

Oversulfated Chondroitin Sulfate and OSCS-Contaminated Heparin Cause Dose- and Route-Dependent Hemodynamic Effects in the Rat

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During 2007–2008, serious adverse events were reported following iv administration of certain batches of commercially available heparin in humans. Anaphylactoid reactions with acute hypotension were the hallmark of these cases. Subsequently, it was shown that a contaminant, oversulfated chondroitin sulfate (OSCS), was responsible for these adverse events. The present study was undertaken to further elucidate the risks related to OSCS-contaminated heparin preparations. Using an anesthetized rat hemodynamic model, marked diastolic blood pressure drops were induced with a single iv injection of a contaminated heparin (1000 IU/kg; 34% wt/wt OSCS). OSCS alone (0.8 and 20 mg/kg) or in combination (0.8–1.7 mg/kg) with uncontaminated heparin produced a similar hypotensive effect, whereas heparin spiked with 0.2 or 0.4 mg/kg OSCS produced no hemodynamic changes. In conscious rats, acute hypotensive effects were seen following single iv administration of OSCS-spiked heparin (1.7 or 3.0 mg/kg). Conversely, no hemodynamic effects were observed with same doses when administered sc. Pretreatment with a bradykinin-2 receptor antagonist (HOE140) fully abolished the hypotensive response after iv OSCS (1.7 mg/kg) administration, whereas pretreatment with the histamine (H1) receptor antagonist cetirizine did not. *In vitro*, OSCS (25 and 250 µg/ml) induced a robust, dose-related increase in kallikrein activity in rat and human plasma with a lower amplitude of response in dog and pig. The data suggest that the adverse events associated with OSCS-contaminated heparin are dependent upon the concentration of contaminant and its route of administration. Furthermore, the kallikrein-kinin system plays a pivotal role in the initiation of OSCS-related vascular effects.

Key Words: OSCS; heparin; cardiovascular; rat.

During the period between the end of 2007 and midyear 2008, a large number of severe adverse events (SAEs) were reported in patients after iv dosing with high-molecular weight heparin, with fatal outcomes in some instances. Hypotension and nausea were the most prominent features, with dyspnea,

vomiting, tingling, angioedema, and flushing representing other common symptoms (Blossom *et al.*, 2008). Suspect heparin products were eventually recalled by the manufacturers (FDA Press Release, 2008a,b). German authorities (Bundesinstitut für Arzneimittel und Medizinprodukte [BfArM]) also initiated a recall of heparin batches after reports of about 80 cases of similar SAEs following iv administration of heparin solution (WHO, 2008). Soon thereafter, the U.S. Food and Drug Administration (FDA) announced that a “heparin-like” compound had been detected as a contaminant in heparin products. Ultimately, the contaminant was identified as oversulfated chondroitin sulfate (OSCS) representing up to about 30% wt/wt in adulterated lots of heparin (Avorn, 2008; Guerrini *et al.*, 2008; Warkentin and Greinacher, 2009). Because OSCS is structurally similar to heparin and possesses its own anticoagulant activity, its presence was undetected using standard chemical and biological methods used for heparin characterization, thus allowing for its introduction into the clinical supply.

Soon after the reports of adverse reactions associated with contaminated heparin began to appear, Kishimoto *et al.* (2008) made a first attempt to identify the biological link between the presence of OSCS in heparin and the adverse clinical events. In this study, OSCS from contaminated heparin lots and synthetically generated OSCS were found to activate the kinin-kallikrein pathway in human plasma, which can lead to the production of the vasoactive mediator bradykinin (BK) (Moreau *et al.*, 2005). Furthermore, OSCS induced generation of the anaphylatoxins C3a and C5a *in vitro* and elicited kallikrein activation and hypotension in the swine when administered by iv infusion. The ability of OSCS to induce both of these biochemical pathways was found to be dependent upon the presence factor XII.

The finding that OSCS-mediated blood pressure (BP) effects resulted from a specific pharmacological mechanism, as opposed to a hypersensitivity reaction as suggested originally, is critical

for a proper risk evaluation and management. Such mechanisms are generally dose or concentration dependent and have a clear threshold below which risk is regarded as limited. Furthermore, a toxic effect mediated through activation of an enzyme cascade in circulating blood may be attenuated by altering the route of administration of the heparin preparation.

This paper describes investigations carried out to replicate OSCS-mediated acute effects on BP, similar to those reported in humans, in a rat hemodynamic model. This model was further used to assess the dose and route dependence of those effects and to strengthen the evidence for a direct activation of the blood contact system cascade as a likely mechanism underlying the serious adverse events reported in patients.

MATERIALS AND METHODS

Drugs and chemicals. OSCS (C16H22NO23S4.5Na) and HOE140 (CAS 138614-30-9; type 2 BK receptor antagonist) were synthesized by the Chemical and Analytical Sciences Department of sanofi-aventis. OSCS-contaminated heparin (Baxter Pharmaceuticals—5000 IU/ml stock solution with 34% OSCS contamination determined using an in-house Nuclear Magnetic Resonance assay) was representative of the commercial product resulting in reported SAEs. Standard heparin was provided by Choay (Notre Dame de Bondeville, France) or American Pharmaceutical Partners (Schaumburg, IL). BK, histamine, cetirizine (histamine receptor 1 antagonist), chromogenic substrate S-2302, and pentobarbital were from Sigma (St Louis, MO). Test substances were diluted or dissolved into saline as necessary to adjust bolus volume to 1 ml/kg.

Animals and biological samples. The animals used in these studies were handled and maintained in accordance with the requirements of European Economic Community guideline (1986) and U.S. Federal Guidelines (1985). Compliance to the above public laws was accomplished by conforming to the standards promulgated in the Guide for the Care and Use of Laboratory Animals, National Academy Press (Institute for Laboratory Animal Research), revised 1996. Male (M) and female (F) Sprague Dawley (SD) rats were supplied by Charles River Laboratories (L'Arbresle Cedex, France or Wilmington, MA).

Human plasma samples were obtained from Instrumentation Laboratory Company, Inc. (München, Germany) for control plasma, Hyphen BioMed (Neuville sur Oise, France) for prekallikrein-deficient plasma, and Precision Bio Logic (Nova Scotia, Canada) for factor XII-deficient plasma. In addition, plasma samples were collected from Harlan-Winkelmann rats, beagle dogs, and Landrace pigs.

Hemodynamic evaluation in the anesthetized rat model. Rats were anesthetized by an ip administration of sodium pentobarbital (60 mg/kg). A catheter filled with a heparinized (1%) aqueous solution of 0.9% sodium chloride was inserted and firmly secured in the artery. A second catheter was inserted in the jugular vein. The free end of the catheter inserted in the carotid artery was connected to a BP amplifier via a specific transducer for BP recording. Data acquisition was performed through a specific software (Hem 3.5, Notocord systems, Paris, France) configured for a continuous recording at a sampling rate of 500 Hz. After a control period of 10 min, the various formulations were administered as an iv bolus injection (1 ml/kg) into the jugular vein. Anesthetized SD rats received the following treatments: an aqueous solution of 0.9% sodium chloride (control group: $n = 7$ M and 7 F), OSCS-contaminated heparin ($n = 6$ M), OSCS at 0.8 ($n = 3$ M and 4 F) and 20 mg/kg ($n = 6$ F), and heparin alone (1000 IU/kg) ($n = 7$ M and 7 F) or in combination with OSCS at 0.2, 0.4, 0.8, 1.2, or 1.7 mg/kg ($n = 7$ M and 7 F

each dose level). All doses and concentrations were expressed in terms of pure active ingredient. The iv route was chosen as it is the clinical route for which the adverse events had been reported in patients. The doses of OSCS were selected based on reported information regarding serious adverse events with OSCS-contaminated heparin (Fareed *et al.*, 2008). The range was chosen to approximate 5 (0.21 mg/kg)–50% (2.09 mg/kg) contamination of a 50,000 IU heparin dose (human dosage). The 1000 IU/kg dose for heparin represents approximately the maximal therapeutic dose for this compound (50,000 IU per patient) in a clinical setting.

BP was recorded via the arterial heparinized catheter, systolic (SBP, mmHg), diastolic (DBP, mmHg), and mean BPs (MBP, mmHg) being automatically measured. The heart rate (HR) was calculated from the pressure signal (beats per min). Measurements were made before, during, and for 40 min after administration. For each time point, each parameter was calculated as the average of the data recorded during the preceding minute. For each parameter and at each selected time point, changes from initial values were calculated as the difference between the value of the parameter measured at time t and the initial predose value. In addition, relative changes were also calculated as $[(\text{parameter at time } t) - (\text{initial value})]/[(\text{initial value}) \times 100]$. No statistical analysis was performed.

BK and histamine studies. To investigate the involvement of BK and histamine pathways in the OSCS-induced hypotension, the anesthetized rats were dosed with HOE140, a highly specific type 2 BK receptor antagonist (Moreau *et al.*, 2005), or cetirizine, a histamine receptor type 1 antagonist (Gillard *et al.*, 2002), prior to treatment with OSCS. The doses of BK and histamine used were determined in anesthetized rats together with a preliminary determination of the potency of HOE140 and cetirizine to block the BK- and the histamine-induced effects, respectively.

For the preliminary BK study, male and female rats received increasing doses of BK (3, 10, and 30 $\mu\text{g/kg}$; under a dosing volume of 1 ml/kg) as bolus iv injections separated by 5 min. After recovery from the effects of the third dose, the dose of HOE140 to be tested (0.0003, 0.001, 0.003, or 0.01 mg/kg; $n = 2$ –5 per dose level) was infused over 30 min. At the end of the infusion, the three doses of BK were readministered in 5-min intervals to each rat. For each rat, a basal BP value was determined for the period without HOE140 (BV_{cont}) and another one for the period with HOE140 (BV_{HOE}). After each dose of BK, BP was evaluated at the peak of the effect and expressed as the mean value calculated over a 4-s period of time. The BK-induced changes from basal values were calculated as the difference between the value of the parameter measured at the peak effect and BV_{cont} in the absence of HOE140 or BV_{HOE} after HOE140 administration.

Following this study, rats received either an aqueous solution of 0.9% sodium chloride ($n = 7$ M and 6 F) or HOE140 at doses of 0.0003 ($n = 5$ M and 7 F), 0.003 ($n = 7$ M and 6 F), 0.03 ($n = 6$ M and 6 F), 0.1 ($n = 6$ M and 4 F), or 0.3 ($n = 7$ M and 6 F) mg/kg administered as a 30-min iv infusion followed by a bolus iv injection of OSCS (1.7 mg/kg). Hemodynamic parameters were recorded over the period up to 40 min postinfusion. For each rat, basal BP was evaluated before OSCS administration and expressed as the mean value calculated over a 30-s period of time. After OSCS administration, BP was evaluated at the peak of the product-related effect and expressed as the mean value calculated over a 4-s period of time. The main response obtained in the control group (receiving OSCS only) was considered as the reference hypotensive response. The hypotensive responses to OSCS after HOE140 pretreatment were calculated relatively to this maximal response.

The following experimental setting was applied to assess the contribution of the histaminergic pathway in OSCS-related hypotension. In a preliminary experiment to confirm the effects of histamine and cetirizine, rats ($n = 2$ M and 2 F) received increasing doses of histamine (1, 3, and 10 $\mu\text{g/kg}$) as bolus iv injections 5 min apart. After recovery of the effects of the third dose, cetirizine (1 mg/kg) was administered as an iv bolus injection. At the end of the bolus, the three same doses of histamine were tested again and the effects of cetirizine were recorded. In the main study, rats ($n = 6$ rats per sex per group) received an iv bolus injection of 10 $\mu\text{g/kg}$ histamine to check for their responsiveness. After recovery, they received an iv bolus injection of either an aqueous solution of

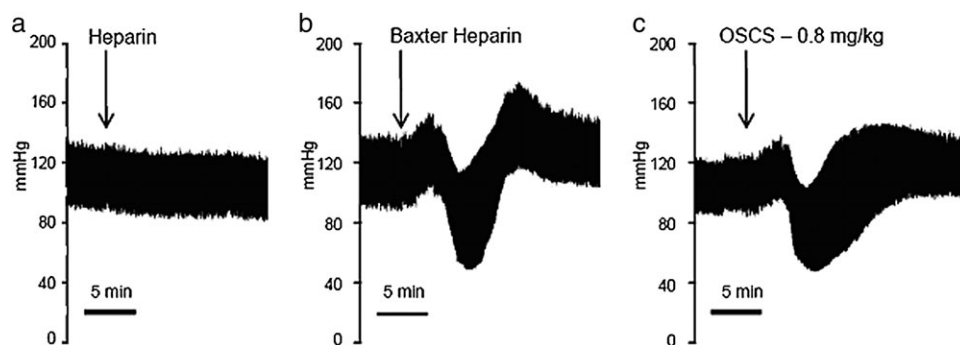


FIG. 1. Typical BP response to heparin (a), Baxter heparin (b), and OSCS (c) following iv bolus administration in the anesthetized rat.

0.9% sodium chloride or cetirizine (1 mg/kg). Thereafter, OSCS (1.7 mg/kg) was administered as bolus injection. For rats receiving cetirizine and OSCS, an additional injection of histamine was performed about 40 min after the injection of OSCS to check for actual histamine receptor blockade. Hemodynamic parameters were recorded from the control period up to 40 min post-OSCS administration.

Hemodynamic evaluation of OSCS in conscious rats. Male and female catheterized SD rats were obtained from Charles River. Rats were catheterized at the jugular vein and at the carotid artery. Six to 10 days after surgery, the rats were used for hemodynamic experiments in the conscious, freely moving state. Before the experiments, the catheters in each rat were drained and refilled with heparinized (100 IU/ml) saline (American Pharmaceutical Partners). During the experiment, each rat was kept in a shoe box-type cage. After a stabilization period of 45 min, rats were injected with saline, heparinized (1000 IU/ml) saline, or heparinized saline spiked with either 1.7 or 3.0 mg/kg OSCS. Solutions were administered as either an iv bolus (through the jugular catheter) or an sc injection. The number of animals was 9–11 per treatment per gender per route of administration. The dosing volume was 1 ml/kg for all rats. The postdosing observation period was 60 min for the iv experiments and 120 min for the sc experiments. The carotid catheter was connected to a pressure transducer (Argon Medical devices, Athens, TX) through which BP was measured continuously with a computerized data acquisition system (Gould/Ponemah, Data Sciences International, St Paul, MN) as 1 min averages. The parameters generated were DBP, SBP, and MBP. HR was derived from the BP waveform. Statistical analysis was carried out with a two-way ANOVA repeated measures (Sigma Stat software) followed by a Student-Newman-Keuls analysis to compare values at concurrent time points (all treatment groups were compared with the heparin-alone-treated group). Differences were considered significant when $p < 0.05$.

Pharmacodynamics of OSCS after a single sc and iv injection in male and female SD rats. The bioavailability of OSCS (1.7 mg/kg) after iv and sc dosing to conscious male and female rats was determined by means of the activated partial thromboplastin time (APTT) in plasma. The dose of 1.7 mg/kg OSCS (in an aqueous 0.9% sodium chloride solution) was administered under a volume of 1 ml/kg by iv bolus injection into the tail vein or by sc injection in the scapula area ($n = 3$ per gender, route, and sampling time). Blood samples (3.5 ml) were withdrawn from the abdominal aorta into citrated tubes under isofluran anesthesia. Blood samples were collected from three animals per sampling time at the following time points: 5 min, 15 min, 30 min, 1, 2, 3, 4, 6, 24, 48, and 72 h. The baseline value was determined on additional plasma samples obtained from control animals ($n = 10$ per sex) sampled in the same conditions as above (abdominal aorta). APTT was measured by chronometric assays using an exploratory method based on the one used in humans (Collignon *et al.*, 1990). The clotting time prolongation (Δt) or difference between the clotting time measured (t_i) and the baseline value before any anticoagulation treatment (t_0), ($\Delta t = t_i - t_0$, expressed in s) was calculated for OSCS pharmacokinetic assessment. The following pharmacodynamic

parameters were calculated from the mean APTT clotting time prolongation at each time point using a noncompartmental approach (WinNonlin version 3.3, Pharsight, Cary, NC): $A_{\max}$ (Δs) = maximum observed APTT clotting time prolongation, AUC_{last} ($\Delta s \cdot h$) = area under the curve for APTT clotting time prolongation from time 0 to time t (t being the last reliable measured APTT value).

Kallikrein activity induced by OSCS in human, rat, dog, and pig plasma. Pooled plasma (human, rat, dog, and pig), prekallikrein- and factor XII-depleted human plasma were incubated with various concentrations (0.025, 0.25, 2.5, 25, and 250 $\mu\text{g/ml}$) of OSCS. One volume of test agents (unfractionated heparin; OSCS; APTT reagent) dissolved in Tris Buffered Saline (TBS) buffer (50 μM Tris-HCl, 150 μM NaCl, pH 7.4) was mixed with one volume plasma (human control plasma, human prekallikrein-deficient plasma, human factor XII-deficient plasma, citrated rat plasma, citrated dog plasma, and citrated pig plasma). Twenty microliters of the diluted plasma samples containing test agents were mixed in a 96-well microtiter plate with 80 μl saline buffer. The amidolytic activity of plasma kallikrein was assessed by adding 50 μl of 2mM chromogenic substrate S-2302 at 37°C. Samples were monitored by continuous spectrophotometric measurement of change in absorbance at 450nm for 10 min. All samples for each individual experiment were run in quadruplicates; means $\pm$ SEMs represent measurements from eight separate experiments. APTT reagent was evaluated as a positive control activator for the contact system. TBS buffer alone was included as negative control.

RESULTS

Hemodynamic Effects of OSCS-Contaminated Heparin, OSCS Alone, or in Combination with Heparin in the Anesthetized Rat Model

A relevant hypotensive response was defined as a reduction in DBP of 15 mmHg or more within 10 min after iv administration. This threshold was set based on magnitude of spontaneous variations of DBP as observed in control animals and predose recordings. Figure 1A shows that a single iv injection of saline or heparin at 1000 IU/kg did not result in any relevant change in BP or HR. Contaminated heparin (34% wt/wt OSCS), tested at 1000 IU/kg, produced in four of six rats a marked hypotensive effect starting 4–8 min after injection. A typical tracing is shown in Figure 1B. In responsive animals, the effect was more pronounced on DBP than on SBP. DBP was maximally decreased by 52 ± 3 mmHg (28–69 mmHg corresponding to -40 to -65% of predose value), whereas

TABLE 1

Hemodynamic Effects of OSCS Alone or in Combination with Heparin (1000 IU/kg) and of OSCS-Contaminated Baxter Heparin (1000 IU/kg). The Corresponding Level of Contamination for Heparin Was Calculated Based on a Strength of 150–200 IU/mg

Treatment	Corresponding level of heparin contamination (%)	Number of animals tested	Number of responsive animals	Range of changes for DBP (mmHg) ^a	Mean + SEM change for DBP (mmHg) ^a
Heparin	0	14	0	NA	NA
Contaminated Baxter heparin	34	6	4	–69 to –28	–52 ± 3
OSCS (0.8 mg/kg)	NA	7	3	–69 to –30	–47 ± 12
OSCS (20 mg/kg)	NA	6	6	–60 to –38	–52 ± 3
OSCS (0.2 mg/kg)-spiked heparin	3–5	14	0	NA	NA
OSCS (0.4 mg/kg)-spiked heparin	6–8	14	0	NA	NA
OSCS (0.8 mg/kg)-spiked heparin	12–20	14	1	–44	–44
OSCS (1.2 mg/kg)-spiked heparin	18–24	14	8	–23 to –60	–44 ± 4
OSCS (1.7 mg/kg)-spiked heparin	25–34	14	13	–18 to –82	–48 ± 5

Note. NA, not applicable.

^aData include responsive animals only (i.e., animals presenting with a change in DBP).

SBP was reduced by 18 ± 6 mmHg only (4–34 mmHg corresponding to –4 to –24%; Table 1). Thereafter, hypotension progressively lessened, and BP returned close to initial values with a transient rebound. No significant compound-related changes in HR were noted.

When administered alone at 0.8 and 20 mg/kg, OSCS induced hypotensive responses very similar to those elicited by contaminated heparin. A typical tracing obtained after administration of OSCS at 0.8 mg/kg is shown in Figure 1C. The number of responsive animals increased with the dose (three of seven at 0.8 mg/kg and six of six at 20 mg/kg), whereas the amplitude of BP changes did not (Table 1).

At 0.8 mg/kg, DBP was reduced by 47 ± 12 mmHg (30–69 mmHg corresponding to –36 to –60% of the predose value) and SBP by 18 ± 6 mmHg (9–44 mmHg corresponding to –8 to –29%). At 20 mg/kg, DBP was reduced by 52 ± 3 mmHg (38–60 mmHg corresponding to –50 to –64%) and SBP by 37 ± 5 mmHg (17–45 mmHg corresponding to –15 to –37%). Among the animals regarded as nonresponsive at 0.8 mg/kg, one of seven developed a moderate increase in BP. OSCS had no relevant effect on HR at any dose administered. Because the decreases in DBP appear to be the most prominent OSCS-related change, we focused our analysis on this hemodynamic parameter for the subsequent *in vivo* studies.

OSCS at 0.2 or 0.4 mg/kg in combination with a fixed dose of heparin (Fig. 2 and Table 1) had no hemodynamic effect. From 0.8 mg/kg OSCS, the incidence of hypotension was dose dependent with 1 of 14 responsive animals at 0.8 mg/kg, 8 of 14 animals at 1.2 mg/kg, and 13 out of 14 at 1.7 mg/kg. Among the responsive animals, the amplitudes of the DBP fall were roughly similar, regardless of the OSCS dose administered: 44 mmHg at 0.8 mg/kg (–36% from baseline), 23–60 mmHg at 1.2 mg/kg (–31 to –61%), and 18–82 mmHg at

1.7 mg/kg (–25 to –63%). In some instances, a mild to moderate increase in BP occurred and persisted during the period of observation. OSCS-spiked heparin showed no relevant effect on HR.

Inhibition of OSCS-Induced Hypotensive Effects by HOE140 (Type 2 BK Receptor Antagonist)

A preliminary experiment was designed to confirm functional antagonism of BK by HOE140. BK was investigated at doses of 3, 10, and 30 μ g/kg prior to and after increasing doses of HOE140 (Fig. 3). BK induced a dose-dependent decrease in BP that was rapidly reversible in the minutes following administration. The effect was more pronounced on DBP compared with SBP. The highest dose of BK tested (30 μ g/kg) elicited a hypotensive response with an amplitude of about –40 mmHg comparable to the one elicited by OSCS. No clear effect on HR was noted.

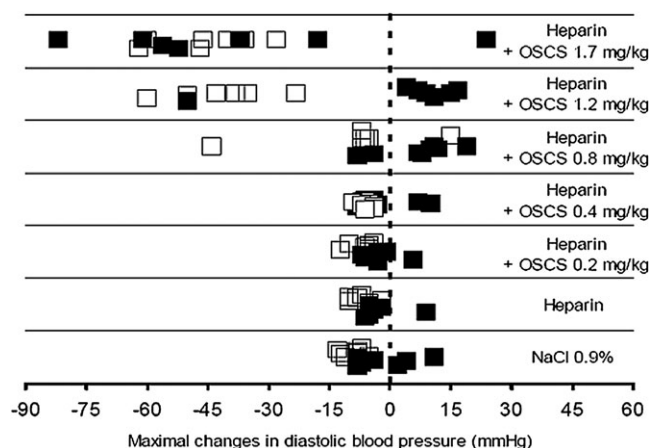


FIG. 2. Magnitude of DBP changes (mmHg) in response to OSCS-spiked heparin in the anesthetized rat. Squares represent the individual maximal changes in DBP in male (■) and female (□) rats.

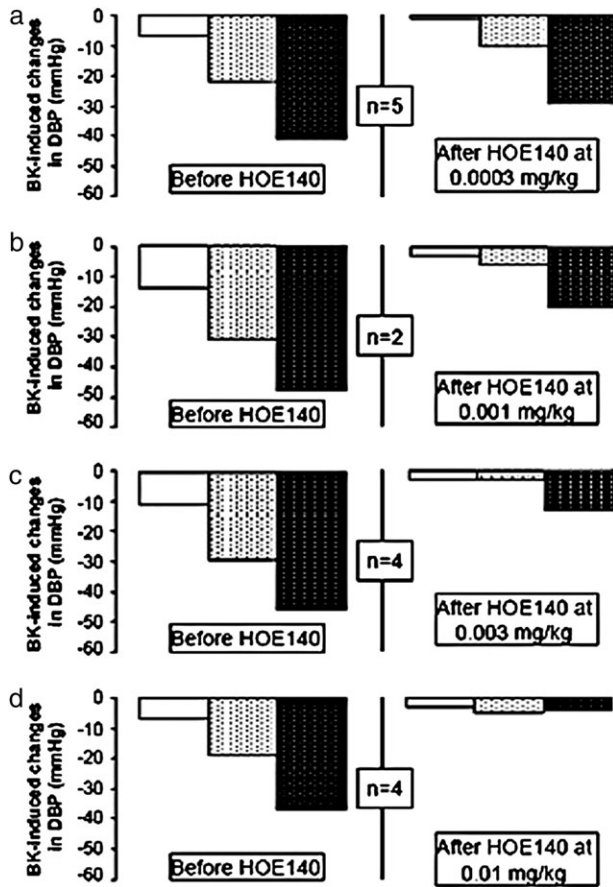


FIG. 3. Inhibitory effect of HOE140 on BK-induced DBP changes in the anesthetized rat. Mean DBP changes after BK at 3, 10, and 30 $\mu\text{g/kg}$ are reported before and after treatment with HOE140 at 0.0003 (a), 0.001 (b), 0.003 (c), or 0.01 mg/kg (d).

HOE140 inhibited the BK effects in a dose-dependent manner. A full inhibition of the response to 30 $\mu\text{g/kg}$ BK was observed at 0.01 mg/kg HOE140.

We next tested the effects of HOE140 pretreatment on OSCS-induced hypotension. With no pretreatment with HOE140, OSCS administered iv at 1.7 mg/kg induced a rapid and major hypotensive response similar to the one described previously with a mean drop in DBP of 61 ± 3 mmHg (-62% of predose level, $n = 13$, Fig. 4). HR was not significantly altered. Pretreatment of the rats with HOE140 (0.0003 and 0.003 mg/kg) failed to modify the hypotensive effects of OSCS (Fig. 4). The OSCS-induced decrease in DBP measured 68 ± 4 mmHg following 0.0003 mg/kg HOE140 and 57 ± 3 mmHg after 0.003 mg/kg HOE140. However, HOE140 dose dependently inhibited the hypotensive effect of OSCS when administered at doses ranging from 0.03 to 0.3 mg/kg. The OSCS-induced decrease in DBP was 36 ± 7 mmHg following administration of HOE140 at 0.03 mg/kg, 29 ± 8 mmHg after HOE140 at 0.1 mg/kg, and 3 ± 1 mmHg after HOE140 at 0.3 mg/kg.

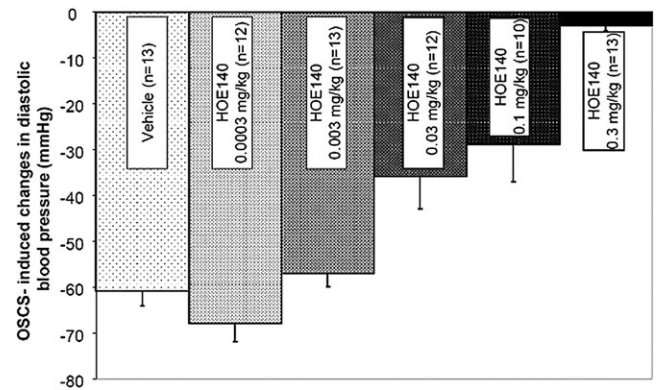


FIG. 4. Effects of an iv pretreatment with HOE140 on the OSCS (1.7 mg/kg)-induced DBP changes in the anesthetized rat. Group mean values $\pm$ SEMs are represented.

These values correspond to inhibition of the OSCS-induced hypotension of 40% at 0.03 mg/kg, 52% at 0.1 mg/kg, and 98.5% at 0.3 mg/kg.

Effect of a Histamine Receptor 1 Antagonist (Cetirizine) on OSCS-Induced Hypotension

Histamine release and histamine receptor 1 activation are key mechanisms for immediate hypersensitivity and anaphylaxis. Involvement of histamine pathway was therefore investigated as evidence for a hypersensitivity reaction. A preliminary set of experiments was undertaken in order to confirm the blockade of histamine receptor activity with cetirizine. Histamine was tested at doses of 1, 3, and 10 $\mu\text{g/kg}$ prior to and after administration of cetirizine at 1 mg/kg ($n = 4$; Fig. 5A). Histamine induced a dose-dependent decrease in DBP, rapidly reversible within minutes after treatment. Cetirizine (1 mg/kg) antagonized the hypotensive effects of histamine at all doses tested (Fig. 5A). OSCS (1.7 mg/kg) induced a rapid and major hypotensive response in the anesthetized rats similar to the one observed in previous experiments, with an average fall in DBP of 60 ± 3 mmHg (-58% of predose level). HR was not modified in a biologically significant way. After pretreatment with cetirizine (1 mg/kg), the response to OSCS was unchanged with DBP being decreased by 68 ± 3 mmHg (-64% of predose level, Fig. 5B).

Effect of the Route of Administration on the Hypotensive Response to OSCS in Conscious Rats

A single iv bolus injection or sc injection of saline or heparin alone (1000 IU/kg) did not result in any significant changes in BP or HR in conscious rats. Conversely, a single iv bolus injection of OSCS (1.7 mg/kg)-spiked heparin induced a transient decrease in BP between the third and fifth min postdosing in a subset of 8 out of 20 rats tested in this group. In these rats, as was the case with the anesthetized rats, the DBP (plotted in Fig. 6A) was reduced to a greater extent than either the SBP or the MBP. The largest average decrease in DBP in

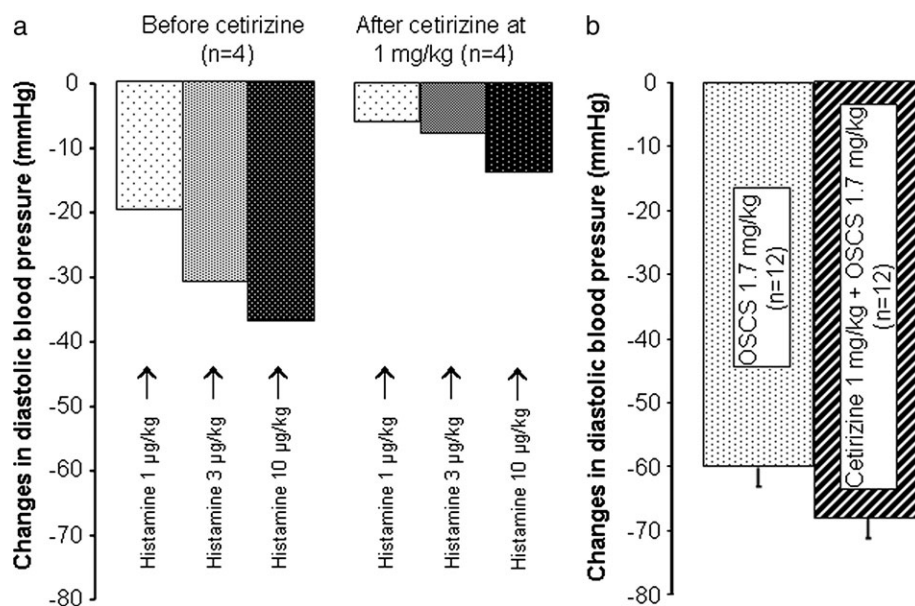


FIG. 5. Effects of a pretreatment with cetirizine (iv bolus; 1 mg/kg) on (a) the histamine-induced and (b) the OSCS-induced changes in DBP in the anesthetized rat. Group mean values $\pm$ SEMs are represented.

these responding rats was -18% (82 ± 6 mmHg vs. an initial average value of 101 ± 3 mmHg). Between the 7th and 50th min postdosing, a significant increase in BP developed in the entire group ($n = 20$). The highest average DBP, SBP, and MBP (all occurring at the 25th min postdosing) represented 19%, 16%, and 17% increases from the baseline values, respectively. HR in this group was also slightly, but significantly, elevated at the 8th, 9th, 12th, and 30th min postdosing. The maximum increase in HR was observed at the eighth min postdosing time point and represented a 7% increase over the baseline value.

Higher iv doses of OSCS-spiked heparin resulted in a more pronounced and a more consistent reduction in BP in conscious rats. A single iv bolus injection of OSCS (3 mg/kg)-spiked heparin (Fig. 6A) resulted in a decrease in BP in all rats in this treatment group ($n = 20$). Average DBP was significantly decreased to 81 ± 5 mmHg (-22% of average baseline) between 3 and 4 min postdose. SBP and MBP were decreased by 9% and 18%, respectively. The 3 mg/kg dose also significantly increased HR ($+12\%$ maximal average) between 2 and 8 min postdosing. In contrast to iv administration, a single sc injection of OSCS (1.7 or 3 mg/kg)-spiked heparin did not result in significant changes in BP or HR in conscious rats when compared with the heparin-alone-treated group (Fig. 6B). This was true during the entire 2-h observation period for this group.

Pharmacodynamics of OSCS after a Single Sc or Iv Injection in Male and Female SD Rats

We next examined the pharmacodynamics of OSCS using APTT. Following iv administration of OSCS, a large increase in APTT was observed (Fig. 7A). $A_{\max}$ occurred at the first

sampling time (0.083 h) in males and at the 0.5 h sampling time in females. A higher $A_{\max}$ was observed in females (92 Δ s) than in males (65.7 Δ s). Additionally, a higher AUC_{last} was observed in females compared with males (103 Δ s.h vs. 51.2 Δ s.h). In both sexes, 24 h after iv administration, APTT clotting time prolongation was returned to baseline values. In contrast to iv administration, the mean APTT clotting times measured after sc administration remained close to baseline values (i.e., $\Delta(t)$ APTT close to zero) in both sexes at all sampling times (Fig. 7B).

Kallikrein Activity Induced by OSCS in Human, Rat, Dog, and Pig Plasma

In order to elucidate the potential mechanism of the marked hypotensive effect of OSCS in rats *in vivo*, we examined the ability of OSCS to activate kallikrein activity in plasma of the rat, human, pig, and dog (Fig. 8). OSCS induced a robust and dose-related increase in kallikrein activity at 25 and 250 μ g/ml in human and rat plasma. The dog appeared to be less responsive with only a slight increase at 25 μ g/ml and a moderate response at 250 μ g/ml but considerably lower compared with rat and human. A clear increase of kallikrein activity was seen in pig plasma at 25 μ g/ml, but this response was somewhat lower at 250 μ g/ml. When Choay heparin was tested as a reference in the same assay (Fig. 9), no relevant increase in kallikrein activity was seen in rat plasma up to 250 μ g/ml heparin concentration. In human, dog, and pig plasma, a small increase in kallikrein activity was observed at the highest dose of 250 μ g/ml only. No effect by OSCS on kallikrein activity was observed in kallikrein- and factor XII-deficient human plasma (data not shown).

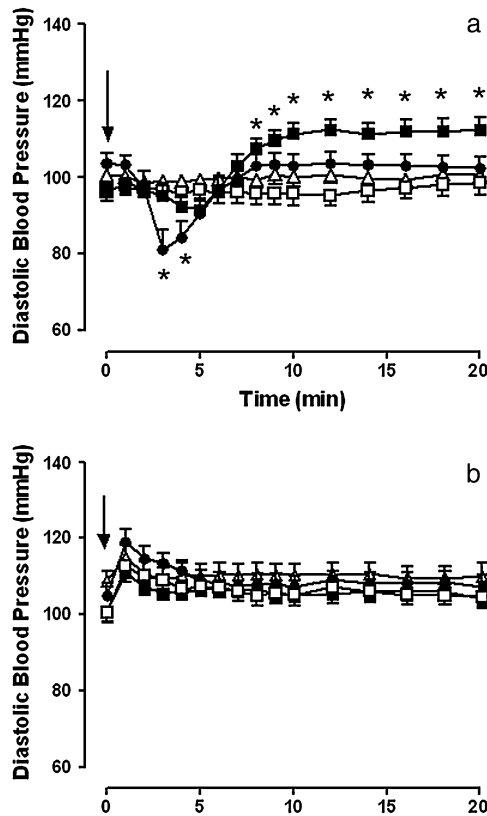


FIG. 6. Time course of diastolic blood pressure in conscious catheterized rats. Diastolic blood pressure following intravenous (a) or subcutaneous (b) treatment with saline (Δ ; $n = 20$ for iv and 20 for sc), heparin-alone ($\square$; $n = 21$ for iv and 20 for sc), heparin + OSCS at 1.7 mg/kg ($\blacksquare$; $n = 20$ for iv and 20 for sc), or heparin + OSCS at 3 mg/kg ($\circ$; $n = 20$ for iv and 19 for sc) are shown. Arrows indicate the administration of the test article. Intravenous administration occurred through the indwelling catheter and did not require handling of the animals. The initial rise in diastolic blood pressure in all of the sc treated animals is a result of the handling and subsequent sc injection of these animals. Asterisks denote $p < 0.05$ (ANOVA) versus the heparin-alone treatment group. Error bars indicate SEM.

DISCUSSION

Heparin contaminated with OSCS has led to serious adverse events in patients in recent years. These adverse events were often referred to as anaphylactic or anaphylactoid reactions given their occurrence within minutes after iv administration of heparin producing symptoms resembling anaphylactic shock (e.g., urticaria, dyspnea, tachycardia, and flushing). However, in an epidemiologic study of 106 patients treated with contaminated heparin, urticaria was rather infrequent (1.9%) among all cases (Blossom *et al.*, 2008). Hypotension was the most prominent feature (58.5%) with variable presentations of nausea (46.2%), shortness of breath (25.5%), vomiting (28.3%), flushing (25.5%), and tachycardia (24.5%). Although some of these symptoms mimic anaphylactic reactions resulting from mast or basophil cell activation, there is now good evidence that the reported reactions are the result of an initiation of the contact system cascade through activation of

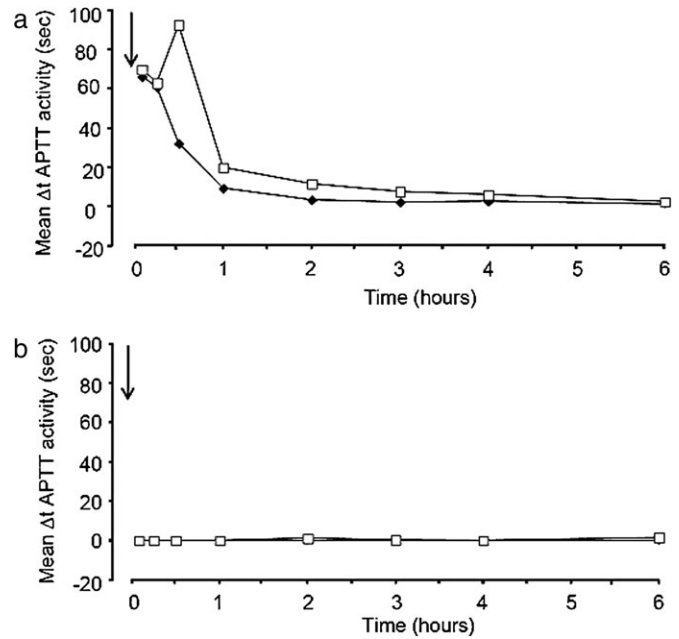


FIG. 7. Time course of APTT changes after intravenous (a) versus subcutaneous (b) treatment with OSCS at 1.7 mg/kg in male ($\blacklozenge$) and female ($\square$) conscious rats. For both routes of administration, $n = 3$ per sampling time.

factor XII by a polyanionic contaminant, leading to the generation of BK (Schwartz, 2008).

Highly sulfated polysaccharides and polyanions (e.g., dextran sulfate and chondroitin A sulfate) have been shown to serve as a negatively charged surface that can initiate fluid-phase activation of the contact system (Hojima *et al.*, 1984; Samuel *et al.*, 1992; Siebeck *et al.*, 1994). Li *et al.* (2009) proposed a mechanism to explain OSCS enhancement of vasoactive BK production through contact activation of factor XII, prekallikrein, and high-molecular weight kininogen. In contrast to heparin, OSCS tightly binds factor XIIa and hampers its inactivation by proteases when freely exposed in plasma, whereas heparin catalyzes inhibition of factor XIIa through antithrombin III. Therefore, although it is structurally similar to heparin, OSCS displays distinct molecular features, which can impact the interplay of plasma factors within the contact system cascade and disturb their intrinsic physiological regulation.

In our first set of studies, we investigated the potential hemodynamic effects of an OSCS-contaminated heparin and of OSCS administered alone or in combination with a fixed dose of heparin. OSCS-contaminated heparin (34% wt/wt OSCS) induced immediate and dramatic hypotensive responses when injected as a bolus to anesthetized rats. The anesthetized rat appears to be a suitable experimental model to replicate the hemodynamic effects of contaminated heparins. The hypotensive responses were similar in magnitude and duration to those obtained in anesthetized pigs treated iv with OSCS or with an OSCS-contaminated heparin (Kishimoto *et al.*, 2008). In addition, the pattern of the BP responses to OSCS in rats

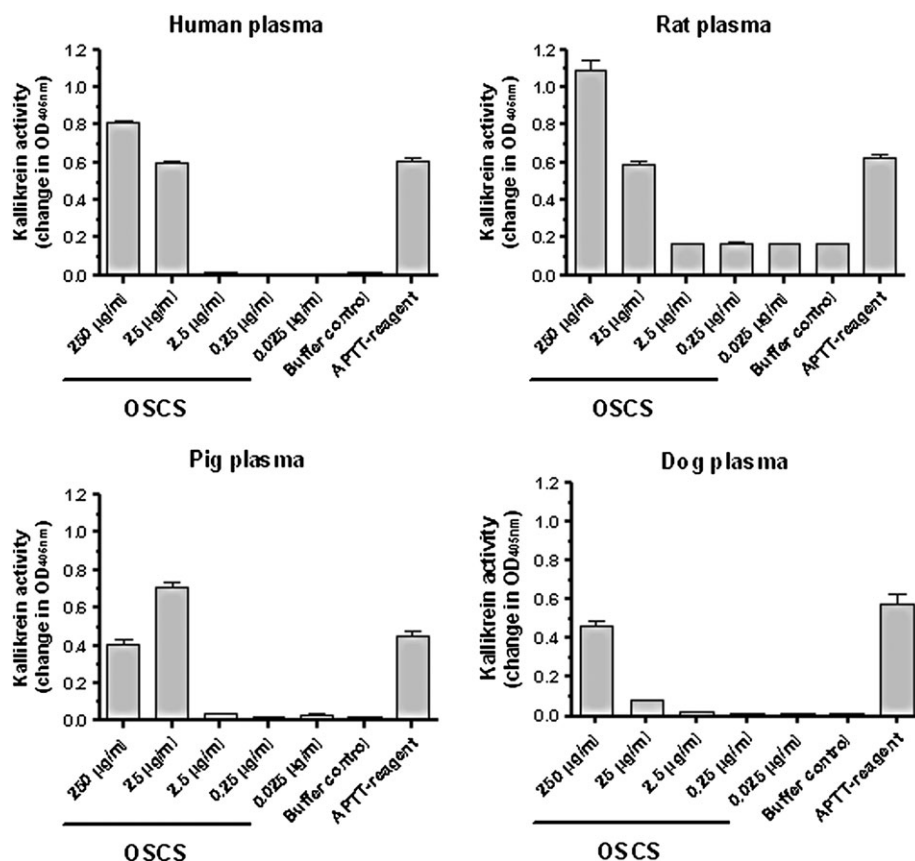


FIG. 8. Evaluation of kallikrein activity *in vitro* in plasma from different species (mean $\pm$ SEM, $n = 8$).

was similar to that obtained with contaminated heparin or with OSCS-spiked high-molecular weight heparin at comparable OSCS dose level, which confirms the pivotal role of OSCS in the hypotensive responses induced by contaminated heparins.

A further goal was to characterize a dose-response relationship for BP changes induced by OSCS. It was shown that the doses of 0.2 and 0.4 mg/kg OSCS administered in association with a fixed dose of heparin (1000 IU/kg) were devoid of hypotensive effects in the rat. A BP fall started to occur from 0.8 mg/kg, with a clear dose-related increase in incidence at 1.2 and 1.7 mg/kg (Table 1). When plotting maximal hypotensive responses against treatment (Fig. 2), the threshold for activity was between 0.4 and 0.8 mg/kg. Although the incidence of response increases with dose, the magnitude of the BP changes remains constant. This finding is consistent with the postulated mechanism of OSCS on the contact system where the end biological effects (i.e., hypotension) are amplified through the enzyme cascade in an all or none fashion.

The route of administration plays an important role in the cardiovascular effects of OSCS. The conscious rat model was applied for comparison of iv and sc routes in order to allow a more prolonged monitoring of BP after sc administration (2 h observation). In contrast to iv administration, single sc injections of OSCS-spiked heparin (1.7 and 3 mg/kg) did not

induce any significant hemodynamic changes (compared with a heparin-alone group) over the course of the experiment. In a follow-up pharmacodynamic study using APTT determination as a biomarker for OSCS (1.7 mg/kg) bioavailability, the mean APTT clotting times remained similar to baseline values after sc administration compared with the large increases in APTT observed after iv administration. The results indicate that systemic OSCS levels following sc administration are insufficient to produce either biological or toxic hemodynamic effects. This is consistent with the long-recognized differences in bioavailability between unfractionated heparin administered iv and sc (Jacques, 1976) and the fact that during the recent outbreak no SAEs were observed in patients treated sc (FDA Press Release, 2008c). Overall, these data suggest that acute hypotensive reaction after sc administration of OSCS-contaminated heparin is unlikely to occur because of the low bioavailability of OSCS through this route and minimal circulating concentrations—as evidenced by lack of biological effect—as compared with iv route.

Previously, it was demonstrated that OSCS-contaminated heparin, as well as synthetically generated OSCS, directly activates the kallikrein-kinin pathway, leading to the generation of BK (Adam *et al.*, 2010; Kishimoto *et al.*, 2008) and thrombin (Qian *et al.*, 2010). BK is an endogenous vasoactive

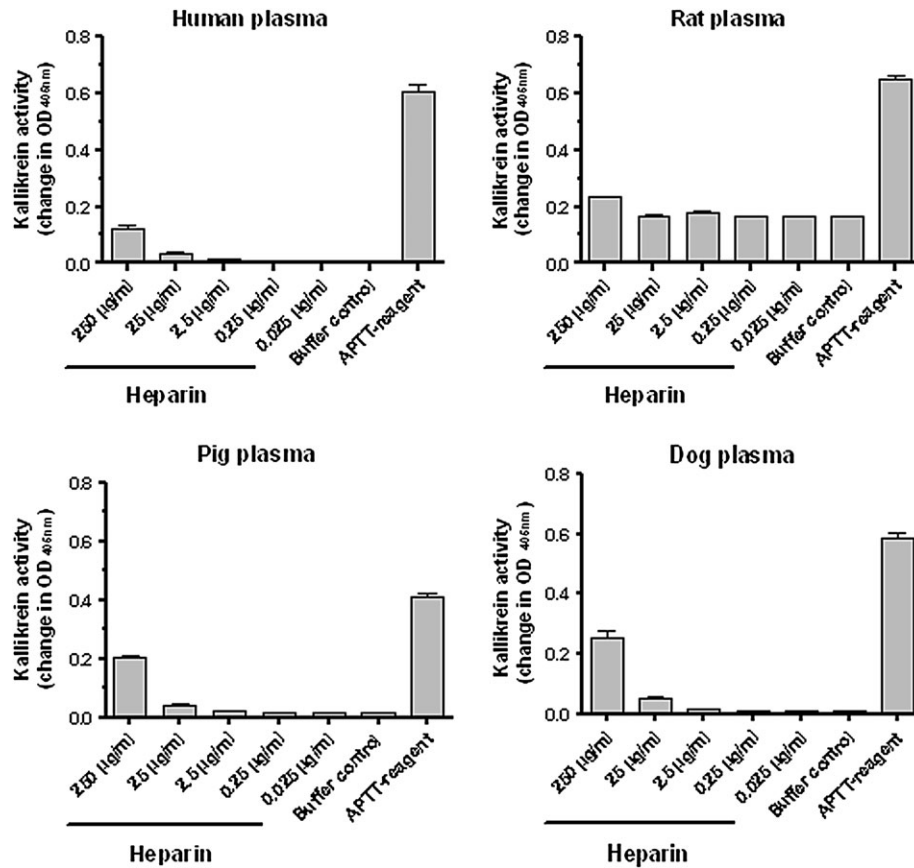


FIG. 9. Effects of Choay heparin on kallikrein activity *in vitro* in plasma from different species (mean $\pm$ SEM, $n = 8$).

compound with a physiological role in both inflammatory and protective processes. The biological activity of BK is mediated through the interaction with two cell surface receptors, designated B_1 and B_2 . The pharmacological effects of BK and BK-related peptides, including vasodilatation, increased vascular permeability, stimulation of sensorial and sympathetic nervous connections, and smooth muscle contraction, derive from the presence of these receptors on various cell types such as the vascular endothelium, primary sensory afferent neurons, vascular and nonvascular smooth muscles, epithelial cells, and perhaps some types of leukocytes (Moreau *et al.*, 2005). The B_2 receptor is involved in acute pain, acute inflammatory reactions, edema formation, regulation of cardiovascular function, and bronchoconstriction, whereas B_1 receptors are mainly implicated in maintaining chronic pain, plasma extravasation, and neutrophil accumulation (Leeb-Lundberg *et al.*, 2005). Our experiment with the potent B_2 receptor antagonist, HOE140, demonstrated that HOE140 dose dependently reduced the amplitude of the hypotensive response observed after iv OSCS (1.7 mg/kg) administration. This functional antagonism of the vascular effects of OSCS indicates that in the anesthetized rat model, most of the vascular effects of OSCS are mediated via activation of B_2

receptors. These results provide further evidence of the pivotal role of the kallikrein-kinin system in the initiation of OSCS-related vascular effects.

In order to further explore the mechanisms of OSCS-mediated hypotension, we conducted experiments using the histamine (H_1) receptor antagonist cetirizine. Histamine is a major vasomediator in Type 1 hypersensitivity reactions like anaphylaxis and mediates systemic vasodilation by binding to the H_1 receptor (Hill *et al.*, 1997). The inability of cetirizine to block OSCS-induced hypotension indicates that this phenomenon is not mediated via activation of the histamine receptor and suggests that histamine release from mast cells and basophils is not a major component of the mechanism underlying the adverse effects seen in humans.

Several authors have used *in vitro* methods to assess kallikrein activity in human and animal plasma after treatment with OSCS or OSCS-contaminated heparin (Blossom *et al.*, 2008; Kishimoto *et al.*, 2008). High kallikrein activities were found after plasma treatment with OSCS-contaminated heparin at concentrations of 2.5 and 25 μ g/ml in human plasma and 2.5, 25, and 250 μ g/ml in pig plasma, but not in rat, rabbit, or horse (Kishimoto *et al.*, 2008). However, the concentration of OSCS in this specific heparin lot was not reported. In our hands, using

the same method, OSCS induced a robust and dose-related increase in kallikrein activity in rat and human plasma at 25 and 250 µg/ml. Kallikrein activity in pig and dog at these concentrations was less robust and not dose related. The similarity of kallikrein activation in rat and human plasma further confirms the suitability of the rat model for the examination of OSCS-related effects.

In summary, the present study further elucidates the potential mechanisms for the adverse events reported in patients treated with OSCS-contaminated heparins. In the rat hemodynamic model, it was clearly shown that the hypotensive effect of OSCS-contaminated heparin is dose and route dependent. It was possible to replicate the hypotensive effects of OSCS-contaminated heparin via iv but not sc administration and establish a threshold dose for the hypotensive effect with OSCS-spiked heparin. It is concluded that the concentration of the contaminant and its route of administration play an important role in the occurrence of adverse events in patients. Further evidence for the involvement of the kinin-kallikrein pathway in the etiology of the hypotensive responses seen in humans is given by the fact that OSCS-induced hypotension was fully abolished by pretreatment with a type 2 BK receptor antagonist in the rat, whereas pretreatment with a histamine antagonist was without effect. In addition, high kallikrein activity was seen after OSCS treatment of rat and human plasma *in vitro*. These data suggest that enhanced BK production, but not histamine release, is responsible for the hemodynamic effects triggered by OSCS-contaminated heparin.

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