Basic fibroblast growth factor and heparin influence coronary arteriolar tone by causing endothelium-dependent dilation

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Abstract

Objective: The strong angiogenic and mitogenic agents acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) share signalling pathways with known vasodilatory agonists. Therefore, we hypothesized that FGF’s produce vasoactive responses. We also proposed that heparin would exert a similar action to FGF, because this proteoglycan not only binds to the FGF receptor, but also facilitates the release of FGF from the cardiac extracellular matrix and promotes its binding to a high-affinity receptor. To test these hypotheses, we examined the vasodilatory reactions of coronary arterioles to aFGF, bFGF, and heparin, and the effects of antagonists to NO synthase L-NMMA, prostaglandins indomethacin, ATP-sensitive potassium (K_{ATP}) channels glibenclamide, FGF and FGF receptors on the vasoactive responses. Methods: Arterioles (70–110 μm, internal diameter) were dissected from pig hearts and cannulated with micropipettes. Diameter was determined with videomicroscopy in response to bFGF and aFGF in concentrations of 1–100 ng/ml and to heparin (5–200 U/ml). Results: Basic FGF, but not aFGF, caused dose-dependent vasodilation with a maximum of 61 ± 4%. Relaxation to bFGF was antagonized by pretreatment with L-NMMA, but was not affected by pretreatment with indomethacin or glibenclamide. Heparin caused dose-dependent vasodilation with a maximum of 100 ± 3% which was partially blocked by either L-NMMA or glibenclamide, but not by indomethacin. Furthermore, the effect of bFGF could be significantly blocked by pretreatment with an FGF receptor antibody as well as with a monoclonal antibody against FGF. Pretreatment with both antibodies significantly inhibited also the effect of heparin. Conclusions: These results indicate that bFGF and heparin cause vasodilation of coronary arterioles via an increase in NO production and heparin additionally by other mechanisms such as by activating K_{ATP} channels. Furthermore, the effect of heparin is partially mediated via FGF and FGF receptors. We therefore speculate that both substances may be involved in the regulation of coronary microvascular tone acting partially through the same signalling mechanisms.

Keywords: Fibroblast growth factor; Heparin; Nitric oxide; Prostaglandins; Potassium channel, ATP-sensitive; L-NMMA; Endothelium; Vasodilation; Pig, coronary artery; Coronary vasculature

1. Introduction

The heparin-binding growth factors—basic and acidic fibroblast growth factor (bFGF and aFGF, respectively)—are potent angiogenic agents and promote growth of vascular smooth muscle cells and endothelial cells [1]. Intracoronary treatment with bFGF increased microvessel numbers in infarcted and non-infarcted myocardium in pigs [2]. Basic FGF reduced the size of acute myocardial infarction and increased the density of arterioles and capillaries in the infarcted area [3]. Chronic application of acidic FGF did not cause angiogenic responses in viable myocardium but induced smooth muscle cell hyperplasia in ischemic areas [4]. Intracoronary injection of bFGF enhanced collateral blood flow via its angiogenic effects in the canine myocardium [5]. Basic FGF also appears to protect vascular function in the setting of ischemia, because chronic administration increased endothelium-dependent relaxation of the collateral perfused coronary microcirculation [6]. Apart from angiogenic and mitogenic effects, there are recent reports about direct vascular effects of FGF. Basic FGF dilated rat pial arterioles [7] and systemic administration of
both aFGF and bFGF caused hypotension in rats [8]. Administration of bFGF to isolated coronary arterioles only produced modest (7%) dilation at doses above the physiological range [6]. Basic FGF has also been demonstrated to induce rat aortic contraction [9]. Little is known about direct vascular effects of aFGF.

The stimuli for the formation and release of FGFs are not known. FGFs are primarily stored in the endothelial basement membrane, extracellular matrix [10] and mast cells [11] and bind to heparin with an unusually high affinity [12]. Heparin refers to a group of negatively charged proteoglycans mainly produced by mast cells. These macromolecules are known to have a variety of biological effects such as inhibition of smooth muscle cell proliferation [13], prevention of thrombosis and induction of hypotension [14]. The role of heparin and heparin-related proteoglycans such as heparan sulfates for the availability and signal transduction of FGF seems to be complex. Heparin protects FGFs from proteolytic degradation [15], increases the release of FGF from the extracellular matrix [16] and mast cells [11], and binds to the FGF receptor itself [17]. Furthermore, binding of FGF to cell-surface heparin-like molecules seems to be crucial for an adequate binding of FGF to its high-affinity receptors to induce a biological response [18,19]. Additionally, heparin-like molecules bind FGFs in the extracellular matrix to store them [20] and act as transporters of FGFs [21]. Interestingly, the concentration of heparin in the endothelial cell is 100 times that in plasma [22] and heparin itself has angiogenic and vasoactive properties. Heparin treatment enhanced NO production in human endothelial cells [23], enhanced coronary collateral development in pigs after coronary artery occlusion [24], and lowered blood pressure in hypertensive patients [14] and rats [25].

Taken together, there is evidence in the literature indicating that FGF’s and heparin possess important roles in angiogenesis. There also is the suggestion that these factors are vasoactive, but there has not been conclusive evidence for this action in the coronary circulation. We, therefore, proposed the following hypotheses: (1) fibroblast growth factors produce vasoactive responses in coronary arterioles; (2) heparin exerts a similar action to FGF, because this negatively charged glycosaminoglycan facilitates both the release of FGF from the cardiac extracellular matrix and its binding to a high-affinity receptor; (3) acidic and basic FGF and heparin signal through the production of nitric oxide.

2. Methods

2.1. General preparation

After sedation (Telazol, 2 mg/kg) and anesthesia (sodium pentobarbital, 35 mg/kg i.v.) pigs were intubated, ventilated and a left thoracotomy was performed. Heparin (1000 U/kg) was administered (i.v.), the hearts electrically were fibrillated, excised and placed in 4°C buffered physiological saline solution (PSS).

2.2. In vitro measurement of coronary arteriolar diameters

After perfusion of the left anterior descending and circumflex coronary arteries with an India ink/gelatin mixture in physiological salt solution (PSS) [26] to facilitate microdissection, coronary arterioles (<100 µm in internal diameter) were carefully dissected from the myocardial tissue at 4°C and were transferred to a lucite vessel chamber containing PSS–albumin solution (containing per liter: 5 mM MOPS buffer, 0.168 g NaH₂PO₄, 0.901 g glucose, 0.22 g pyruvate, 0.0074 g EDTA(Na), 200 ml 2 mM Ca²⁺ Ringer, 10 g albumin; pH 7.4). Both ends of each arteriole were cannulated with a glass micropipette with an external tip diameter of approximately 40 µm and secured with 11-0 ophthalmic suture. The India ink/gelatin PSS solution was flushed out at low pressure (20 cmH₂O) and the other end of the microvessel was secured to a second micropipette. After the vessels were cannulated, the chamber was transferred to the stage of an inverted microscope (IM35, Carl Zeiss, Thornwood, NY; objective Zeiss 40×, numerical aperture 0.75) fitted with a Cohu TV camera (Model 4915, San Diego, CA) and video micrometer (Texas A and M Microcirculation Research Institute, College Station, TX). Arterioles were pressurized to 60 cmH₂O by adjusting the height of a reservoir connected to each micropipette. This pressure approximates the estimated intraluminal pressures for microvessels of this size in vivo [27]. By setting both reservoirs to the same height, the vessels were pressurized without flow. Leaks were detected by closing off the system to the reservoirs and examining for a decline in intraluminal pressure. Vessels with leaks were excluded from further study. Internal diameters were recorded continuously during experiments. The microvessels were set to their in situ length and were bathed in PSS–albumin solution with the temperature maintained at 36–37°C by an external circulating heat bath. Arterioles prepared in this manner developed spontaneous tone of 25–30% of maximal diameter. After equilibration of the vessels, vasodilatory responses to the endothelium-dependent vasodilator, serotonin (5HT; 10⁻⁵ M), and to the endothelium-independent vasodilator, sodium nitroprusside (10⁻⁵ M), were obtained to assess normal endothelial and smooth muscle function of the vessels. We elected to use 5HT because we have previously found that the dilation to 5HT is mediated by the production of NO, inasmuch as the response can be blocked by inhibitors of NO production [26].

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).
2.3. Drugs

The following drugs were used in this study: acidic and basic FGF from Life Technologies dissolved in sterile water and diluted in PSS (without albumin). Heparin from Elkins/Sinn; 5HT, nitroprusside, L-NMMA, glibenclamide and indomethacin from Sigma diluted in PSS (without albumin). Antibodies against FGF and its receptor were obtained from Upstate Biotechnology, purified via immunoprecipitation and used after dilution in PBS.

2.4. Experimental protocols

Before application of FGFs, heparin (5 U/ml) was added to the bath to prevent binding of FGF to the extracellular matrix. This dose of heparin did not affect vascular tone.

Three different doses of bFGF (1, 10 and 100 ng/ml), aFGF (1, 10 and 100 ng/ml) or heparin (5, 10, 25, 50, 100 and 200 U/ml) were administered extraluminally (in the organ chamber) under control, baseline conditions and after pretreatment with indomethacin (blockade of prostaglandin synthesis, 10^{-5} M), L-NMMA (blockade of NO synthesis, 10^{-4} M) or glibenclamide (blockade of ATP-sensitive potassium channels, 10^{-6} M). Of these 3 antagonists only L-NMMA caused a reduction in the baseline diameter.

Additionally, responses to the doses of bFGF, aFGF and heparin were obtained under baseline conditions and after pretreatment with a monoclonal anti-FGF antibody (10 mg/ml) and after pretreatment with an FGF-receptor antibody (10 mg/ml).

In all experiments, vascular responses to 5HT and nitroprusside (both 10^{-5} M) were obtained under baseline conditions and after pretreatment with the different inhibitors. Although we did not establish the effectiveness of the specific blockade to indomethacin and glibenclamide with challenges by arachidonic acid or cromokalim, respectively, it was previously established that the doses of these antagonists that we employed produce efficacious blockade of the desired enzyme or channel in isolated coronary arterioles [26,28]. Moreover, we believed the blockades to be specific because the responses to serotonin and nitroprusside were not affected by these two antagonists.

2.5. Data analysis

Responses to the various agonists were assessed as percent dilation over baseline using the calculation:

\[(\text{Diameter after agonist} - \text{baseline diameter}) / (\text{maximal diameter} - \text{baseline diameter}) \times 100.\]

Baseline diameter was defined as that prior to administration of the vasodilatory agonist (e.g., that occurring with spontaneous tone or that during L-NMMA). The percent dilation values were compared by two-way repeated measures ANOVA. All statistics were computed using Statview 4.1 on a Macintosh 8100 computer. A probability level of 5% was used in all studies as the criterion of statistical significance.

3. Results

3.1. Vessel diameter

Arterioles investigated in this study had a maximal diameter of 89 ± 5.1 μm and after developing spontaneous tone 65.5 ± 3.8 μm. Neither pretreatment with indomethacin (10^{-5} M), glibenclamide (10^{-5} M), the monoclonal anti-FGF antibody (10 mg/ml) or the FGF receptor antibody (10 mg/ml) did significantly affect basal vascular tone. L-NMMA (10^{-4} M) caused in all vessel preparations a significant vasoconstriction of about 15%. Vascular relaxation to both 5HT and nitroprusside was between 90 and 100% in control experiments as well as after pretreatment with indomethacin, glibenclamide, the FGF receptor antibody or the monoclonal anti-FGF antibody. The response to 5HT, but not that to nitroprusside, was significantly reduced after pretreatment with L-NMMA (7 ± 6%), indicating significant inhibition of NO synthesis.

3.2. Effects of bFGF (Fig. 1)

Under baseline conditions (n = 14), basic FGF caused dose-dependent dilation with a maximal response of 61 ± 4% at 100 ng/ml (P < 0.001). The vasodilating effect of basic FGF (1, 10 and 100 ng/ml) on coronary arterioles under control conditions and after pretreatment with indomethacin, glibenclamide and L-NMMA. Pretreatment with L-NMMA significantly inhibited the vasodilating effect of bFGF. Baseline diameters were 66 ± 4, 64 ± 5, 64 ± 4 and 56 ± 5 under control conditions and during administration of indomethacin, glibenclamide, and L-NMMA, respectively.
bFGF was not significantly influenced by pretreatment with indomethacin (n = 8; maximal response 56 ± 7% relaxation; P = 0.997) or glibenclamide (n = 9; 54 ± 7%; P = 0.799). Pretreatment with L-NMMA caused a significant reduction in baseline diameter (n = 11; −16 ± 6%). The effect of bFGF was completely inhibited after L-NMMA pretreatment.

In contrast, aFGF did not have any significant effect but rather tended to cause a minor vasoconstriction (n = 13; −5 ± 3%; n.s.) under baseline conditions. Following administration of the inhibitors, aFGF still did not elicit any significant vasoactive responses.

### 3.3. Effects of heparin (Fig. 2)

Under baseline conditions (n = 15), heparin induced dose-dependent dilation. The threshold dose was 10 U/ml; with 200 U/ml maximal relaxation could be observed. The response to heparin could not be influenced by pretreatment with indomethacin (n = 11; maximum response 99 ± 1%; n.s.). However, both pretreatment with L-NMMA (n = 12) and glibenclamide (n = 10) significantly attenuated the vasodilatory effect of heparin.

### 3.4. Effects of monoclonal anti-FGF antibodies (Fig. 3)

Incubation with monoclonal anti-FGF antibodies (n = 9) for 20 min significantly inhibited the response to bFGF (maximal response 30 ± 7%; P = 0.0198 versus control response). The response to aFGF was not significantly changed (1 ± 3%; n.s.). In addition, the vasodilating response to heparin was significantly reduced (78 ± 5%; P = 0.0132).

The effects of 5HT and nitroprusside were unaltered after pretreatment with monoclonal anti-FGF antibodies.

### 3.5. Effects of FGF-receptor antibodies (Fig. 4)

After incubation with specific FGF-receptor antibodies (n = 10), the response to bFGF was completely inhibited (maximum 5 ± 3%; P < 0.001 versus control response). Again, there was no significant effect of aFGF (0 ± 3%; n.s.). Interestingly, application of the FGF-recep-
tor antibodies significantly blunted the vasodilatory action of heparin response (P < 0.01). The effects of 5HT and nitroprusside were not influenced by pretreatment with FGF receptor antibodies.

4. Discussion

In this study we have made the following new observations: (1) Heparin and basic FGF are potent vasodilators of coronary arterioles; however, acidic FGF is not vasoactive. (2) The vasodilatory effects of heparin and bFGF on coronary arterioles are primarily mediated via NO synthesis and not via release of vasoactive prostaglandins. (3) The vasodilation is receptor-mediated, inasmuch as pretreatment with a monoclonal anti-FGF antibody or an FGF-receptor antibody significantly decreased the effect of bFGF and of heparin. (4) The vasodilatory actions of heparin appear to be modulated by FGF and the FGF-receptor, and, in part, by the KATP channel. Taken together, these data indicate that bFGF and heparin are potent coronary vasodilators, acting primarily via the production of nitric oxide. These results suggest that heparin and bFGF may impact on the tone of coronary resistance vessels and effect coronary blood flow. Our findings and conclusions bear upon previous investigations and coronary vascular regulation.

Apart from angiogenic and mitogenic effects, different growth factors such as VEGF [26], TGF-β [30] and bFGF [6] have been demonstrated to have direct vasoactive effects such as inducing hypotension [8] and causing arterial [29] and arteriolar [6, 7] dilation. Our study significantly extends these findings into coronary resistance vessels by showing the potent vasodilatory effect of bFGF on coronary arteriolar tone. The signalling pathways have not been systematically investigated; possible transduction mechanisms have been suggested to include production of NO [1, 8], increase in intracellular calcium [29] and activation of ATP-sensitive potassium channels [8]. Basic FGF caused dose-dependent relaxation in our study which was inhibited by pretreatment with 1-NMMA, but not by pretreatment with indomethacin or glibenclamide. Importantly, none of these substances altered smooth muscle function, inasmuch as dilation to sodium nitroprusside was not affected by these antagonists. Furthermore, the effect of bFGF could be significantly inhibited by application of monoclonal anti-FGF antibodies as well as specific FGF receptor antibodies, indicating a specific effect of bFGF mediated via specific FGF receptors. These results are in agreement with findings of Rosenblatt et al. [7] who showed a direct vasodilating effect of bFGF in rat pial arterioles and Cuevas [8] who found a hypotensive effect of bFGF in rats which could be inhibited by either 1-NAM or glibenclamide. Furthermore, in a study by Ku et al. [29], the effects of VEGF could also be inhibited by 1-NAM and not by indomethacin. Sellke et al. [6] showed an enhancement of flow in collateral perfused coronary microvessels by chronic bFGF treatment in a pig model of chronic coronary artery occlusion. In contrast to our observations that bFGF is a potent vasodilator, a maximal dose of bFGF caused only a small relaxation (about 7%) which was completely inhibited by pretreatment with L-NAME (10^-5 M). Although it is not easy to resolve differences between the two studies, we are compelled to point out that Sellke et al. preconstricted arterioles with the thromboxane agonist, U 44619, which in our experience makes vessels less susceptible to vasodilators. Also, the fact that these arterioles were isolated from control areas of hearts with chronic occlusion of a coronary artery may contribute to the differences in results—i.e., is the non-ischemic area truly representative of a control area? And finally, we used a subthreshold dose of heparin to act as chaperone for FGF through, and prevent its binding to, the extracellular matrix. This maneuver is important because without heparin we had found extreme variation in vasodilatation to the mitogens. Taken together, there are many reasons to account for the differences between the vasodilatory potency of bFGF in our study versus that of Sellke et al.

Heparin and heparin-related proteoglycans are widely distributed in the body and are associated with extracellular matrix, basement membranes, mast cells, endothelial cells and almost all cell surfaces [14]. They interact with many biological proteins and have a plethora of vascular effects. The proteoglycan lowers blood pressure in rats [14, 31] and in hypertensive patients [32], interacts with the renin–angiotensin system [32, 33], modulates arachidonic acid metabolism [25], increases collateral flow via promoting angiogenesis [24] and induces NO synthesis in endothelial cells [14]. We cannot help but mention that none of these studies elucidated a vascular action of heparin on resistance vessels, and any hypotensive effect could be modulated by a plethora of hemodynamic actions (e.g., vasodilation) affecting levels of pressor hormones, negative inotropic influences, venodilation, etc. Our results impact on these observations by showing that one of the hypotensive effects of heparin may be attributed to a direct vasodilatory effect on resistance vessels.

In this study, we demonstrated for the first time a direct vascular effect of heparin causing dose-dependent vasodilation in isolated coronary arterioles. This effect is partially mediated by NO synthesis as well as by activation of ATP-sensitive potassium channels as demonstrated by significant inhibition of the heparin-induced vasodilation by pretreatment with both 1-NMMA and glibenclamide. These data are supported by results of Yokokawa et al. [23], showing an enhanced cGMP production via an NO-mediated pathway by heparin in cultured endothelial cells as well as by findings of Ito et al. [34] demonstrating an interaction between heparin and potassium channels in isolated myocytes. We are compelled to point out that our conclusion regarding the involvement of NO in heparin-induced dilation is not totally decisive. The large decrease in
baseline diameter produced by L-NMMA could have potentially skewed the results. In the presence of the antagonist, the percent relaxation produced by heparin was significantly attenuated. However, the absolute magnitude of dilation was less affected. One could argue that the effects of L-NMMA are exclusively due to the changes in baseline diameter. We do not support this contention, because a component of heparin-induced dilation was blocked by the antibodies to FGF or its receptor, of which the dilation was mediated exclusively by NO. We still maintain that heparin induces NO relaxation via this mechanism.

Heparin and its derivatives either free in solution or bound to the cell surface are known to be essential for the effects of FGF. FGFs have been shown to possess FGF-receptor binding- and heparin-binding domains [35,36]. In our experiments, the vasodilatory effect of heparin was significantly reduced by an antibody to FGF and, even more pronounced, significantly blocked by pretreatment with specific FGF receptor antibodies. These data most likely indicate that part of the heparin-induced vasodilation is mediated via bFGF. This is supported by results demonstrating that: (1) heparin is involved in the release of bFGF from mast cells and extracellular matrix [11]; (2) heparin acts as a transporter of FGF to the cells [21]; (3) interaction of FGF with cell-membrane-bound heparin derivatives is an essential requirement for the binding of FGF to the high-affinity receptor [18,19]; (4) heparin itself binds to the high-affinity FGF receptor [17]; and (5) FGF can be internalized directly via cell-surface heparin derivatives independent of the FGF receptor [36]. From these observations in the literature and our own findings, we speculate that anti-FGF antibodies decrease the amount of bFGF which can be released by heparin and/or transported to the cell binding sites. This impairs vasodilation. The heparin effect is not completely inhibited because the response to heparin is most likely only partially mediated by FGF and pretreatment with anti-FGF antibodies does also not completely inhibit the response to FGF itself. Pretreatment with the FGF-receptor antibodies allows FGF to bind to the low-affinity heparin-like receptors, but prevents binding to the specific FGF-tyrosine kinase receptors on the endothelial cell. In this case, the heparin response is mainly mediated by FGF-independent pathways, because the response to FGF itself was nearly completely inhibited after pretreatment with the receptor antibodies. Additionally, via blockade of the high-affinity FGF receptor, a possible binding of heparin to these receptors is prevented as well.

Taken together, our data demonstrate that basic FGF causes vasodilation of coronary arterioles by increasing endothelial NO production via specific FGF receptors. Heparin-dependent vasodilation is, in part, mediated by NO and also by activation of ATP-sensitive potassium channels. The effect of heparin is partially mediated via FGF receptors. In contrast to basic FGF, acidic FGF does not have vasodilating properties in isolated perfused coronary arterioles, but induces vasoconstriction when NO and prostaglandin synthesis is inhibited. We conclude from these data that basic fibroblast growth factor and heparin may be involved in the regulation of coronary microvascular tone acting partially through the same signalling mechanisms. The physiological and pathophysiological roles of these substances in the control of coronary resistance, however, remain to be elucidated.

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