced arteriolar constrictions.

We have previously shown that RGD-mediated vasodilation occurs through interaction of the peptides with $\alpha_v\beta_3$ integrin expressed by VSMCs (20). To determine whether the $\alpha_5\beta_1$-mediated effects were localized to VSMC or EC, we performed experiments in arterioles denuded of EC. Vessels successfully denuded of a functional endothelium were unable to maintain the constriction induced by GRGDNP, indicating that the maintained response results from the interaction of the RGD sequence with EC $\alpha_5\beta_1$ integrin, unlike the RGD-induced vasodilations that are mediated by VSMC $\alpha_v\beta_3$ integrin expressed by the VSMCs (20).

Endothelial cells are known to produce vasoconstrictive factors that can play a role in the local control of blood flow including endothelin-1 (31, 39). Because endothelin-1 is capable of producing long-lasting arterial constrictions in humans and rats (28, 31), we hypothesized the GRGDNP-induced vasoconstriction was due to release of endothelin-1 from the endothelium and subsequent interaction with the ET_A receptors expressed by VSMCs. The ET_A receptor is the predominant endothelin receptor expressed by VSMCs (27, 28), and shows selectivity for endothelin-1 (40). Pretreatment of isolated arterioles with BQ-610, a highly specific blocker of ET_A receptors (30), significantly inhibited the maintenance of the GRGDNP-induced vasoconstriction. Successful blockade of ET_A receptors was demonstrated by a lack of arteriolar constriction to ET-1 (10 nM), a concentration that produces strong, persistent constrictions of the isolated arterioles (data not shown). Other receptor-operated vasoconstrictors do not appear to be affected by BQ-610 since the arterioles responded to the α_1 adrenergic receptor agonist phenylephrine (1 μM) to a similar extent as untreated vessels. Removal of BQ-610 by extensive washing restored the constrictor response of the arterioles to 2.1 mM GRGDNP. Thus, the endothelium-dependent response to GRGDNP appears to result from $\alpha_5\beta_1$-mediated release of endothelin.

There are two possible mechanisms that could explain the effect of soluble RGD peptides on the behavior of adherent cells. First, the peptides may induce cellular responses by causing detachment of RGD-dependent integrin–ligand interactions, or second, the peptides may interact with free integrins not engaged by an ECM component. While it is not possible to distinguish between these two mechanisms, some insight may be provided by the observation that treatment of the arterioles with the α_5 integrin function–blocking antibody did not alter arteriolar tone. Function-blocking antibodies are thought to stabilize the nonfunctional, or unengaged, conformation of integrins (41), thus preventing ligand-induced conformation changes that may ultimately lead to cellular responses. That the antibody blocked the maintained constriction induced by the peptides without itself altering arteriolar tone argues that the response results from peptide interaction with free, unengaged receptors and not from detachment of engaged $\alpha_5\beta_1$ integrin. Further support for an agonist effect of the peptide is that the response occurs within 10 s, whereas in cultured cells detachment by RGD peptides requires on the order of minutes (42, 43). This argument implies that at least two populations of $\alpha_5\beta_1$ integrin can be expressed by a cell at the same time: one involved in binding of insoluble ECM fibronectin and a separate set of unengaged receptors. Evidence in support of this comes from a study showing that both high-affinity and low-affinity states of $\alpha_5\beta_1$ coexist on K562 erythroleukemic cells based on affinity for soluble fibronectin (44). The high-affinity receptors bind soluble ligand readily while the low-affinity receptors remain engaged with immobilized fibronectin, even in the presence of soluble ligand. Presumably, the two affinity states of $\alpha_5\beta_1$ represent different conformations of the receptor. Interestingly, cultured EC exposed to physiological levels of shear stress have been shown by immunocytochemical techniques possibly to express two populations of $\alpha_5\beta_1$ distinguished by associated focal adhesion proteins. One group of receptors was found primarily at the upstream portion of the cells, and colocalized with vinculin and the terminations of stress fibers, while another group colocalized with talin, and was found to be diffusely expressed along the length of the stress fibers on the EC basolateral surface.

$\alpha_5\beta_1$ integrin has also been reported to be expressed on the luminal surface of EC in situ (33, 34). Under normal conditions, these receptors would be exposed to circulating soluble fibronectin at a concentration of ~ 1 μM (45). Although binding between luminal $\alpha_5\beta_1$ and plasma fibronectin is reported to not occur (33, 34), it is possible that the presence of the soluble ligand could influence the peptide-induced constrictions. To examine this possibility, we added rat plasma fibronectin to the arteriole perfusion solution. The presence of the soluble luminal fibronectin did not alter the basal level of spontaneous tone, nor did it alter the arteriolar response to GRGDNP, suggesting that the luminal receptors are not involved in the peptide-induced constrictor response. To more directly address this possibility, the RGDN peptide was luminally introduced and was found to be ineffective at producing the vasoconstrictor response. Collectively, these observations suggest that the constrictor response we observed to RGDN is being mediated by abluminal EC $\alpha_5\beta_1$ integrin, and does not involve $\alpha_5\beta_1$ integrin expressed on the EC luminal surface.

In summary, we show that soluble RGDN peptide interacts with endothelial cell $\alpha_5\beta_1$ integrin in isolated rat skeletal muscle arterioles to cause sustained endothelin-dependent vasoconstriction. These responses are opposite to the RGD-induced vasodilation previously reported to result from peptide interaction with VSMC $\alpha_v\beta_3$ integrin. This study provides further support for the role of integrins as novel signaling receptors that can alter vascular tone, and thus may play an important role in vascular control.

Acknowledgments

Dr. Gerald A. Meininger is supported by National Institutes of Health grant HL33324 and Texas A&M University, FMG 96-41. Dr. George E. Davis is supported by the American Heart Association, TX affil. grant 94R025.

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