Integrin-binding peptides containing RGD produce coronary arteriolar dilation via cyclooxygenase activation

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Received 12 April 2001; accepted in final form 17 August 2001

Hein, Travis W., Steven H. Platts, Kelli R. Waitkus-Edwards, Lih Kuo, Shaker A. Mousa, and Gerald A. Meininger. Integrin-binding peptides containing RGD produce coronary arteriolar dilation via cyclooxygenase activation. Am J Physiol Heart Circ Physiol 281: H2378–H2384, 2001.—Integrin binding by Arg-Gly-Asp (RGD)-containing peptides has been shown to alter vascular tone in a variety of blood vessels and has been implicated as a mechanism of vasoregulation during tissue injury. However, the effect of these peptides in the coronary circulation has not been examined. Thus the purpose of our study was to test the hypothesis that integrins act as receptors linked to the regulation of coronary vasomotor function. In particular, the ability of RGD-containing peptides to influence vascular tone by interacting with the αβ3- and αβ1-integrins was studied in isolated pig coronary arterioles. All vessels developed basal tone and dilated in a concentration-dependent manner in isolated pig coronary arterioles. All vessels developed basal tone and dilated in a concentration-dependent manner in isolated pig coronary arterioles.

Intracellular signaling mediated by integrins in vascular smooth muscle has been well described, but surprisingly little known about the role of integrins in coronary vasoregulation. The present study demonstrates an important role for integrins in the regulation of coronary vascular tone and provides a mechanism for mediating the response to cyclooxygenase inhibitors. These results have important implications for the use of cyclooxygenase inhibitors as antithrombotic agents in the treatment of myocardial ischemia.

INTEGRINS ARE A LARGE FAMILY of heterodimeric transmembrane glycoproteins that play an important role in mediating attachment to the extracellular matrix (ECM) and in serving as receptors for signaling mole-

icles (11, 27). They are composed of one α-subunit and one β-subunit. A majority of integrins, including αβ3 and αβ1, recognize a conserved amino acid sequence, Arg-Gly-Asp (RGD) (27). This tripeptide sequence is found in a variety of ECM molecules, including fibronectin and collagen (3, 24). Interestingly, in vitro studies have shown in isolated vessels from a variety of vascular beds that synthetic RGD peptides (20–22, 28, 31, 34) and fragments of fibronectin (17) and type I collagen (22) alter vascular tone. However, there appears to be a heterogeneous distribution of vascular responses to RGD-containing peptides. For example, Mogford et al. recently demonstrated that interaction of synthetic RGD peptides with the smooth muscle αβ3 (22) and endothelial αβ1-integrins (21) mediates vasodilation and vasoconstriction, respectively, in the rat skeletal muscle microvessels. In contrast, Yip and Marsh (34) showed that an RGD peptide produced smooth muscle-mediated constriction of isolated rat kidney afferent arterioles. Furthermore, some studies report an endothelium-dependent vasodilation to RGD peptides (17, 20, 31), whereas others show that smooth muscle-mediated vasodilation predominates (22, 28).

REGARDLESS OF THE DISPARATE RESULTS, these findings suggest that it is plausible to postulate a role for integrins in contributing to the local control of blood flow and regulation of coronary vascular tone.

It is likely that an in vivo source of soluble RGD sequences is fragments of ECM proteins such as collagen that are frequently generated following tissue injury (9). Type I collagen is a likely candidate because it is the major ECM protein in cardiac tissue (2) and it contains seven RGD sequences per molecule (1). However, it remains to be determined whether integrin-mediated vascular responses to such soluble RGD peptides will occur in the coronary circulation where collagen has been shown to be degraded in response to myocardial infarction (7) and cardiomyopathy (23). Because the control of blood flow within a vascular bed is determined predominantly by the resistance vessels, we deemed it important to understand the ability of
RGD-binding integrins to influence vascular tone in coronary resistance arterioles. Specifically, we tested the hypothesis that integrin-binding, RGD-containing peptides affect coronary microvascular tone by interacting with the αβ3- and αβ1-integrins. To test this hypothesis, the effects of synthetic cyclic GPenGRGDSPCA (cyclic RGD), highly specific and novel αβ3- and αβ1-binding peptides (29, 32), and collagen fragments on isolated and pressurized coronary resistance vessels were examined. Studies were also undertaken to determine the involvement of endothelial versus smooth muscle integrins.

MATERIALS AND METHODS

Isolated vessel preparation. The techniques for identification and isolation of pig coronary microvessels were described previously (14). Pigs (8–12 wk old of either sex) were anesthetized with pentobarbital sodium (20 mg/kg). After a left thoracotomy, the heart was electrically fibrillated, excised, and placed in cold (5°C) saline solution. The procedures followed were in accordance with guidelines set by the Laboratory Animal Care Committee at Texas A&M University. Each arteriole (60–130 μm in internal diameter in situ) was dissected from the subepicardium and then cannulated with physiological salt solution (PSS)-filled micropipettes in a Lucite vessel chamber. After cannulation of arterioles, the chamber was transferred to the stage of an inverted microscope (model IM35, Zeiss). Arterioles were bathed in PSS at 36.5°C and pressurized without flow at 60 cmH2O by a reservoir system to allow development of basal tone. Internal diameter of an arteriole was measured using video microscopic techniques, as described previously (13).

Experimental protocols. After arterioles developed stable basal tone (~40 min) at 60 cmH2O, the concentration-diameter relationships for cyclic RGD (0.2 μM–0.2 mM; GIBCO-BRL), GRGESP (0.2 μM–0.2 mM; Life Technologies), an αβ3-cyclic-binding peptide (XJ735, 0.07 μM–0.2 mM; DuPont Pharmaceuticals), an αβ1-binding peptide (DMP7677, 0.07–70 μM; DuPont Pharmaceuticals), and protease-generated (neutrophil elastase) fragments of denatured collagen type I (0.2–4.0 μM) were established. Peptides were added at 3-min intervals to the vessel bath (abluminal addition) without causing fluctuations in temperature. Purified rat-tail collagen type I (Becton-Dickinson) was dialyzed into PSS, and collagen fragments were prepared as described previously (22); the concentration of fragments was determined out causing fluctuations in temperature. Purified rat-tail collagen type I (Becton-Dickinson) was dialyzed into PSS, and collagen fragments were prepared as described previously (22); the concentration of fragments was determined from the molecular weight of intact collagen. We have previously shown (22) that the dilation of skeletal muscle arterioles to collagen type I fragments can be inhibited after treatment with a β3-integrin function-blocking antibody (F11) and that these fragments competitively interfere with the effects of RGD in a cell binding assay. These findings strongly implicate an RGD-dependent mechanism for the action of collagen fragments.

The specificity of the involvement of αβ3- or αβ1-receptors in the vasomotor responses to the integrin-binding peptides was addressed by pretreatment for 15 min with function-blocking monoclonal antibodies directed against either β3-integrins (clone F11; 100 μg; PharMingen) or αβ1-integrins (clone HMα5–1; 50 μg; PharMingen). XJ735 and DMP7677 were then added to the vessel bath to assess the blocking effects of the antibodies.

The role of endothelium in these vascular responses was evaluated by comparing the response before and after endothelial removal. A nonionic detergent, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (0.4%), was perfused into the arteriole to remove endothelial cells, as reported previously (12). In another series of studies, the involvement of prostaglandins was examined by comparing the vascular responses before and after extraluminal incubation (30 min) of arterioles with the cyclooxygenase inhibitor indomethacin (10 μM). This treatment completely inhibited arachidonic acid-induced dilation in our previous studies (10, 13).

To assess whether the observed vascular responses of coronary arterioles to integrin peptides under luminal pressure also occur in the presence of flow, cyclic RGD-mediated vasomotor responses were examined in microvessels perfused with flow. Vascular response to increased flow was studied under constant luminal pressure using dual-reservoir techniques as demonstrated previously (15). In brief, the luminal flow was produced by simultaneously moving the pressure reservoirs in opposite directions of the same magnitude, generating a pressure gradient of 10 cmH2O (mean volumetric flow of ~7.5 nl/s) (15). Because we (10, 13) have previously demonstrated that flow induces endothelium-dependent nitric oxide (NO)-mediated dilation in pig coronary arterioles, the vascular response to cyclic RGD was studied in perfused vessels pretreated with the NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME, 10 μM, 30 min). The cyclic RGD-mediated vascular response was also examined in the L-NAME-pretreated vessels after incubation with indomethacin (10 μM, 30 min).

Cyclic RGD, GRGESP, and DMP7677 were dissolved in PSS. Indomethacin and XJ735 were dissolved in ethanol and 0.1 N HCl, respectively, as stock solutions (10 mM). Subsequent concentrations of indomethacin and XJ735 were diluted in PSS. The final concentrations of ethanol and HCl in the vessel bath were 0.1% and 0.01 N, respectively. Vehicle control studies indicated that these solvent concentrations did not affect arteriolar function.

Data analysis. At the end of each experiment, vessels with basal tone were relaxed with 100 μM sodium nitroprusside to obtain their maximal diameter at 60 cmH2O. All diameter changes in response to agonists were normalized to the vasodilation to 100 μM sodium nitroprusside and expressed as a percentage of maximal dilation. All data are presented as means ± SE. Statistical comparisons of vasomotor responses under various treatments were performed with ANOVA and tested with Fisher protected least significant difference test. Differences in resting diameter before and after pharmacological interventions were detected with Student’s paired t-tests. Significance was accepted at P < 0.05.

RESULTS

In this study, all isolated coronary arterioles (n = 47) developed a similar level of basal tone (constricted to 66 ± 1% of maximal diameter) at 36.5°C bath temperature with 60 cmH2O intraluminal pressure without flow. The average resting and maximal diameters of the vessels were 91 ± 3 and 137 ± 4 μm, respectively. The abluminal addition of cyclic RGD to the vessel bath caused concentration-dependent vasodilatation. Each concentration of cyclic RGD produced vasodilatation, which was developed within 3 min, and the highest concentration (0.7 mM) elicited 90% of maximal dilation (Fig. 1A). In contrast, GRGESP, used as a negative control, did not affect arteriolar diameter (Fig. 1A). We also examined the effect of a highly specific αβ3-cyclic-binding peptide (XJ735) on coronary arteriolar tone. XJ735 elicited concentration-dependent vasodilatation...
The vasodilations to the cyclic RGD and XJ735 were almost completely blocked by endothelial removal and by the cyclooxygenase inhibitor indomethacin (10 μM) (Fig. 1A and B). Endothelial denudation did not alter basal tone but did completely block vasodilation to endothelium-dependent vasodilator bradykinin (1 nM) (12). Although the efficacy of the indomethacin blockade on coronary arteriolar dilations was reduced to 18% by both treatments.

The ability of another RGD-binding integrin, α5β1, to alter coronary arteriolar tone was studied. A specific α5β1-binding peptide (DMP7677) caused concentration-dependent dilation of coronary arterioles (Fig. 1C). In contrast to cyclic RGD and XJ735, α5β1 ligation with DMP7677 following endothelial removal resulted in a concentration-dependent vasoconstriction (Fig. 1C), which indicates a vascular smooth muscle involvement. A comparable level of vasoconstriction to DMP7677 was observed in another set of vessels after incubation with indomethacin (Fig. 1C).

The specific involvement of the α5β3- and α5β1-integrins in mediating vasodilation to the integrin-binding peptides was evaluated by pretreatment with function-blocking monoclonal antibodies directed against either β3-integrins (F11) or α5-integrins (HMo5-1). The vasodilation to the α5β3-cyclic-binding peptide DMP7677 (21 μM) was significantly inhibited by F11 but not by HMo5-1 (Fig. 2). The vasodilation to the α5β1-binding peptide DMP7677 (7 μM) was abolished by HMo5-1, but was not altered by F11 (Fig. 2). The antibodies did not affect the vasodilation to sodium nitroprusside (data not shown) or the resting vessel diameter.

The influence of flow on integrin-mediated vasodilation was examined in isolated coronary arterioles in the presence of L-NAME (10 μM). In the absence of flow, coronary arterioles pressurized at 60 cmH2O dilated in a concentration-dependent manner to cyclic RGD. After washout of cyclic RGD, establishment of a pressure gradient of 10 cmH2O elicited dilation in control vessels (i.e., control diameter, 88 ± 6 μm vs. flow diameter, 115 ± 9 μm, Fig. 3A). L-NAME abolished the flow-induced vasodilation (flow + L-NAME diameter, 89 ± 7 μm, Fig. 3A). In the presence of both flow and L-NAME, coronary arteriolar dilation to cyclic RGD was maintained (Fig. 3B). However, as in the nonperfused vessels (Fig. 1A), the cyclic RGD-mediated dilation in the perfused vessels was inhibited by indomethacin (10 μM, Fig. 3B). Indomethacin did not alter the resting vessel diameter (88 ± 4 μm, Fig. 3A).
A putative in vivo source of soluble RGD sequences is fragments of ECM proteins that are frequently generated following tissue injury (8). In support of this idea, protease-generated (neutrophil elastase) fragments of denatured collagen type I (4 μM) produced a robust dilation of an isolated arteriole from the baseline diameter of 84 to 142 μm (Fig. 4A). The diameter gradually returned to the baseline level after the vessel bath was replaced with control solution (Fig. 4A). After removal of the endothelium, vasodilation to collagen fragments (4 μM) was reduced from 80 to 94 μm (Fig. 4B). The diameter returned to the baseline level following washout, and then sodium nitroprusside produced maximal dilation of the vessel from 82 to 146 μm (Fig. 4B). Further study showed that collagen fragments elicited concentration-dependent vasodilation of endothelium-intact vessels (Fig. 4C). In general, the vasodilation to each concentration of collagen fragments was developed within 3–4 min, and dilation to the highest concentration (4 μM) elicited 92% of maximal dilation (Fig. 4C). The vasodilatory response was significantly inhibited by endothelial removal and by incubation with indomethacin (Fig. 4C). Vasodilations to the highest concentration under these conditions were reduced to 22% and 35% of maximal dilation, respectively (Fig. 4C). It is important to note that concentration-dependent vasodilation to sodium nitroprusside (1 nM–10 μM) was not altered by either endothelial denudation or indomethacin (data not shown), which indicates that these treatments did not affect smooth muscle vasodilatory function.

**DISCUSSION**

The present study provides the first evidence that coronary microvessels dilate in the presence of RGD-containing peptides. The synthetic peptide cyclic RGD and novel α,β3 and α5β3-binding peptides XJ735 and DMP7677 elicit dilations of isolated coronary arterioles by interaction with integrin receptors on the endothelium. The endothelium-dependent vasodilations appear to be mediated by activation of the cyclooxygenase pathway because cyclooxygenase blockade inhibited the vasodilatory responses. In a similar manner, neutrophil elastase-generated collagen fragments produced endothelium-dependent, cyclooxygenase-mediated dilation of these microvessels.

The tripeptide RGD is a target amino acid recognition sequence for many integrins. Recent studies have shown that integrin-binding, RGD-containing peptides alter vasomotor tone in a variety of vascular preparations, including skeletal muscle (21, 22), mesentery (17), kidney (34), and aorta (17, 20, 28, 31). However,
the influence of these RGD-containing peptides on the coronary circulation has not been examined. In the present study, ligation of $\alpha_3\beta_3$ or $\alpha_5\beta_1$ produced dilation of isolated and pressurized porcine coronary arterioles. Because this vasodilatory response was almost completely blocked by endothelial removal, it is suggested that the vasodilation is mediated by activation of endothelial integrins. This finding is consistent with those reported in the rat aorta (20, 31). In contrast, Mogford et al. (22) previously demonstrated that the cyclic RGD peptide elicits endothelium-independent dilation of rat skeletal muscle microvessels. These disparate results suggest that the mechanisms of integrate-mediated control of vascular tone vary with the vascular tissue studied.

RGD-containing peptides have been shown to bind to various integrins, including $\alpha_3\beta_3$, $\alpha_5\beta_1$, $\alpha_3\beta_8$, and $\alpha_5\beta_1$ (27). Because the RGD-containing peptides used in previous studies are nonselective, the possibility that multiple integrins may be involved cannot be ruled out. To overcome this specificity problem, we utilized two novel compounds, XJ735 and DMP7677, which are selective for binding $\alpha_3\beta_3$ (29) and $\alpha_5\beta_1$ (32) receptors, respectively. Both of these compounds produced concentration-dependent dilation of coronary arterioles. Activation of the selective $\alpha_3\beta_3$- and $\alpha_5\beta_1$-receptors was confirmed by the ability of a $\beta_3$-function-blocking antibody to inhibit the vasodilation to XJ735 and an $\alpha_5$-function-blocking antibody to abolish the vasodilation to DMP7677. After removal of the endothelium, vasodilation to XJ735 was almost completely blocked, whereas the vasodilatory response to DMP7677 was reversed to concentration-dependent vasoconstriction. Taken together, these results suggest that activation of endothelial $\alpha_3\beta_3$- and $\alpha_5\beta_1$-receptors can elicit coronary arteriolar dilation. In addition, the $\alpha_5\beta_1$-receptor-dependent vasodilation appears to override a vasoconstrictor response to smooth muscle $\alpha_5\beta_1$-receptors. In smooth muscle cells from rat cremaster arterioles, electrophysiological evidence suggests that coupling between the $\alpha_5\beta_1$-integrin and L-type Ca$^{2+}$ channel results in enhanced Ca$^{2+}$ entry (33). This selective integrin-signaling mechanism is consistent with the vasoconstriction observed in the present study.

The activation of endothelial $\alpha_3\beta_3$- and $\alpha_5\beta_1$-receptors may stimulate the production and release of endothelium-derived vasodilators. Previous studies have shown that RGD-containing peptides evoke endothelium-dependent vasodilation via release of NO (17, 31) or of prostaglandins (17). In the present study, administration of indomethacin to the coronary arterioles, with a concentration (10 $\mu$M) sufficient to block prostaglandin synthesis in our previous studies (10, 13), inhibited the vasodilations to cyclic RGD, XJ735, and DMP7677 to the same extent as did endothelial removal. This result indicated that $\alpha_3\beta_3$- and $\alpha_5\beta_1$-induced vasodilations are mediated by the endothelial release of prostaglandins. Our conclusion is supported by evidence showing that fibrin interaction with the $\alpha_5\beta_1$-integrin on human umbilical vein endothelial cells increases release of a prostaglandin prostacyclin (5). However, it is important to note that the integrin-mediated vasodilatory response of pig coronary microvessels is different from that in rat skeletal muscle microvessels, which is induced by smooth muscle $\alpha_3\beta_3$-integrins (22) linked to potassium channels (25) and the L-type Ca$^{2+}$ channel (33). Despite the possible species or tissue differences, it appears that activation of the cyclooxygenase pathway in coronary arterioles is responsible for the endothelium-dependent component of vasodilation to the nonspecific cyclic RGD and specific $\alpha_3\beta_3$- and $\alpha_5\beta_1$-binding peptides.

A potential caveat of our findings is that the isolated microvessels were studied in the absence of flow, a
condition that would not occur in the heart in vivo. Thus we studied the coronary arteriolar response to cyclic RGD in the presence of flow. To study the RGD-mediated response in the presence of flow, we had to abolish the flow-mediated dilation in coronary arterioles to preserve vascular tone. This NO-dependent vasodilatory response was blocked by incubating the vessels with L-NAME, which is consistent with results reported in our previous studies (10, 13). In addition, these perfused vessels dilated in response to abuminal cyclic RGD in the same fashion as those in the nonperfused vessels, suggesting a physiological relevance of the observed vasomotor responses. Importantly, because the impaired coronary flow regulation observed in patients with heart disease has been suggested to be a result of NO deficiency (26, 35), the integrin-binding peptides (XJ735 and DMP7677) could be used clinically to ameliorate vascular function by providing an alternative form of coronary vasodilation through the release of prostanoglandins.

Physiological mediators for the activation of integrin receptors in the vasculature may be fragments of ECM proteins, such as collagen, that are frequently generated following tissue inflammation and injury (8). The binding of specific integrins to collagen is recognized to change following conversion of collagen from its native state to a denatured condition (8). This is proposed to result from exposure of cryptic RGD sequences (matrix- cryptic sites) (9). Subsequent proteolytic cleavage of the denatured collagen by enzymes of tissue and inflammatory cell origin may release soluble RGD-containing fragments or matricryptins (9). Indeed, neutrophil elastase-generated collagen fragments bind $\alpha_2\beta_3$-receptors to elicit dilation of rat skeletal muscle microvessels in an RGD-dependent fashion (22). In our present study, these collagen fragments dilated coronary arterioles in a concentration-dependent manner. Because this vasodilation was significantly inhibited by endothelial removal and by indomethacin, it is suggested that activation of endothelial integrins and subsequent release of prostanoglandins are involved in mediating this vasodilatory response. The inability of these treatments to completely block the collagen fragment-induced vasodilation, as well as the integrin peptide-induced vasodilations described above, indicates that smooth muscle integrins may also be involved in vasodilation to higher concentrations of these fragments/peptides. On the other hand, it is possible that the release of other endothelium-derived relaxing factors such as endothelium-derived hyperpolarizing factor is involved in the residual vasodiations. An involvement of NO is not supported by our data because the vasodilation at higher concentrations persisted in the L-NAME-pre treated perfused microvessels. Regardless of these interpretations, because collagen degradation is associated with a number of coronary complications such as atherogenesis (19), myocardial infarction (7), and cardiomyopathy (23), our current data indirectly support the hypothesis that fragments of ECM proteins may act as wound recognition signals and play a role in the vascular response to these pathologies. However, the rate and formation of such vasoactive matrix fragments remains to be determined.

It is important to note that type I collagen is not a major basement membrane component of blood vessels like type IV collagen (1). However, because type I collagen is the major ECM protein in the heart (2), it could be a major source of RGD for coronary microvessels. This is not to underestimate the involvement of basement membrane collagen type IV. However, in the present study, the application of collagen type I fragments or integrin-binding peptides to the vessel bath mimics the abluminal exposure of microvessels to these peptides derived from the interstitial space of the heart. Interestingly, we have estimated the concentration of collagen type I fragments in the interstitial space of the heart using data reported in the literature. Our calculations are as follows. The wet weight of pig hearts used in our studies was $\sim$100 g. The heart dry weight, which has been reported to be $\sim$20% of the wet weight, would be 20 g (16). Because the collagen content of the myocardium has been shown to be $\sim$4.0% of the heart dry weight (16), the pig heart would contain $\sim$0.8 g of total collagen. It is estimated that 0.7 g of collagen are type I collagen in the pig heart because type I collagen constitutes 85–90% of myocardial collagen (2). The fraction of collagen that undergoes extracellular degradation daily in the heart has been reported to be $\sim$2.5% (18). This would produce 18 mg of degraded collagen fragments in the interstitial space (interstitial fluid volume = 20 ml because interstitial fluid volume has been reported to be 20% of heart wet weight) (2, 6). Thus it is possible that 0.9 mg/ml of collagen type I fragments are present in the interstitial fluid under normal conditions. This level of collagen fragments is nearly equivalent to the highest concentration of collagen fragments used in our study (1 mg/ml = 4 \mu M), which could provide numerous RGD-binding sites because type I collagen contains 7 RGD sequences per molecule (1). Interestingly, the concentration of collagen fragments are probably even much higher under conditions of myocardial infarction, where the collagen content has been shown to be degraded by as much as 25–50% (4, 30). Thus the experimental design of our study allowed us to assess the effect of a physiological level (i.e., representative of myocardial interstitium) of collagen type I fragments on vasomotor function in isolated coronary arterioles. Future studies will investigate in vivo evidence for the influence of collagen type I degradation products on coronary microvascular tone.

In conclusion, the present study demonstrates that selective $\alpha_2\beta_3$- and $\alpha_5\beta_1$-peptides XJ735 and DMP7677 elicit dilations of isolated coronary microvessels. It appears that these vasodilatory responses are a result of the compounds binding endothelial $\alpha_2\beta_3$- and $\alpha_5\beta_1$- receptors and subsequent production of cyclooxygenase-derived prostanoglandins. RGD-containing peptide cyclic RGD and protease-generated collagen fragments also induce endothelium-dependent prostanoglandin-mediated dilations of these microvessels. Collectively,
these data support a role for endothelial integrins in the regulation of vascular tone in the coronary circulation.

G. A. Meininger is supported by National Heart, Lung, and Blood Institute (NHLBI) Grants HL-55050 and HL-58810. L. Kuo is supported by NHLBI Grants HL-55524, HL-48179, and HL-03693.

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