Heparin Induces Rat Aorta Relaxation via Integrin-Dependent Activation of Muscarinic M₃ Receptors


Abstract—Previous reports have shown that heparin may promote human hypotension and vascular relaxation by elevation of NO levels through unclear mechanisms. We hypothesized that endothelial muscarinic M₃ receptor activation mediates the heparin-induced vasodilation of rat aortic rings. The experiments were carried out using unfractionated heparin extracted from bovine intestinal mucosa, which elicited an endothelium and NO-dependent relaxation of aortic segments with maximal potency and efficacy (EC₅₀: 100±10 μmol/L; Eₘₐₓ: 41±3%). Atropine and 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide inhibitors reduced the heparin-dependent relaxation, indicating that M₃ muscarinic receptor is involved in this phenomenon. However, no direct binding of heparin to muscarinic receptors was observed. More importantly, studies performed using the arginine-glycine-aspartic acid peptide and 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine, an Src family inhibitor, reduced by 51% and 73% the heparin-dependent relaxation, respectively, suggesting the coupling of heparin and M₃ receptor through extracellular matrix molecules and integrin. Furthermore, unfractionated heparin induced activation of focal adhesion protein kinase, Src, and paxillin. Finally, fluorescence resonance energy transfer approach confirmed the interaction of the M₃ receptor to integrin. Taken together, these data demonstrate the participation of M₃ receptor and integrin in heparin-dependent relaxation of vascular smooth muscle. These results provide new insights into the molecular mechanism and potential pharmacological action of heparin in vascular physiology. (Hypertension. 2010;56:713-721.)

Key Words: heparin ■ muscarinic receptors ■ M₃ receptor ■ integrin ■ smooth muscle relaxation ■ aorta

The successful use of heparin in the treatment of thromboembolic diseases has been associated with numerous pharmacological actions of this complex polysaccharide. Alterations in the blood vessel and associated cells, such as platelet hyperactivity, inflammatory processes, endothelial dysfunction, and angiogenesis, lead to the development of diseases related to clot formation, which can be efficiently modulated by heparin and its derivatives.¹⁻³ Heparin is a glycosaminoglycan composed of repeating 1→4-linked α-D-glucosamine, mainly N-sulfated, and uronic acid, either β-D-glucuronic acid or α-L-iduronic acid; also, O-sulfation occurs at different positions of the disaccharide units.⁴⁻⁵ Although heparin usually acts as an anticoagulant,⁶ other biological activities have also been described, including antiviral,⁷ antibacterial,⁸ and antithrombotic effects. This antithrombotic action has been related, at least in part, to the increased production of a peculiar heparan sulfate proteoglycan produced by the endothelium.⁹⁻¹¹ In addition, heparin modulates the function of many proteins, for example, the myosin from skeletal muscle,¹² the inositol 1,4,5-trisphosphate receptor inhibition,¹³ and the activation of Na⁺/Ca²⁺ exchanger by enoxaparin and heparan sulfate/heparan disaccharides.¹⁴ The hypotensive effect of heparin has been described in patients submitted to diverse clinical procedures.¹⁵⁻¹⁷ In addition, it induces a decrease in the blood pressure in patients with glomerulonephritis,¹⁸ as well as in hypertensive humans¹⁹ and rats.²⁰⁻²² In addition, hypotension caused by an increased level of NO was observed during hemodialysis of heparinized patients.²³ Further investigation of heparin mechanisms of action showed an endothelium-dependent relaxation and endothelial NO synthase activation by heparin and N-acetylated heparin in rat aortic smooth muscle.²⁴ Low molecular weight heparins (LMWHs), such as enoxaparin and nadroparin, also induced endothelium-dependent relaxation in the human thoracic artery.²⁵ Nevertheless, neither the site of action in blood vessels nor the molecular mechanisms involved in heparin-induced vascular relaxation were described. Because of the wide clinical use of heparin, it is important to understand its secondary actions, as those observed in...
patients treated with heparin. Muscarinic M₃ receptor is coupled to G protein and induces aortic relaxation by increased levels of NO in rat aortic rings. Considering that activation of endothelial muscarinic receptors and subsequent NO release are important mechanisms of vasodilatation, in the present study we hypothesized that endothelial muscarinic M₃ receptor activation mediates the heparin-induced vascular relaxation of rat aortic rings. Among the different heparins tested, unfractionated heparin (UFH) extracted from bovine intestinal mucosa was selected because of its efficacy in the rat aortic ring model. Our data suggest that the heparin-induced relaxation occurs by activation of the muscarinic acetylcholine M₃ receptor in an integrin-dependent fashion, and this interaction leads to the phosphorylation of focal adhesion proteins, which, in turn, increases NO levels.

Materials and Methods
Detailed methods and chemicals used in this study are available in the online Data Supplement (http://hyper.ahajournals.org).

Chemicals
UFHs were isolated from bovine intestinal mucosa (Kim Master), porcine intestinal mucosa (Roche), and bovine lung (Proquimio), and enoxaparin was from an LMWH from Sanofi-Aventis. Arginine-glycine-aspartic acid (RGD) peptide was synthesized as described previously.³⁷

Isolated Vascular Ring Studies
Male normotensive Wistar rats (200 to 250 g) from the Wistar Institute and inbred at Centro de Desenvolvimento de Modelos Experimentais from the Federal University of São Paulo were used. All of the experiments were approved by the animal care ethics committee of the Federal University of São Paulo (No. 0658/05) and were conducted in accordance with the Brazilian Guide for the Care and Use of Laboratory Animals (federal law 11794/2008), which is equivalent to the National Institutes of Health guidelines. All of the animals (n=45) were killed under anesthesia with a mixture of ketamine (80 mg·kg⁻¹) and xylazine (10 mg·kg⁻¹). Aortic rings were cut and placed in a jacketed organ chamber. Acquisition and analysis of the isometric contractions were conducted with KitCad8 software (Software & Solutions).

Confocal Microscopy
Rat aortic rings were incubated with Texas red-conjugated heparin probes, embedded in tissue freezing medium and frozen. Aortic ring slices were labeled with anti-M₃ and/or anti-α₁ integrin and stained with appropriated fluorescent-labeled secondary antibodies and 4',6'-diamidino-2-phenylindole. Slides were examined using a laser scanning confocal microscope LSM 510 META (Zeiss).

Immunoblot Analysis
An endothelial cell line derived from rabbit thoracic aorta was used in this study. After stimulation, cell lysates and Western blotting were carried out as described previously.²⁸

Fluorescence Resonance Energy Transfer Assay
The aortic rings were labeled with α₁ chain and M₃ antibodies. Appropriately, secondary antibodies Alexa Fluor 488 or 546 were used. Microscopy analyses were performed with a confocal laser scanning microscope (Zeiss, LSM 510 META). Fluorescence resonance energy transfer (FRET) ratios were calculated as described previously.²⁹

NO Measurement in Rat Aortic Rings
The NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate) was used to measure intracellular NO concentration in rat thoracic aorta.³⁰,³¹ Aortic rings embedded in 3% agarose were sliced in vibrotome (1000 Plus Sectioning System, myNeuroLab).³² Images were captured with a confocal microscope (Zeiss, LSM 510 META).

[^3H]Methyl Quinuclidinyl Benzilate Binding Assays
[^3H]methyl Quinuclidinyl Benzilate ([³H]mQNB) binding assay was performed to evaluate the possible interaction of heparin with a muscarinic receptor, using membrane fraction from a rat hippocampus, as described previously.³³

Statistical Analysis
The effect of heparins was represented as relaxation after precontracted aorta by 1 μmol/L of norepinephrine. The DAF-FM fluorescence intensity was normalized with reference to basal intensity (F₀/F₁) and shown as a representative pseudocolored image, according to a fluorescence intensity scale ranging from 0 (black) to 255 (white). All of the data represent ≥3 independent experiments and were expressed as mean±SEM. Statistical analyses were performed using Student’s t test for comparison between 2 groups, and ANOVA and Dunnett post hoc test for multiple comparisons among groups. A P value of P<0.05 was considered significant.

Results
Different Heparins Induce Endothelium-Dependent Vascular Relaxation
Initially, the relaxation effects of different heparins were evaluated on rat aortas precontracted with 1 μmol/L of norepinephrine. As shown in Figure 1B, heparin-dependent relaxation was slower than the acetylcholine (ACh)-induced one (Figure 1A and 1B). A 30-minute incubation period was necessary to observe maximal heparin-dependent relaxation of the aortic rings (Figure 1B), with major effects obtained with UFH extracted from bovine intestinal mucosa (Figure 1C and 1D). Cumulative concentration-response curves for heparins of distinct sources were constructed (Figure 1C). UFH from bovine intestinal mucosa was the most potent and effective heparin (EC₅₀: 100±10 μmol/L; Eₘₐₓ: 41±3%; n=8), whereas UFH from porcine intestinal mucosa exhibited a similar potency but less efficacy than UFH from bovine intestinal mucosa (EC₅₀: 105±6.6 μmol/L; Eₘₐₓ: 32±1%; n=5). Moreover, UFH from bovine lung (EC₅₀: 135±4 μmol/L; Eₘₐₓ: 27±4%; n=5) and LMWH enoxaparin (EC₅₀: 206±4 μmol/L; Eₘₐₓ: 26±3%; n=5) were less potent and effective than UFH from bovine intestinal mucosa. To evaluate the actual contribution of endothelium on heparin effects, the heparin-dependent relaxation was also tested in endothelium-denuded aortic rings using 400 μmol/L of UFH and 1 mmol/L of LMWH enoxaparin. As observed in Figure 1D, endothelium removal reduced the relaxation effect of all of the heparins tested.
Heparin Binds to Endothelium to Promote an Increase in NO Production

The next step was to evaluate how heparin induces the relaxation in aorta. Assays were then performed using the heparin preparation that showed the higher relaxation effect (UFH extracted from bovine intestinal mucosa). Initially, a possible interaction of heparin with endothelium was investigated. To confirm the binding of heparin to blood vessels, Texas red-conjugated heparin and anti-M3 receptor antibody were used. The muscarinic receptor M3, found on the surface of endothelial cell was used as a marker of endothelium surface. As shown in Figure 2A, the M3 receptor and heparin were overaid on the endothelium, suggesting the binding of heparin on the endothelial surface.

Because heparin-induced vascular relaxation was shown to be an endothelium-dependent process, the participation of NO in relaxation by UFH extracted from bovine intestinal mucosa was studied. For this purpose, aortic rings were incorporated with DAF-FM diacetate, which emits increased fluorescence after reaction with an active intermediate of NO formed during the spontaneous oxidation of NO to nitrogen dioxide. As expected, UFH from bovine intestinal mucosa was able to increase NO production. As observed in Figure 2B and 2C, the NO produced by endothelial cells in the vessel lumen spread into smooth muscle cells, thus inducing vascular vasodilation. Unstimulated and endothelium-denuded samples stimulated with heparin were used as negative controls (Figure 2B and 2C). In addition, the endothelial NO synthase inhibitor, NG-nitro-L-arginine, abolished heparin-dependent relaxation of thoracic aorta (Figure 2D), confirming the involvement of NO production in heparin-induced vascular relaxation. In addition, NO production also was observed in the supernatant of endothelial cells after 10 minutes of stimulation with heparin (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Heparin Induces Vascular Relaxation in Rat Aorta Through M3 Receptor Activation

Subsequently, different approaches were used to identify the site of heparin action. The cumulative concentration-response curve of UFH from bovine intestinal mucosa was compared to that of carbachol (CCh; Figure 3A). UFH from bovine intestinal mucosa (EC50: 100±10 μmol/L; Emax: 41±3%; n=8) is less potent and less effective than CCh (EC50: 0.62±0.1 μmol/L; Emax: 63±4%; n=6). Because muscarinic and P2 receptors are expressed in the rat aortic ring, their participation in heparin-induced relaxation was investigated. A comparison of the maximal relaxation caused by heparin, CCh, and uridine-5′-triphosphosphate is shown in Figure 3B. In addition, the nonselective muscarinic inhibitor atropine decreased this effect (Figure 3C). Furthermore, relaxation was inhibited by the specific M3 inhibitor 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide, whereas the selective M2 antagonist 11-[[2-(diethylamino)methyl]-1-piperidinyl]acetyl]-5,1, 1-dihydro-6H-pyrido[2,3-b] [1,4]benzodiazepine-6 was notable to modify the heparin effect (Figure 3C). In addition, suramin, a nonselective P2 receptor inhibitor, did not decrease the action of heparin (Figure 3C).

Taking into account that heparin shows lower efficacy compared with CCh, we next considered a possible competitive effect of heparin for ACh/CCh binding sites, which may lead to a reduction in CCh efficacy. To verify this possibility, cumulative concentration-response curves to ACh and CCh were constructed using rat aorta, in the absence and presence of 100 μmol/L of heparin (EC50). Interestingly, heparin did not modify the ACh and CCh curves, suggesting no direct interaction with muscarinic receptors (Figure 4A and 4B). The lack of interaction of heparin with the orthosteric site (ACh binding site) of muscarinic receptors was also evaluated using the [3H]mQNB binding assay in hippocampus membrane fractions. Figure 4C shows the saturation binding curve of [3H]mQNB (Bmax: 727±58 fmol/mg of protein; dissociation constant: 1.1±0.3). In contrast to the effect of
atropine, a muscarinic receptor antagonist (positive control), UFH from bovine mucosa fail to inhibit \(^{3}H\)mQNB binding (Figure 4D), when a concentration of \(^{3}H\)mQNB close to dissociation constant (50% receptor occupancy) was used.

**Heparin Binds to M\(_3\) Receptor Through Integrin**

Because UFH from bovine mucosa did not interact directly with muscarinic receptors, an indirect coupling through extracellular matrix proteins and integrin was hypothesized. To test this idea, we evaluated the relaxation effect induced by heparin on the presence of the peptide RGD. This peptide sequence is present in many extracellular matrix proteins and can interact with the integrin at the focal adhesion points.\(^{35}\) As can be observed in Figure 5A, the RGD peptide was able to reduce by 50% the heparin-dependent relaxation. The RGD binding to integrin leads to activation of several intracellular pathways associated with focal adhesion proteins, such as Src, focal adhesion kinase, and paxillin.\(^{35}\) Thus, the activation of these kinases by heparin

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**Figure 2.** UFH from bovine intestinal mucosa binds to the vascular endothelium inducing an increase in NO production in rat aortic rings. A, Heparin-Texas red conjugated (red) binding to the endothelium region. Localization of heparin (red) and the M\(_3\) muscarinic receptor (green) expression on the endothelial cell surface: (i) negative control of the secondary antibodies; (ii) presence of M\(_3\) receptor and heparin binding on the endothelial surface; and (iii) insert of the image shown in (ii). B and C, Heparin increased NO production in aortic rings measured by DAF-FM. We show sequential images of control (unstimulated) and 400 \(\mu\)mol/L of UFH-stimulated endothelium intact and denuded samples. C, Fluorescence intensity versus time of the images shown in B corresponding with control and heparin-stimulated samples. A and B, Bar, 20 \(\mu\)m. D, The endothelial NO synthase inhibitor NG-nitro-L-arginine (LNNA) abolished the heparin-dependent relaxation. Data are expressed as mean±SEM of 6 independent experiments. *\(P<0.05\).
was investigated using an endothelial cell line that expresses the M₃ receptor (Figure S2). Stimulation of endothelial cells with heparin for 10 minutes induced activation of focal adhesion kinase, Src, and paxillin, as demonstrated by Western blot analysis of total protein extracts (Figure 5B and 5C). To further verify the participation of integrin in heparin-dependent relaxation, the Src inhibitor 1-(1,1-dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP1) was used (1 μmol/L). The Src inhibitor attenuated 73% the relaxation induced by heparin (Figure 5D). Furthermore, the physical interaction between M₃ receptor and integrin α₅ chain was confirmed by FRET assay (Figure 6). The observed FRET signal occurred only in the presence of α₅ (donor) and M₃ receptor (acceptor; Figure 6A). FRET signal is observed when the donor and the acceptor are in proximity, typically <100 Å, because of the interaction between the 2 molecules. The spectra are shown in Figure 6B. The negative control (α₅ integrin) showed a reduced FRET ratio, whereas α₅M₃ presented a maximum FRET ratio (Figure 6C), indicating that α₅ and M₃ receptors are closely connected.

Figure 3. Heparin-dependent relaxation occurs by activation of muscarinic receptors. A, Relaxation induced by UFH from bovine intestinal mucosa and CCh. Cumulative relaxation curves values are expressed as percentage of reversal precontraction with norepinephrine (10 minutes) in the presence of CCh (5 minutes) or heparin (30 minutes). B, Maximal relaxation induced by CCh, uridine-5'-triphosphate (UTP), and UFH. C, Heparin-dependent relaxation was specifically abolished by atropine, a nonselective muscarinic inhibitor, and by 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), a selective M₃ receptor inhibitor. Neither 11-[2-[(diethylamino)methyl]-1-piperidinyl][acetyl]5,1-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6 (AF-DX 116), a selective M₂ receptor antagonist, nor suramin, a P₂ receptor inhibitor, was able to abolish the heparin-dependent relaxation. Data are expressed as mean ± SEM of 6 independent experiments. Statistical comparisons were made vs the UFH from bovine intestinal mucosa effect. *P<0.05.

Figure 4. Heparin did not interact with muscarinic receptors. We show the cumulative concentration-response curves of (A) ACh and (B) CCh in the presence and absence of 100 μmol/L of heparin. Data are expressed as mean ± SEM of 4 independent experiments. C and D, Saturation curves obtained from rat hippocampi membranes incubated with 0.05 to 7.00 nmol/L of [³H]mQNB. C, [³H]mQNB binding curve. D, UFH from bovine intestinal mucosa was added in the presence of 1 nmol/L of [³H]mQNB. Heparin did not alter the binding of [³H]mQNB to muscarinic receptors. Atropine, which binds to muscarinic receptors, was used as a positive control.
Discussion

UFH and LMWH are the only sulfated polysaccharides currently used as anticoagulant drugs. Heparin still is the therapeutic drug of choice for management of thrombotic disorders, despite the recent design and development of new drugs. Several studies correlate heparin effects in thrombosis with their action on the blood coagulation cascade and on blood vessels through interaction with many proteins. Several reports in the literature have demonstrated hypotension in some patients receiving therapeutic doses of heparin. Previous studies conducted in human arteries and rat aortic rings have shown different potencies for heparin-induced vasodilation. In addition, in the clinical setting, heparin can be administered in intravenous bolus at doses \( \approx 1 \text{ mg/kg} \); thus, immediate effects in human vessels occur at high concentrations of heparins.

Endothelium-dependent relaxation induced by heparin is a known effect. The heparin concentration needed to cause vasorelaxation was 10-fold higher in rat aortic rings than in human arteries. In addition, the novel findings presented in this study clarify the possible binding site and downstream signaling molecules involved in this activity. The effects of P2 and muscarinic receptor antagonists were evaluated to clarify the mechanism of heparin relaxation. The results suggested that heparin-dependent relaxation of rat aortic rings occurs by activation of M3 receptors (Figure 3). Heparin-binding motifs were described in proteins with sequences such as XBBBXXBX and XBBXBX (where B and X are basic and hydrophobic residues, respectively). M3 receptors do not have heparin-binding sites on the extracellular loops, which is in accordance with the absence of heparin binding to muscarinic receptors (Figure 4C and 4D). In addition, other possibilities to explain heparin-induced relaxation were evaluated. Because heparin binds to acetylcholinesterase, a possible effect would be the inhibition of acetylcholinesterase, causing an increase in the physiological cholinergic agonist ACh. To discard this possibility, the acetylcholinesterase inhibitor ambenonium dichloride was used to reduce heparin-dependent relaxation, but no effect was observed (data not shown). Another possibility is that heparin could act as a partial muscarinic agonist or as an enhancer of a cholinergic agonist because of its low potency and efficacy. However, as shown in Figure 4A and 4B, heparin did not alter the ACh and CCh cumulative concentration-response curves.

Several reports have shown that the activation of muscarinic receptors leads to the phosphorylation of focal adhesion proteins, such as focal adhesion kinase and paxillin. Apparently, the activation of focal adhesion proteins by muscarinic receptor activation occurs by an intracellular mechanism involving cytoskeleton proteins, the small Rho GTPase, phosphoinositide 3-kinase, and integrins in different cells, such as neuroblastoma cells, rat pancreatic acinar cells, and HEK cells transfected with \( \alpha \)-receptor. Barkalow and Schwarzbauer and others have previously reported the binding of heparin to fibronectin and with other molecules of extracellular matrix. Based on this literature, we hypothesized that heparin could interact indirectly with \( \alpha \)-receptor through integrin. Our results showed that heparin induced activation of focal adhesion proteins involved in integrin signaling (Figure 5A through 5C). Moreover, integrin and M3 receptors are in close proximity, as demonstrated by the FRET approach (Figure 6). Previous information and our own data suggest a close relationship between muscarinic...
receptors (and probably other G-coupled protein receptors) with extracellular matrix molecules/integrin/focal adhesion proteins. Therefore, it is likely that the interaction of heparin to extracellular matrix molecules/integrin could favor the active state of the M3 receptor, triggering NO production. In fact, the presence of the muscarinic inhibitor atropine and the M3 receptor antagonist 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide, which favor the inactive state of the receptor, abolished heparin relaxation response. A schematic model of the mechanism involved in heparin-induced relaxation is presented in Figure 7.

**Perspectives**

Our findings extended and complemented previous studies on NO-dependent relaxation of blood vessels induced by heparin. Herein, we demonstrate the role of the muscarinic M3 receptor in the endothelium-dependent relaxation elicited by
heparin. Furthermore, new data are provided showing the interaction between integrin and M3 receptor activation, as well as the downstream signaling mediated by the focal adhesion complex. These findings bring new insights to our current knowledge on the molecular mechanisms of heparin-induced hypotension in humans. Studies are in progress in our laboratory to further understand the biochemical structural requirements of heparins to elicit vasodilation. Deciphering the structural characteristics and the pharmacological and molecular aspects of heparin-induced vascular relaxation may create new potential applications of heparin in medicine.

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Disclosures
None.

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43. Paredes-Gamero et al. Heparin Activates Muscarinic M3 Receptor
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SUPPLEMENTARY MATERIAL AND METHODS

RGD synthesis

Arginine-glycine-aspartic acid (RGD) peptide was synthesized manually by the solid-phase method using the t-Boc strategy on a 4-methylbenzhydrylamine-resin (0.4 mmol/g) (1). Full deprotection and cleavage of the peptide from the resin were carried out using anisole and dimethyl sulfide as scavengers and an anhydrous HF treatment at 0°C for 1 h. Immediately, after its extraction, the resulting peptide solution was lyophilized. Crude peptides were purified by preparative Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) on a Vydac C_{18} column (25x250 mm, 300 Å pore size, 15 µm particle size) in two steps. The first step was performed by using triethyl-ammonium phosphate pH 2.25 as solvent A and 60% acetonitrile in A as solvent B. The second one was carried out using 0.1% trifluoroacetic acid/H_{2}O as solvent A and 60% acetonitrile in A as solvent B. Pure peptides were characterized by amino acid analysis and by Liquid Chromatography/Electrospray Ionization Mass Spectrometry.

Isolated vascular ring studies

Male normotensive Wistar rats (200-250 g) from the Wistar Institute, (USA) inbred at Centro de Desenvolvimento de Modelos Experimentais (CEDEME) from the Federal University of São Paulo were used. The animals were killed and the thoracic aortas removed and placed in Krebs-bicarbonate solution (in mmol/L) (NaCl, 118.4; KCl, 4.7; CaCl_{2}, 2.5; KH_{2}PO_{4},1.2; MgSO_{4}; NaHCO_{3}, 25; C_{6}H_{12}O_{6}, 11.6; pH 7.4, at 37°C). All experiments were approved by the Animal Care Ethics Committee of the Federal University of São Paulo (number 0658/05) in accordance to the Brazilian federal law which takes into consideration the 3Rs. All animals (n=45) were killed under anesthesia with a mixture of ketamine (80 mg kg\(^{-1}\)) and xylazine (10 mg kg\(^{-1}\)). Rings of 1 cm length were cut and placed in a 5 mL jacketed organ chamber, aerated with a mixture of 5% CO\(_{2}\) and 95% O\(_{2}\). After an equilibration period of 2 h under an optimal resting tension of 1 g, the rat aortas were stimulated. Responses were measured with a force displacement transducer (TRI 210, Letica, Barcelona, Spain) connected to an amplifier (model AECAD-0804, Solução Integrada, Brazil). Acquisition and analysis of the isometric contractions were conducted with KitCad8 software (Software & Solutions, Brazil). The presence of a functional endothelium was tested in all preparations by checking the acetylcholine (1 µmol/L) relaxation response, which is characteristic of vessels with an intact endothelium. The inhibitors NG-Nitro-L-arginine (LNNA) and 1-Naphthyl PP1 (PP1) were added to Krebs-bicarbonate solution containing rat aortas previously to stimulation with epinephrine.
Confocal microscopy

Rat aortic rings obtained as described above, were washed and incubated with 10 µg/mL Texas red-conjugated heparin probes in Kreb’s solution. After 30 min, the aorta was washed, embedded in tissue freezing medium and frozen. Aortic rings were sliced in 10-15 µm sections using a Cryostat Chamber (Leica Instruments, Germany), and collected on silanized slides for immunofluorescence staining. Aortic rings sections were fixed with 2% paraformaldehyde for 30 min, washed with 0.1 mol/L glycine, and permeabilized with 0.01% saponin. After 15 min, the sections were washed with phosphate buffered saline (PBS) and incubated with rabbit anti-M₃ and/or rat anti-α₅ integrin antibodies in PBS/1% bovine serum albumin (BSA) for 2 h. Samples were then incubated with 2 µg/mL appropriated fluorescent-labeled secondary antibodies anti-rat or anti-rabbit IgG conjugated with Alexa Fluor 488 or 546 for 40 min. After washing, nuclei were stained with 20 µg/mL 4’,6-diamidino-2-phenylindole (DAPI) for 20 min. Finally, the slides were mounted in fluoromount-G and examined using a laser scanning confocal microscope LSM 510 META (Zeiss, Germany).

Immunoblot analysis

An endothelial cell line derived from rabbit thoracic aorta was used in this study (2-5). Cells were grown in Ham’s Nutrient Mixture F-12 supplemented with 10% fetal calf serum (Cultilab, Brazil), streptomycin (100 mg/mL) and penicillin (100 IU/mL) (Sigma Chemical Co., USA) at 37°C in a humidified atmosphere with 2.5% CO₂.

Endothelial cells grown in 100 mm diameter plates were stimulated with 100 µg/mL heparin for 10 min, at 37°C in a humidified atmosphere with 2.5% CO₂. After stimulation, cells were lysed in 150 µL buffer [50 mmol/L Tris–HCl (pH 7.4), 1% Tween 20, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, and protease inhibitors (1 µg/mL aprotinin, 10 µg/mL leupeptin and 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride)] for 2 h in ice. Protein extracts were cleared by centrifugation and protein concentration determined using the Lowry protein assay. An equal volume of 2x sodium dodecyl sulphate (SDS) gel loading buffer [100 mmol/L Tris–HCl (pH 6.8), 200 mmol/L dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue and 20% glycerol] was added to the samples, which were subsequently boiled for 10 minutes. Equal quantities of protein (50 µg) were loaded onto SDS-PAGE and blotted onto nitrocellulose membranes (Millipore, USA). Membranes were blocked in 1% fat-free dried milk or 2% BSA in Tris buffered saline (TBS) with 0.05% Tween 20 (TBST) and incubated overnight at 4°C with appropriate primary antibody (anti-M₃ receptor, anti-phospho-FAK Tyr925, anti-phospho-Src Tyr473, anti-phospho-Paxillin Tyr118 and anti-β-actin) at 1:1,000 dilution. After washing in TBST, membranes were incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies, at 1:2,500 dilutions in blocking buffer for 1 h. Detection was performed using enhanced chemiluminescence (ThermoFisher Scientific, USA).
Fluorescence resonance energy transfer (FRET) assay

Rat aortic rings were fixed with 2% formaldehyde for 30 min, washed with 0.1 mol/L glycine and permeabilized with 0.01% saponin for 15 min. The cells were then incubated for 2 h with 4 µg/mL goat anti-α5 chain. Anti-goat IgG Alexa Fluor 488-conjugated antibody was used for 40 min as secondary antibody. Subsequently, rabbit IgG anti-M3 was used, followed by incubation with anti-rabbit IgG Alexa Fluor 546-conjugated antibody for 40 min. Light microscopy analyses were performed with a confocal laser scanning microscope equipped with a Plan-Apochromat x63 objective (Zeiss, LSM 510 META). The pinhole device was adjusted to capture fluorescence of one airy unit in one focal section.

For FRET analysis, FRET signal was obtained at 488 nm excitation employing an argon laser and the emitted fluorescence was detected from 560 nm to 650 nm in 10.6 nm steps using the lambda modus. FRET ratios were calculated using the following equation: FRET ratio = Intensity at 575 nm/Intensity at 521 nm (6).

Nitric oxide measurement in rat aortic rings

The NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate) was used to measure intracellular NO concentration in rat thoracic aorta (7, 8). To quantify the nitric oxide (NO) production induced by heparin, rat aorta was embedded in 3% agarose. Aortic rings were sliced in vibratome (1000 Plus Sectioning System, myNeuroLab, USA) into 100-150 µm sections and placed on cover glass (25 mm) in Krebs solution as described for rat aorta and other tissues (7, 9). For NO measurements, aortic rings were incubated for 40 min at room temperature with 10 µmol/L DAF-FM and washed with Krebs solution, similarly to previously described by De Angelis et al. (7). Images were captured with a microscope (Zeiss, Axiovert 100 M) equipped with a laser scanner (Zeiss, LSM 510 META) and a 40x objective (Plan-Neofluor, 1.4 numerical aperture) under oil immersion. The DAF-FM probe was excited with an argon laser (λEx = 488 nm), and light emission was detected using online emission fingerprint spectrum of DAF-AM indicator. Online emission fingerprint spectrum of DAF-FM indicator was obtained from 505 nm to 558 nm in 10.6 nm steps using the lambda modus. The use of online emission fingerprint helps to exclude autofluorescence. The pinhole device was not used for NO measurements. Images were collected at approximately 10 s intervals. For each experiment, fluorescence intensity was normalized to the basal fluorescence using the Examiner 3.2 (Zeiss, Germany) and Spectralyzer (USA) software.

[3H]methyl quinuclidinyl benzilate ([3H]mQNB)-binding assays

Hippocampi obtained from adult male normotensive Wistar rats were homogenized in PBS containing 10 mmol/L EDTA (PBS-EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L pepstatin A and 5 mmol/L bacitracin, using a Dounce tissue-grinder. The samples were centrifuged twice at 20,000 g for 10 min and the final pellet (membrane
fraction) was diluted in 20 mmol/L Tris buffer containing 1 mmol/L MgCl₂, (Tris-Mg), pH 7.4, to reach 200 µg protein/mL, as previously described (10). Samples were, then, incubated with 0.05–10 nmol/L [³H]mQNB for 60 min at 25°C, in a final volume of 100 µL, using MultiScreen HTS 96-well filtration System (Millipore Corporation, USA). The reaction was stopped by 3 successive vacuum filtration using ice-cold Tris-Mg (100 µL). Radioactivity was determined by scintillation counting (Micro Beta Jet 1450, Perkin Elmer, Finland) and the values were expressed as fmol of [³H]mQNB binding sites per mg of protein. Nonspecific binding was obtained in the presence of 1 µmol/L atropine.

Nitric Oxide measure

NO production in the supernatant of endothelial cells was measure after 10 min of stimulation with heparin. Aliquots of 100 µL of supernatant were collected and injected in 2.0 mL of Griess reactant (0.01 M sulfanilamide, 0.01 M N-(1-naphthyl)ethylene diamine dihydrochloride, 4.0 M HCl) contained in a quartz cuvette. The absorbance of the Griess solution was measured directly in the cuvette at 540 nm. Absorbance at this wavelength provides a quantitative measurement of NO release after its conversion to NO₂⁻.

SUPPLEMENTARY REFERENCES


SUPPLEMENTARY RESULTS

Figure S1: Nitric oxide production in the supernatant of endothelial cells after 10 min of stimulation with heparin.

Figure S2: Endothelial cell lineage expresses the M₃ receptor.