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Chemical Reduction of Carboxyl Groups in Heparin Abolishes its Vasodilatory Activity

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ABSTRACT

Previous studies have shown that heparin induces vascular relaxation via integrin-dependent nitric oxide (NO)-mediated activation of the muscarinic receptor. The aim of this study was to identify the structural features of heparin that are necessary for the induction of vasodilatation. To address this issue, we tested heparin from various sources for their vasodilatation activities in the rat aorta ring. Structural and chemical characteristics of heparin, such as its molecular weight and substitution pattern, did not show a direct correlation with the vasodilation activity. Principal component analysis (PCA) of circular dichroism (CD), ^{1}H-nuclear magnetic resonance (NMR) and vasodilation activity measurements confirmed that there is no direct relationship between the physico-chemical nature and vasodilation activity of the tested heparin samples. To further understand these observations, unfractionated heparin (UFH) from bovine intestinal mucosa, which showed the highest relaxation effect, was chemically modified. Interestingly, non-specific *O*- and *N*-desulfation of heparin reduced its anticoagulant, antithrombotic, and antihemostatic activities, but had no effect on its ability to induce vasodilation. On the other hand, chemical reduction of the carboxyl groups abolished heparin-induced vasodilation and reduced the affinity of heparin toward the extracellular matrix (ECM). In addition, dextran and dextran sulfate (linear non-sulfated and highly sulfated polysaccharides, respectively) did not induce significant relaxation, showing that the vasodilation activity of polysaccharides is neither charge-dependent nor backbone unspecific. Our results suggest that desulfated heparin molecules may be used as vasoactive agents due to their low side effects. J. Cell. Biochem. 113: 1359–1367, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: HEPARIN; AORTA; VASCULAR RELAXATION; SULFATION; CARBOXYLGROUP

The hypotensive effect of heparin has been described in human patients [Waser and Keller, 1954; Keller, 1956; Abbott et al., 1966; Kutyrina et al., 1985; Kongsgaard et al., 1991] and rats [Wilson et al., 1981; Susic et al., 1982; Kentera et al., 1985]. Heparin's ability to induce vascular relaxation is due to an increase in nitric oxide (NO) levels [Yokokawa et al., 1995; Kouretas et al., 1998; Buzun et al., 2004]. In addition, the participation of the extracellular matrix (ECM) in heparin-dependent effects on the endothelium has also been demonstrated [Trindade et al., 2008]. Recently, we demonstrated that heparin-dependent vasodilation via NO was due to the activation of the muscarinic M_3 receptor as a result of heparin binding to ECM components and subsequent

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activation of integrin signaling [Paredes-Gamero et al., 2010]. However, the structural requirements of heparin necessary for the induction of vascular relaxation are still unknown.

Heparin is a sulfated glycosaminoglycan present in several mammalian and other vertebrate and invertebrate tissues [Gomes and Dietrich, 1982; Hovingh et al., 1986; Medeiros et al., 2000]. It consists of a linear, highly sulfated polysaccharide chain comprised of repeating disaccharide units of 1,4 *O*-linked α-L-iduronic or β-D-glucuronic acid, and α-D-glucosamine. The predominant substitution pattern comprises *N*- and 6-*O*-sulfation of the glucosamine and 2-*O*-sulfation of the iduronate residues [Perlin et al., 1971; Lindahl, 1977; Rudd et al., 2009]. The distribution of these sulfate groups in the polysaccharide chain provides heparin with an average of 3.5 negative charges per disaccharide unit [Dietrich et al., 1980]. Other substitution patterns are also possible, including *N*-acetylation and 3-*O*-sulfation of the glucosamine, as well as non-sulfated iduronate and 2-*O*-sulfation of the β-*D*-glucuronate moiety [Esko, 2001].

In this study, we investigated whether heparin from various sources have different vascular relaxation activities due to the differences in their molecular and chemical characteristics. Carboxyl-reduction and desulfation of heparin was performed to determine the role of spatial conformation of the uronic acid moiety and the negative charge due to sulfate groups, respectively, on the vasodilatation activity of heparin. Our results showed that the integrity of carboxyl groups, which are chemical moieties associated with chain flexibility, is more important in the vasodilatory effects than in the extent of heparin sulfation. Moreover, sulfate groups are critical for the anticoagulant, antithrombotic and hemorrhagic activities of heparin, but they are not important for its vasodilatation activity. These findings could open up future therapeutic opt ions for the use of heparin.

MATERIALS AND METHODS

HEPARIN SAMPLES

Unfractionated heparin (UFH) samples from the following sources were used: Bovine intestinal mucosa (Kim Master; Brazil; lot # 02904), porcine intestinal mucosa (Roche; Brazil; lot # 0715723), bovine lung (Proquimio; Brazil; lot # 300-P), and enoxaparin, a low molecular weight heparin (LMWH), purchased from Sanofi-Aventis (Brazil; lot # 63911). Dextran sulfate was purchased from Sigma (lot # 99F0458). Dextran T-10 was purchased from Pharmacia Fine Chemical AB (Sweden, Lot # 6930). All other chemicals were purchased from Merck (Brazil) and Sigma-Aldrich (Saint Louis, MO).

ISOLATED VASCULAR RING STUDIES

Male normotensive Wistar rats (200–250 g) from the Wistar Institute, inbred at the Centro de Desenvolvimento de Modelos Experimentais (CEDEME) of the Federal University of São Paulo, were sacrificed and the thoracic aortas removed and placed in a jacketed organ chamber containing Krebs-bicarbonate solution (pH 7.4 at 37°C, containing 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, $MgSO_4$ 1.1 mM; 25 mM $NaHCO_3$, 11.6 mM $C_6H_{12}O_6$). All animals were sacrificed under anesthesia using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Rings of 1 cm length were cut from the thoracic aorta and placed in a 5 ml jacketed organ chamber aerated with a mixture of 5% CO_2 and 95% O_2. Responses were measured with a force displacement transducer (TRI 210, Letica, Spain) connected to an amplifier (model AECAD-0804, Solução Integrada, Brazil). The presence of a functional endothelium was tested in all preparations by checking the acetylcholine (1 μM) relaxation response, which is characteristic of vessels with an intact endothelium. All experiments were approved by the Animal Care Ethics Committee of the Federal University of São Paulo (approval/protocol number 0658/05) in accordance with Brazilian federal law, which takes into consideration the 3Rs.

HEPARIN MODIFICATIONS AND MOLECULAR WEIGHT DETERMINATION

Heparin carboxyl reduction was performed as described by Taylor and Conrad [1972]. Partially and non-specifically desulfated heparin was obtained by solvolytic desulfation by DMSO of the heparin pyridium salt [Nagasawa et al., 1979]. The amount of sulfate in heparin was determined by 2D Nuclear Magnetic Resonance (NMR) and other chemical measurements as previously described [Dodgson and Price, 1962; Nader and Dietrich, 1977; Lima et al., 2011a]. Molecular weight was determined by gel permeation chromatography using a HPLC system (Shimadzu, Kyoto, Japan) with the outlet of the joint columns (TSK G3000SW and TSK G2000SW, TosoHaas, Tokyo, Japan) attached to an UV detector. The fractions were eluted using 0.3 M sodium sulfate as the mobile phase at a flow rate of 1 ml/min.

MULTIVARIATE ANALYSIS

Principal component analysis (PCA) was used to uncover the latent structure (dimensions) of a complex data set and extract the crucial information while eliminating noise. PCA performs the optimum coordinate rotation to align the axes thus maximizing variance within the data. This transforms a set of previously correlated variables into a set of uncorrelated ones, which are linear combinations of the original variables. The linear combination that extracts the maximum variance from the data is termed the principal component. Once determined, this component is removed, and the process is repeated to identify the next principal component. This process is continued until all the variance in the data has been explained (in practice, this is not achieved because of residual noise, and the process is terminated). PCA and Cluster analysis were performed using the software R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Viena, Austria. http://cran.r-project.org/) with prior mean centering. Components represent the underlying dimensions that account for the original set of observed data. Component loadings are the correlation coefficients between variables and factors. The squared factor loadings indicate the percentage of the variance in the original variable explained by a factor. Component scores are a composite measure created for each observation on each factor extracted in the factor analysis [Lima et al., 2011b].

Far-UV CIRCULAR DICHROISM MEASUREMENTS

Far-UV (190–250 nm) circular dichroism (CD) spectra were measured for heparin, carboxyl-reduced heparin and desulfated heparin on a Jasco J810 spectropolarimeter (Tokyo, Japan) at 25°C. All measurements were performed using a quartz cell of 1 mm path length and 1.0 mg/ml of the glycosaminoglycan. All spectra were recorded after accumulation of two runs, and a smoothing Fast Fourier Transform (FFT) filter was used to eliminate background effects. All dichroism spectra were corrected for background by subtraction of the water spectrum from the sample spectrum [Lima et al., 2011b].

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

Proton (^{1}H), carbon (^{13}C) and 2D HSQC spectra were recorded using a Bruker AVANCE III 400 MHz spectrometer equipped with a 5 mm inverse probe. Approximately 10 mg of each sample was deuterium-exchanged by lyophilization (three times), dissolved in 99.97% D_2O (Sigma Chemical Co.) and then analyzed. All spectra were recorded at 308 K with solvent (HOD) suppression by presaturation as described by Guerrini et al. [2001].

ANTICOAGULANT AND ANTITHROMBOTIC ACTIVITIES

The anticoagulant activity was measured using whole sheep plasma as described by USP [2008]. The inhibitory effect of heparin on venous thrombosis following venae cavae ligation was measured by the method of Reyers et al. [1980]. Briefly, approximately 1 cm of the inferior venae cavae of rats (below the left renal vein) was exposed and a ligation was performed with a piece of cotton thread (number 8) 5 min after intravenous injection of the test substance. The abdominal cavity was then closed. After 1 h, the cavity was re-opened and the thrombi formed were removed from the vein, washed, blotted with filter paper, dried under vacuum for 24 h, and weighed. At specific times, the heparin samples were intravenously injected in 0.2 ml saline. Ten measurements were performed for each sample. Unmodified UFH from bovine intestinal mucosa was used as a control.

ANTIHEMOSTATIC EFFECTS OF HEPARIN

Hemorrhagic activity was evaluated in the rat-tail model as described previously [Dietrich et al., 1999]. Three month-old male rats were anesthetized with nembutal (40 mg/kg) and urethane (0.8 g/kg). Using a razor blade, a scarification (1–2 mm deep and 5 mm long) was made 15 mm from the distal part of the tail. The tail was then immersed in an isotonic NaCl solution, scraped with gauze, and immersed again in fresh saline to observe bleeding. The duration of bleeding measured without heparin treatment ranged from 30 to 60 s. Grazed tails were further immersed for 2 min in heparin saline solution and washed extensively with saline. The treated tails were then immersed in isotonic saline solution, and the amount of blood released was measured by protein quantitation. The results are expressed as the sum of protein amount in each tube minus the amount of blood present before exposure to the test substance.

BIOTINYLATION OF HEPARIN

GAGs were biotinylated by a method developed and described by Boucas et al. [2008]. Briefly, GAG (10 mg, ~20 μM of uronic acid) and biotin-hydrazide (200 μM) were dissolved in 0.1 M HCl (20 ml), and the pH of the mixture was adjusted to 4.8. EDC (200 μM) was then added and the pH of the reaction maintained at 4.8 by drop-wise addition of 0.01 M HCl for 60 min with continuous stirring. The reaction was stopped by the addition of sodium acetate (pH 4.8) to a final concentration of 0.5 M, and the mixture was stirred for an additional 60 min. The solution was then dialyzed against distilled water and lyophilized. This method conjugates biotin to the carboxyl groups of the uronic acid residues.

BINDING OF HEPARIN TO THE ECM

This assay was performed as previously described [Boucas et al., 2008]. Briefly, 8×10^4 cells per well in 96 well plates were cultured for 3 days. All solutions used in this assay were prepared in PBS and all steps were performed at 4°C. After rinsing several times, the cells were incubated in 1% (w/v) BSA for 20 min, washed and exposed to different concentrations of biotinylated heparins (1.0–6.0 μM) in the presence or absence of 100 times molar excess of each individual heparin-like compound for 60 min. The samples were then washed several times, and the bound biotinylated heparin was detected by incubating with europium-conjugated streptavidin (1:5,000) for 40 min [Martins et al., 2003]. A time-resolved fluorometer was used to measure free europium, and the data (counts s^{-1}) were processed automatically in the MultiCalc software program (Perkin-Elmer Life Sciences).

STATISTICAL ANALYSIS

The ability of the various heparin samples to induce vasodilation was measured after pre-contracting the aorta with 1 μM norepinephrine. The data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using analysis of variance (ANOVA) and Dunnett's post-hoc test for multiple comparisons among groups. A probability value of $P < 0.05$ was considered significant.

RESULTS

CORRELATION BETWEEN VASCULAR RELAXATION ACTIVITY AND BIOCHEMICAL CHARACTERISTICS OF HEPARIN FROM DIFFERENT SOURCES

We have previously shown the effect of four different heparin preparations on rat aorta pre-contracted with norepinephrine [Paredes-Gamero et al., 2010]. UFH from bovine intestinal mucosa showed the highest activity, whereas enoxaparin was the least active form of heparin (Table I). No evidence for a direct correlation

TABLE I. Relative Relaxation Efficacy and Molecular Weight for Heparins of Distinct Sources

Heparins	Relative relaxation* efficacy	Average MW (kDa)
UFH from bovine intestinal mucosa	1.00	12.5
UFH from porcine intestinal mucosa	0.78	13.5
UFH from bovine lung	0.66	13
LMWH enoxaparin	0.63	4.5

*The values for relative relaxation efficacy were calculated from data previously published, where the value for acetylcholine equals 1.7 [Paredes-Gamero et al., 2010].

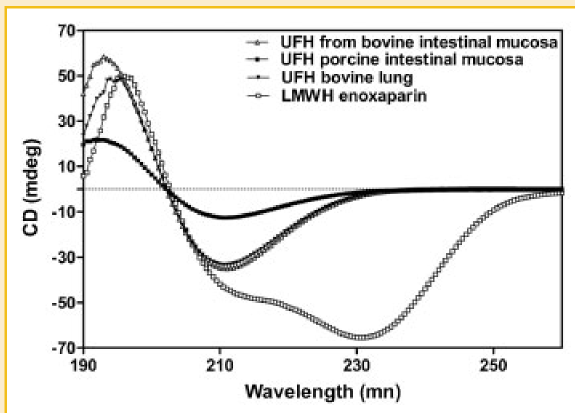

Fig. 1. Heparin Far-UV circular dichroism spectra. All spectra were recorded after accumulation of two runs, and a smoothing FFT filter was used to eliminate background effects. No differences in the spectra were observed between heparin samples at different concentrations (0.5, 1.0, and 10 mg/ml). Spectra were recorded using samples at 1 mg/ml.

between the molecular weight of heparin and its vascular relaxation activity was observed (Table I).

Subsequently, structural analyses were performed to correlate the pharmacological observations with the chemical and conformational characteristics of heparin. Polarized light spectroscopy of heparin samples was measured by CD (Fig. 1). The spectra for UFHs showed a positive maximum at ~190 nm corresponding to the electronic transitions ($n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$) of the oxygen atoms (present in the ring, glycosidic linkage, and hydroxyl moieties) and *N*-sulfate groups, and a negative minimum at 211 nm ($n \rightarrow \pi^*$ transition) arising from the electronic transitions in the carboxylate and *N*-acetyl chromophores. Despite the fact that the various UFHs had similar spectra, they showed different relaxation efficacy. In contrast, enoxaparin showed a negative CD value of approximately 230 nm arising from the unsaturated uronic acid at the non-reducing end of the molecules, but enoxaparin was found to have a vasodilation activity similar to that of bovine lung UFH.

It is generally assumed that the biological effects of heparin are due to the negative charges of the molecule. Therefore, the monosaccharide composition and the degree of sulfation of the heparin samples were determined using 2D NMR spectroscopy (Table II). Among the heparins, enoxaparin and UFH from bovine lung showed the highest degree of sulfation, but displayed the lowest relaxation effect. Moreover, UFH from bovine intestinal mucosa that exhibited the lowest degree of sulfation showed higher relaxation activity. These results support the hypothesis that the relaxation induced by heparin is not associated with the overall charge content of the molecule alone.

To further investigate the relationship between the overall heparin structure and relaxation activity, PCA analysis was performed for spectroscopic data and vasodilatory activity (Fig. 2). Cluster analysis performed on the PCA loading plot for the CD and NMR spectra generated two closely related families (Fig. 2). However, the same analysis performed for the relaxation activity generated distinct families, again suggesting that the overall structure is not directly correlated with the relaxation activity of heparin.

EFFECT OF CHEMICAL MODIFICATIONS ON UFH HEPARIN FROM BOVINE INTESTINAL MUCOSA ON AORTA RELAXATION

To evaluate the role of charge density and chain flexibility in heparin-induced relaxation, UFH from bovine intestinal mucosa was desulfated and carboxyl-reduced. These modifications resulted in approximately 60 and 80% decrease in sulfation and carboxyl groups. Desulfation of the heparin chain can be easily detected in the ^{1}H-NMR spectrum (Fig. 3A). The increased signal intensities at 4.94 and 3.4 indicate 6-*O*-desulfation and *N*-desulfation of the glucosamine moiety, respectively, and the increase at 5.04 corresponds to desulfation of the iduronate residues. The monosaccharide composition of desulfated heparin is shown in Table II. The reduction of carboxyl groups was confirmed by ^{13}C spectra (Fig. 3B). Subsequently, these modified heparin samples were assessed for their antithrombotic (Fig. 4A) and anticoagulant (Fig. 4B) activities as well as their effect on bleeding (Fig. 4C). Desulfation and carboxyl reduction of all the modified heparin samples led to similar decreases in the in vitro anticoagulant activity and in their effect on bleeding. However, the results obtained using aortic rings indicate that desulfated heparin induces similar levels of relaxation (Fig. 5A)

TABLE II. Percent Substitution of Glucosamine, Iduronic Acid, Glucuronic Acid, and Uronic Acid (U) for Heparin Preparations Determined by ^{1}H, ^{13}C and HSQC NMR Spectra

Sample	A_{NS}	A_{NAC}	A_{3S}	A_{6S}	I_{2S}	I	G	A_{NH2}	$M_{NS\text{-}\alpha red}$	A_{NSred}	1.6-an.A	1.6-an.M	ΔU_{2S}	ΔU	Degree of sulfation
UFH															
bovine intestinal mucosa	87.0	10.8	2.2	61.3	63.3	12.5	24.2	–	–		–	–	–	–	2.16
porcine intestinal mucosa	79.5	15.3	5.2	82.0	59.7	15	25.4	–	–		–	–	–	–	2.31
bovine lung	94	2	4	98	86	5	9	–	–		–	–	–	–	2.82
desulfated	1.12	9.07	2.2	58.4	56.3	16.6	27.4	85.4	-		–	–	–	–	1.2
LMWH															
Enoxaparin	74.2	4.9	3.7	90	63.1	3.5	13.4		1.4	10.3	1.4	3.7	18.7	1.1	2.65

a, Percent substitution values were obtained by integration of HSQC spectra as described by Lima et al., 2011a. UFH, unfractionated heparins; LMWHs, low molecular weight heparins; A_{NS}, 2-deoxy-2-sulfoamino-D-glucopyranose; I_{2S}, 2-*O*-sulfo-iduronic acid; G, glucuronic acid; A_{3S}, 2-deoxy-3-*O*-sulfo-2-amino-D-glucopyranose; A_{Nac}, 2-deoxy-2-acetylamino-D-glucopyranose; αred, terminal reducing residue with a configuration; 1,6-an.A, 2-amino-1,6-anhydro-2-deoxy-b-D-glucopyranose; 1,6-an.M, 2-amino-1,6-anhydro-2-deoxy-b-D-mannopyranose; M_{NS}, 2-deoxy-2- sulfamino-D-mannopyranose; ΔU_{2S}, 2-*O*-sulfo-4-deoxy-a-L-threo-hex-4-enopyranosil uronic acid;U, Δ4-deoxy-a-L-threo-hex-4-enopyranosil uronic acid.

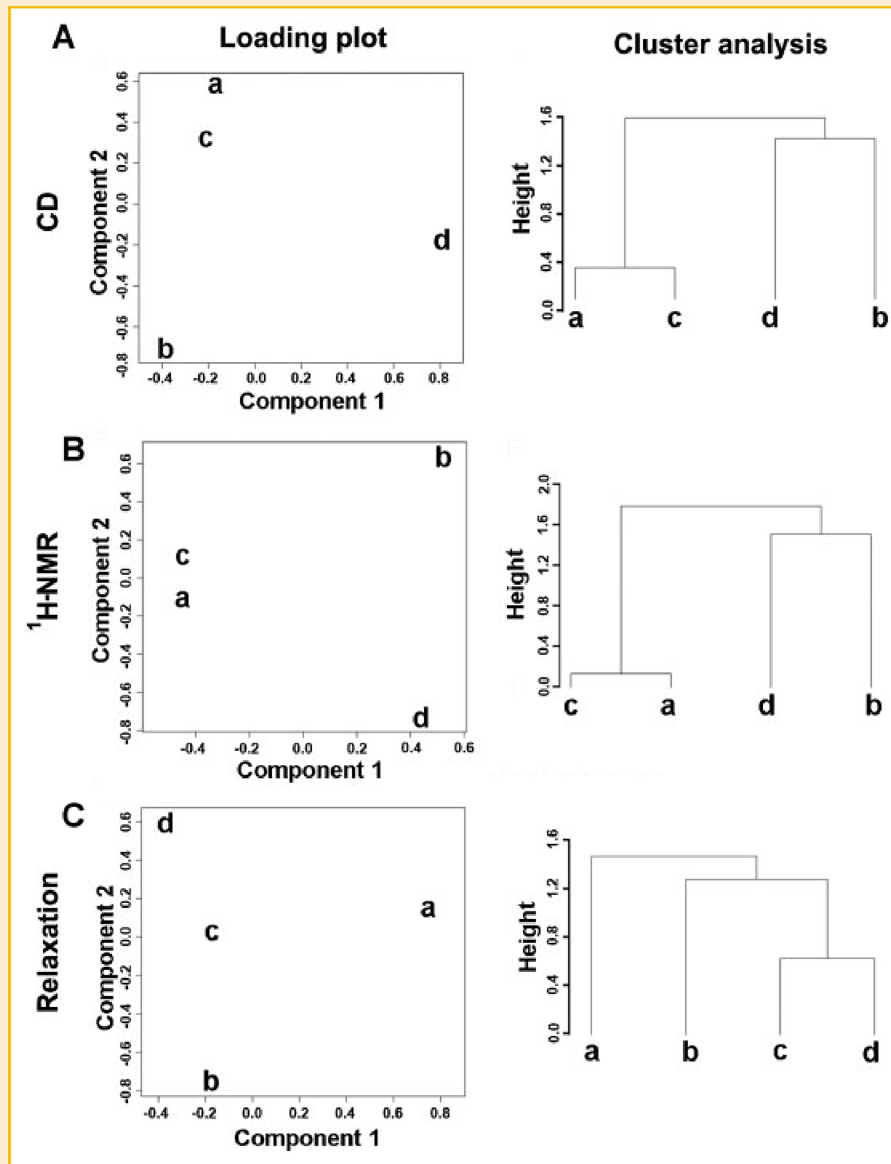

Fig. 2. A–C: Loading plot and hierarchical cluster analysis performed on the load plot derived from PCA of (A) circular dichroism spectra (CD), (B) ^{1}H-NMR spectra, and (C) vascular relaxation activity. The letters were as follows: a, UFH from bovine intestinal mucosa; b, porcine intestinal mucosa; c, bovine lung; d, LMWH enoxaparin.

even though the anticoagulant activity dropped to 50% of that of the parent molecule (Fig. 4B). These results reinforce the hypothesis that heparin-induced relaxation activity is not related to net charge on the molecule.

To further prove this hypothesis, dextran and dextran sulfate, which are non-sulfated and highly sulfated polymeric chains of D-glucose, respectively, were analyzed. At concentrations similar to that of heparin (400 μM), neither dextran nor its sulfated counterpart showed relevant relaxation activity. This demonstrates that sulfate groups alone are not responsible for heparin-induced relaxation effects (Fig. 5A).

CD measurements were then performed for the native and chemically modified heparins to try to correlate the influence of molecular conformation and heparin relaxation activity. The carboxyl-reduced heparin showed a different CD spectrum (Fig. 5B) compared to unmodified samples. The loss of the positive band at 194 nm suggests conformational changes throughout the heparin chain. This band arises from oxygen atoms located in the sugar rings and the glycosidic bonds, which are features critical for chain conformation and are not chemically modified by the carboxy-reduction reaction. In addition, the appearance of a positive band at 235 nm suggests conformational changes in the carboxyl-reduced heparin. On the other hand, CD spectra obtained for the desulfated heparin samples demonstrated a profile similar to that of native heparin, which showed two characteristic bands: A positive band at 194 nm and a negative band at 211 nm (Fig. 5B). It is important to note that the carboxyl-reduced heparin lost its ability to induce aorta relaxation (Fig. 5A) suggesting that heparin-induced relaxation is dependent on the spatial structure rather than net charge of heparin.

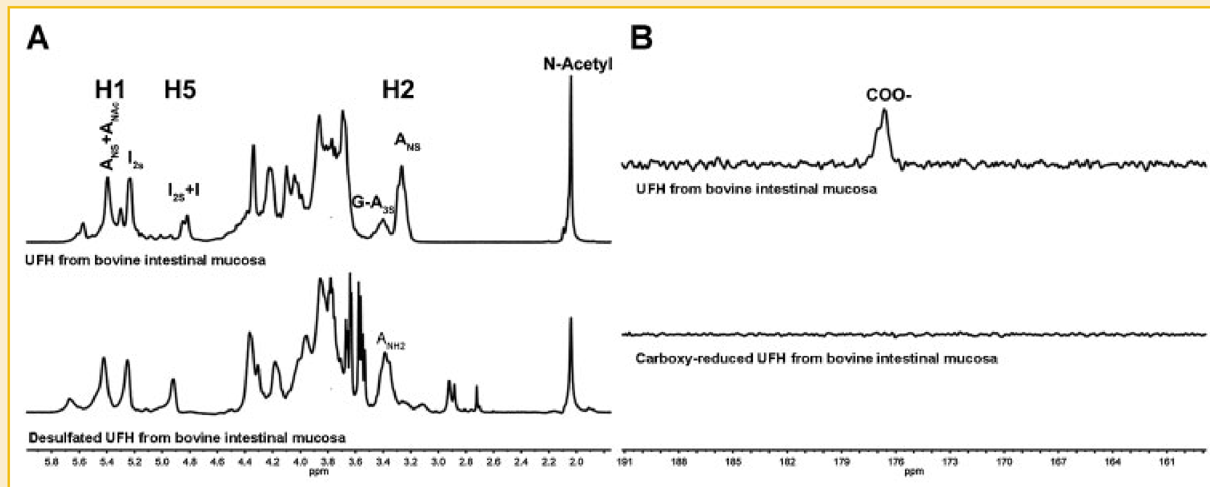

Fig. 3. A: ^{1}H-NMR spectra of heparin samples recorded on a 400 MHz NMR spectrophotometer. The spectra were recorded at 308 K. B: ^{13}C NMR spectra of heparin and its carboxy-reduced form.

As shown previously, the induction of relaxation by heparin is dependent on its binding to the ECM. Therefore the ECM binding affinity of the modified heparin samples was determined. As expected, unmodified heparin showed the lowest apparent Kd of 54 nM, whereas desulfated heparin showed a Kd of 71 nM and carboxyl-reduced heparin showed the highest apparent Kd of 83 nM (Fig. 5C), which is consistent with the pattern of relaxation induced by these samples.

DISCUSSION

Several studies have demonstrated a correlation between the effects of heparin on thrombosis and its action on the blood coagulation cascade and on blood vessels [Nader et al., 2004; Fareed et al., 2008]. The hypotensive effects of heparin have been described in patients undergoing a variety of clinical procedures [Waser and Keller, 1954; Keller, 1956; Kongsgaard et al., 1991]. Moreover, heparin induces a decrease in blood pressure in patients with glomerulonephritis [Kutyrina et al., 1985] and in hypertensive humans [Abbott et al., 1966] and rats [Wilson et al., 1981; Susic et al., 1982; Kentera et al., 1985]. Other experiments showed an endothelium-dependent induction of relaxation by heparin in the smooth muscle of the rat aorta [Kouretas et al., 1998]. Recently, we described the molecular and pharmacological aspects of heparin-induced vascular relaxation in the rat thoracic aorta [Paredes-Gamero et al., 2010]. We showed that the interaction between heparin and ECM molecules of the endothelial cells led to the activation of integrin-associated focal adhesion proteins, which help maintain the active state of the muscarinic M_3 receptor, thereby triggering NO production [Paredes-Gamero et al., 2010; Medeiros et al., 2011]. However, the structural requirements of heparin-induced vascular relaxation are unknown. In this study, the role of the structural characteristics of heparin in its pharmacological activities was analyzed. Our results showed that neither average molecular weight nor overall sulfation of heparin seems to have a direct correlation with its relaxation-inducing effect (Fig. 2, Table I and Table II). Therefore, other factors such as chain flexibility of heparin, which is determined by the β-D-glucuronic acid α-L-iduronic acid content, as well as glycosidic linkages and substitution patterns in the molecule could contribute to this effect.

The α-L-iduronic acid in the heparin chain is known to possess great flexibility, thereby increasing the versatility of the chain by allowing it to assume conformations that are necessary for certain molecular interactions. The monosaccharide composition analysis (Table II) revealed that higher levels of non-sulfated iduronic acid are associated with a higher relaxation activity, indicating that heparin-induced relaxation may be dependent on heparin chain flexibility rather than overall sulfation. Interestingly, because the amount of 3-*O*-sulfation on the glucosamine moiety does not seem as important for this effect as it is for the anticoagulant activity, heparin-induced relaxation is most likely anticoagulant-independent. Moreover, *N*-acetyl levels are higher in heparin samples that induce more relaxation. Carboxyl-reduced heparin lost its ability to induce aorta relaxation (Fig. 5A) and showed a decreased affinity toward the ECM (Fig. 5C). Carboxyl groups are important for the spatial conformational of heparin due to the flexibility of the pyranose ring in the iduronic acid residue [Mulloy and Forster, 2000; Rudd and Yates, 2010]. Also, it has been shown that orientation of the carboxylate groups on uronate residues are important factors for protein biding, hence, biological activity [Guerrini et al., 2008]. Therefore, it is most likely that heparin-induced relaxation is dependent on the conformation rather than the overall net charge of heparin.

On average, heparin chains have 3.5–4 negative charges per disaccharide unit, and the carboxyl groups are responsible for approximately 30% of the heparin anionic character. But carboxyl groups are more pH sensitive due to their higher pKa values (4.5–5) compared to the sulfate groups (pKa < 2). As a result, the levels of carboxylate protonation near physiological pH are much greater, leading to removal of some of the negative charges on the molecule. Consistent with this observation, desulfation, which alone reduces

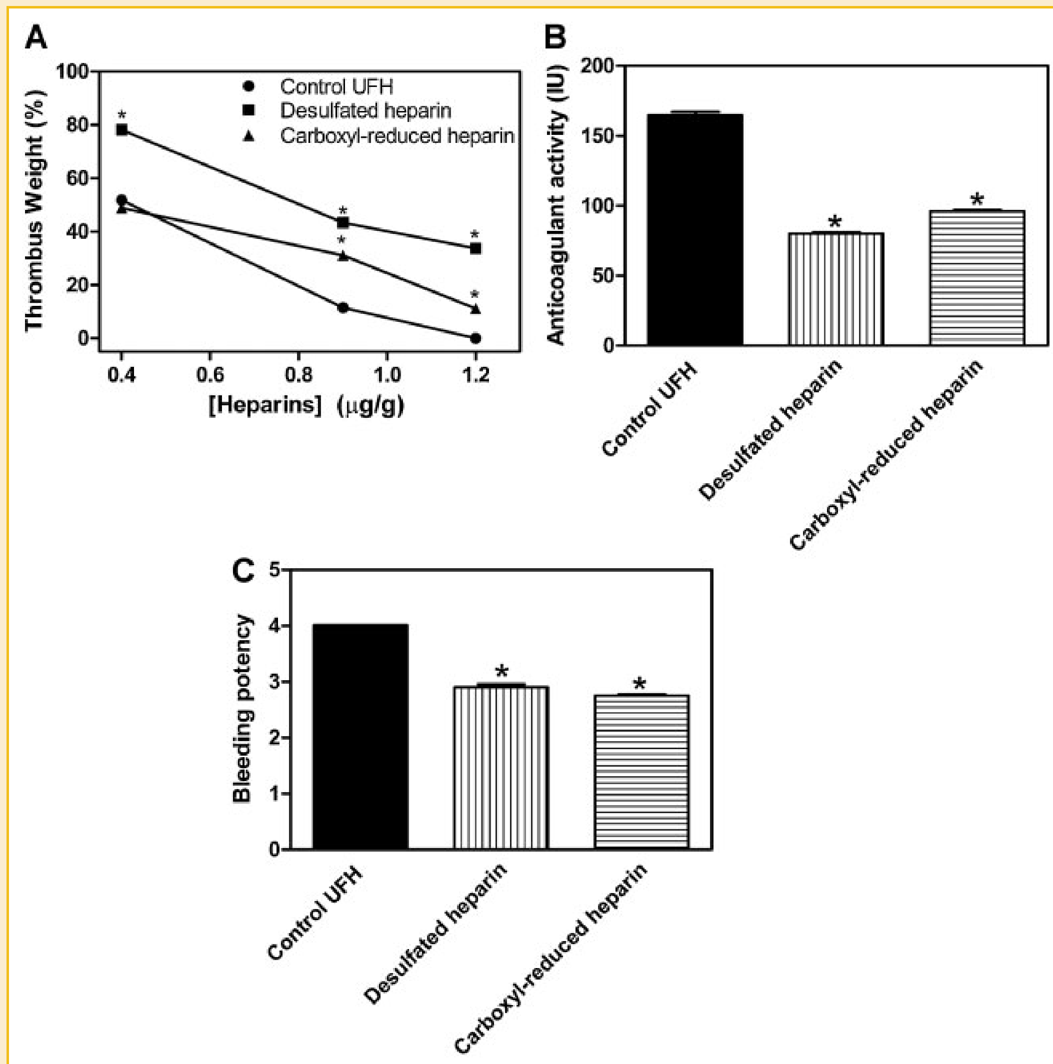

Fig. 4. Antithrombotic, anticoagulant, and antihemostatic activities of modified heparin samples and UFH from bovine intestinal mucosa. A: In vivo antithrombotic activities. The values were normalized as percentage of thrombus weight in absence of heparin (100%). B: Anticoagulant activity of heparin (IU, International units). C: Antihemostatic activity of heparin. Data are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$. A–C: The modified heparin samples used had 60% desulfation (desulfated heparin) and 80% reduction of the carboxyl groups (carboxyl-reduced heparin).

approximately 60% of the overall net charge, rules out the possibility that the chemistry (negative charge itself) rather than structure is more important for induction of relaxation by heparin. Indeed, other sugars such as dextran and dextran sulfate did not possess high relaxation activity (Fig. 5A).

CD analysis was carried out for native and chemically modified heparin samples from bovine intestinal mucosa to determine the effect of molecular conformation on heparin activity. Both native and desulfated heparin samples showed similar CD spectra in which two characteristic bands were visible (Fig. 5B). These results are in agreement with optical models of disaccharide units of heparin [Stone, 1985] and other heparin CD spectra [Park and Chakrabarti, 1977; Villanueva and Allen, 1984]. Although the various UFHs showed similar spectra, they presented different relaxation efficacies. In contrast, the carboxyl-reduced heparin spectrum was quite unique. The loss of the positive band at 194 nm and the appearance of a positive band at 235 nm (Fig. 5B) suggest a strong spatial alteration.

The results obtained in aortic ring experiments indicate that desulfated heparin still induces levels of relaxation comparable to that of native heparin (Fig. 5A). This is important because the desulfated heparin exhibits reduced antithrombotic, anticoagulant, and antihemostatic activity (Fig. 4). In addition, the absence of relaxation activity of dextran sulfate supports the hypothesis that sulfate groups alone are not responsible for this effect (Fig. 5A). Moreover, previous results showed that a non-anticoagulant *N*-acetylated heparin retained its aorta relaxation activity [Kouretas et al., 1998], reinforcing the hypothesis that the relaxation activity of heparin is indeed net charge- and anticoagulation-independent.

Our results also showed that the carboxyl group, which affects the spatial conformation of heparin, is important for heparin function. Modification of the uronic acid residues profoundly affects the

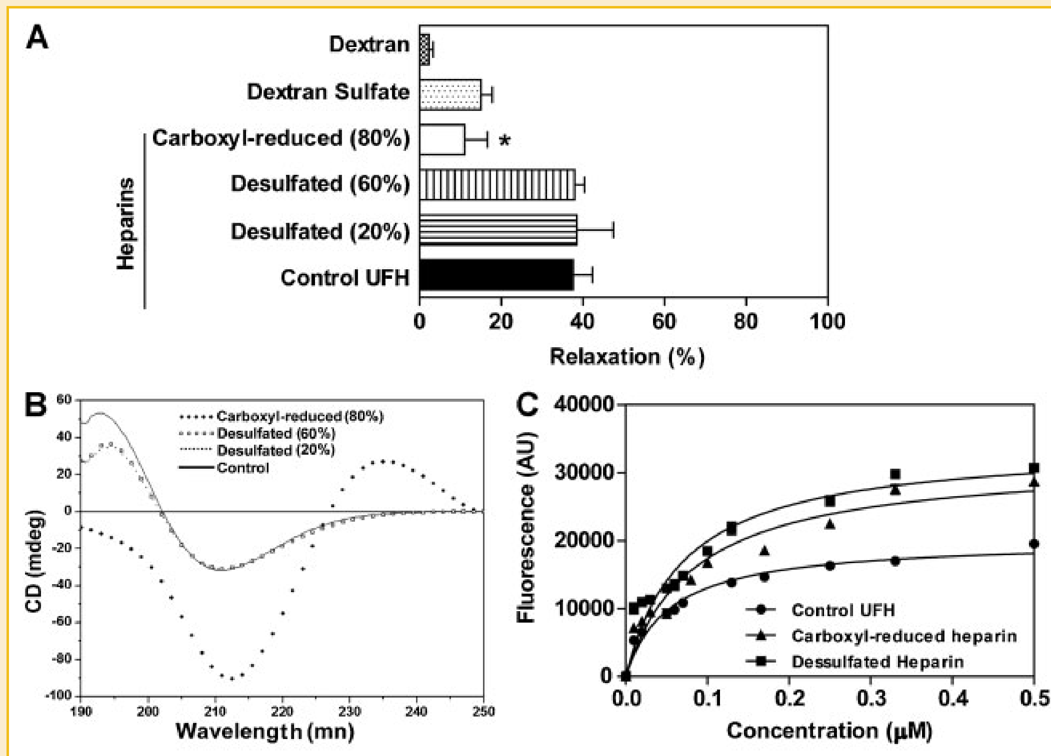

Fig. 5. Carboxyl-reduction, but not desulfation, decreases efficacy of heparin in inducing relaxation of pre-contracted aorta. A: Relaxation values are expressed as percentage of reversal of pre-contraction with norepinephrine (10 min) in the presence of heparin for 30 min. Data are expressed as the mean ± SEM of four independent experiments. $^{*}P < 0.05$. B: Far-UV CD spectra of native heparin, carboxyl-reduced heparin and desulfated heparin. UFH from bovine intestinal mucosa was desulfated and carboxyl-reduced. The heparin concentration used was 1 mg/ml for all samples. C: Kinetics of biotinylated heparin binding to the extracellular matrix. The bound BiotHep was detected by incubation with europium-conjugated streptavidin and quantified in a time-resolved fluorometer. The results are expressed as the mean ± SEM of three different experiments performed in triplicate.

conformational and dynamic properties of the resulting heparin molecule. The loss of sulfate groups in heparin affects its anticoagulant, antithrombotic, and antihemostatic activity but does not alter its vascular relaxation activity. Therefore, we suggest a new potential use for heparin as a vascular relaxation molecule. Characterization of the structural requirements necessary for heparin-induced vascular relaxation can be useful for the development of novel therapeutic agents based on heparin derivatives.

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