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NITROPHORIN 2, A FIX(a)-DIRECTED ANTICOAGULANT, INHIBITS ARTERIAL THROMBOSIS WITHOUT IMPAIRING HEMOSTASIS

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Summary

Nitrophorin 2 (NP2) is a 20 kDa lipocalin identified in the salivary gland of the blood sucking insect, *Rhodnius prolixus*. It functions as a potent inhibitor of the intrinsic pathway of coagulation upon binding to factor IX (FIX) or FIXa. Herein we have investigated the *in vivo* antithrombotic properties of NP2. Surface plasmon resonance assays demonstrated that NP2 binds to rat FIX and FIXa with high affinities ($K_D = 43$ and 47 nM, respectively), and prolongs the aPTT without affecting the PT. In order to evaluate NP2 antithrombotic effects *in vivo* two distinct models of thrombosis in rats were carried out. In the rose Bengal/laser induced injury model of arterial thrombosis, NP2 increased the carotid artery occlusion time by ≈ 35 and $\approx 155\%$, at doses of 8 and 80 $\mu\text{g}/\text{kg}$, respectively. NP2 also inhibited thrombus formation in an arterio-venous shunt model, showing $\approx 60\%$ reduction at 400 $\mu\text{g}/\text{kg}$ (*i.v.* administration). The antithrombotic effect lasted for up to 48 h after a single *i.v.* dose. Notably, effective doses of NP2 did not increase the blood loss as evaluated by tail-transection model. In conclusion, NP2 is a potent and long-lasting inhibitor of arterial thrombosis with minor effects on hemostasis. It might be regarded as a potential agent for the treatment of human cardiovascular diseases.

Keywords

Animal models; Arterial thrombosis; Coagulation inhibitors; Haemostasis; Nitrophorin

Introduction

Factor IXa (FIXa) is a vitamin K-dependent coagulation serine protease, which upon binding to the non-enzymatic cofactor, factor VIIIa, on membrane surfaces in the presence of Ca^{2+} forms the intrinsic Xase complex and rapidly activates factor X to factor Xa (FXa) during the blood coagulation process (1–3). FIXa plays a key role in hemostasis, and its deficiency is associated with the life-threatening disease hemophilia B (4). The zymogen FIX circulates in plasma as a single-chain glycoprotein with 415 amino acids. It can be converted to FIXa by either one of two physiological activators, the factor VIIa(FVIIa)-

tissue factor complex or factor XIa (FXIa), upon cleavage of Arg145-Ala and Arg180-Val peptide bonds (1,3,5).

Under pathologic conditions, excessive clot formation may occlude blood vessels, resulting in a variety of thromboembolic disorders, such as deep vein thrombosis, pulmonary embolism and stroke, which are the main causes of morbidity and mortality (6). Anticoagulation therapy with heparin and oral vitamin K antagonists is currently the mainstay of treatment and prevention of thrombosis (6–8). Unfortunately, both classes of drugs have well-documented limitations, pointing toward need for the development of novel efficacious and safe anticoagulants (8). In practice, this involves identifying drugs capable of preventing thrombus formation without increasing the risk of hemorrhage (9). In this context, it has been proposed that targeting the intrinsic pathway of coagulation would be advantageous for treating thromboembolic diseases (10,11).

A number of specific inhibitors from exogenous sources have been identified from salivary glands of blood sucking arthropods (12,13). Those molecules have been regarded as important tools for the study of blood clotting system, and provided a valuable source of antithrombotic compounds. Nitrophorin 2 (NP2), or prolixin S, is a 20 kDa lipocalin identified in the salivary gland of the blood sucking insect, *Rhodnius prolixus* (14). It has been characterized as a potent anticoagulant due to its ability to inhibit the intrinsic Xase complex (15–18). Further studies have shown that NP2 binds with high affinity to either FIXa or FIX (19,20), most likely by targeting the γ -carboxyglutamic acid-containing Gla domain (19). Thus, the interaction between NP2 and FIXa may interfere with enzyme association with phospholipids, resulting in failure to assemble into the intrinsic Xase and reducing its catalytic activity. In addition, complex formation between NP2 and FIX decreases the zymogen activation by both the FVIIa-TF complex and FXIa, possibly due to decreased zymogen association with phospholipids (19).

In the present study, we evaluated the *in vivo* effects of NP2 on thrombus formation using two distinct thrombosis models in rats. After intravenous administration, NP2 showed effective and long-lasting antithrombotic activity. Remarkably, NP2 produced no excessive bleeding at effective antithrombotic doses.

We conclude that NP2 is a potent and long-lasting inhibitor of arterial thrombosis with minor effects on hemostasis. It might be regarded as a potential agent for the treatment of human cardiovascular diseases.

Materials and Methods

Materials

Recombinant NP2 was produced in *Escherichia coli*, purified, and quantified as previously described (21). Rat FIX and FIXa were purchased from Haematologic Technologies (Essex Junction, VT). Activated partial thromboplastin time (aPTT) (cephalin plus kaolin) and prothrombin time (PT) (thromboplastin with calcium) reagents were from BioMériaux (Rio de Janeiro, Brazil). Anasedan (Xylazin) and Dopalen (Ketamin) were from Agribrands (Rio de Janeiro, Brazil). Chromogenic substrate for thrombin (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline dihydrochloride, S-2238) was purchased from Diapharma (Westchester, OH). Gly-Pro-Arg-Pro amide (GPRP) from Sigma Chemical Co. (St. Louis, MO). Rose bengal dye was from Fischer Scientific Co. (Fair Lawn, NJ).

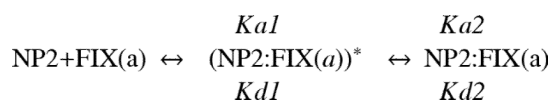
Animals

Adult Wistar rats (both sexes) were housed under controlled conditions of temperature ($24 \pm 1^\circ\text{C}$) and light (12 h light starting at 7:00 a.m.), and all experiments were conducted in

accordance with standards of animal care defined by the Institutional Committee (Institute of Medical Biochemistry, Federal University of Rio de Janeiro).

Surface plasmon resonance (SPR) analysis

All experiments were carried out in a T100 instrument (GE Healthcare Inc., Piscataway, NJ) following the manufacturer's instructions. Sensor chip CM5, amine coupling reagents, and buffers were also purchased from GE Healthcare. HBS (10 mM Hepes, pH 7.4, 150 mM NaCl supplemented with 5 mM CaCl₂) was used as the running buffer for all SPR experiments. Each experiment was carried out in duplicate. FIX or FIXa (1–3 µg/ml) in acetate buffer pH 4.0 containing 5 mM CaCl₂ were immobilized on a CM5 sensor chip using amine coupling methodology resulting in a final immobilization of 300–500 RU. Blank flow cells were used to subtract the buffer effect on sensorgrams. Kinetic experiments were carried out with a contact time of 200–300 s at a flow rate of 30 µl/min at 25°C. NP2-FIX/FIXa complex dissociation was monitored for 600 s, and the sensor surface was regenerated by a pulse of 30 s of 10 mM EDTA at a flow rate of 30 µl/minute. After subtraction of the contribution of bulk refractive index and nonspecific interactions with the CM5 chip surface, the individual association (k_a) and dissociation (k_d) rate constants were obtained by global fitting of data using the two-state reaction (conformational change) interaction model using BIAevaluation™ (Biacore, Inc.) (22):



Values were then used to calculate the dissociation constant (K_D).

$$K_D = kd1.kd2/ka1(ka2+kd2)$$

The values of average squared residual obtained were not significantly improved by fitting data to models that assumed other interactions. Conditions were chosen so that the contribution of mass transport to the observed values of K_D was negligible. In addition, the models in the T100 evaluation software fit for mass transfer coefficient to mathematically extrapolate the true ka and kd .

Effect of NP2 on aPTT and PT

The effect of NP2 on coagulation tests aPTT and PT was evaluated on an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany). Human blood samples were collected from healthy donors in 3.8% trisodium citrate (9:1, v/v), and platelet poor plasma were obtained by further centrifugation at 2000×g for 10 min. Rat plasma samples were obtained following the same procedure after blood collection by cardiac puncture. Plasma (50 µl) was incubated with NP2 (10 µl) for 2 min at 37°C, followed by addition of the aPTT reagent (50 µl, 1 min) and then 25 mM CaCl₂ (100 µl) or the PT reagent (100 µl). Time for clot formation was then recorded. For ex vivo assays in rats, PBS, NP2 or heparin were given I.V. 30 min before cardiac puncture. Blood collection, plasma preparation and aPTT procedure were performed as above.

Measurement of thrombin generation in plasma

Thrombin formation in plasma was followed after activation of the intrinsic pathway, as previously described (23). Rat plasma (50 µL) was incubated with different concentrations of NP2 diluted in 50 µl TBS and 10 µL of APTT reagent (cephalin plus kaolin) for 2 min at

room temperature. Reaction was started by addition of 100 μL of 12.5 mM CaCl_2 and aliquots of 10 μL were transferred every 10 s into microplate wells containing 40 μL of TBS-EDTA buffer. In order to prolong plasma clotting, reactions were carried out in the presence of 10 mM GPRP. Thrombin generation was determined by the addition of 100 μM of the chromogenic substrate S-2238. Hydrolysis of the substrate was detected using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA), equipped with a microplate mixer and heating system. Reactions were recorded continuously at 405 nm, for 20 min, at 37°C. The total volume of the reactions was 100 μL .

Thrombus formation in the arterio-venous shunt model

Antithrombotic activity in an extracorporeal shunt was investigated as described (24). Rats (both sexes, 200–250 g body weight) were anesthetized with intramuscular xylazine and ketamine (16 and 100 mg/kg, respectively). Through a longitudinal incision in the skin over the trachea along the midline, the left jugular vein and the right carotid artery were cautiously freed from the surrounding tissue. The right carotid artery and the left jugular vein were cannulated; a solution of NP2 was injected on the artery and allowed to circulate at various times (15 min to 72 h) before initiating the surgery for thrombosis induction.

In the meantime an extracorporeal shunt was prepared. This shunt consisted of two 6 cm pieces of polyethylene tubing (1.3 mm inner diameter) with both ends cut at an angle; one end of each piece of tubing was forced into the end of a central, 6 cm tube (1.6 mm inner diameter) with a 5 cm length of silk thread drawn through it, so as to leave 1.0 cm outside the tubing at one end and 4.0 cm inside the tubing. This shunt was filled with a heparin solution (50 U/mL). One end of the shunt was inserted into the left jugular vein, and subsequently the other end was inserted into the right carotid artery and the circulation of the blood was established. After 20 min of blood circulation through the shunt, the blood flow was stopped at the arterial side of the tubing with a pinch-clamp and the middle of the extracorporeal shunt was isolated. From this segment the silk thread coated with the thrombus was carefully pulled out with the help of the piece of thread that remained external to the shunt. The wet thrombus weight was immediately determined, subtracting the weight of the wet thread.

Photochemically-induced carotid artery thrombosis in rats

The effect of NP2 on arterial thrombosis was evaluated in a rat model of arterial thrombosis (25). Animals were anesthetized as described above. The right common carotid artery was isolated through a midline cervical incision, and the blood flow was monitored continuously using a 1PR doppler flow probe and a TS420 flowmeter (Transonic Systems, Ithaca, NY). Rose bengal dye (90 mg/kg body weight) was diluted to 60 mg/mL in PBS and administered via slow injection (over 2 min) through the cava vein. Just before injection, the common carotid artery was transilluminated with a 1.5-mV, 540 nm green laser (#25-LGR-193–249, Melles Griot Inc., Carlsbad, CA) immediately proximal to the doppler probe from a distance of 3 cm. Mean carotid artery blood flow was monitored until stable occlusion occurred (defined as a blood flow of 0 ml/min for ≥ 5 min), at which time the experiment was terminated. NP2 or PBS (control) was injected in the cava vein 15 min before initiating the rose bengal administration.

Bleeding effect

Rats (both sexes, ~100 g body weight) were anesthetized as described above and a cannula was inserted into the right carotid artery for administration of NP2. After 60 min, tail was cut at 2 mm of diameter and carefully immersed in 40 ml distilled water at room temperature. The hemoglobin content in water solution (absorbance at 540 nm) was used to

estimate the blood loss (26). Appropriate controls (i.v. injection of PBS or heparin at 1mg/kg) were run in parallel.

Statistical analysis

Results were expressed as mean \pm standard error. Statistical analysis was performed with one-way ANOVA, followed by a post-hoc test (Dunnett) using the statistical package GraphPad Prism 4.0 (GraphPad software, USA).

Results

Previous studies have demonstrated that NP2 interacts with human FIXa and FIX with high affinity, as demonstrated by SPR analysis (19,20). To further investigate binding kinetics of NP2-rat FIX/FIXa interactions, SPR experiments were performed. Typical sensorgrams obtained upon NP2 interaction with rat FIX and FIXa are shown in Figs 1A and 1B, respectively. In both cases, the best fit was attained using a two-state reaction model (Table 1) (22). Using this model, K_D values of 43 nM for FIX and 47 nM for FIXa were calculated (Table 1).

Consistent with its mechanism of action, Fig. 2A shows that NP2 prolonged aPTT in human plasma in a concentration-dependent manner. No effect was observed in the extrinsic pathway, as evaluated by PT. We have also tested the anticoagulant properties of NP2 in rat plasma. Fig. 2B shows that aPTT was prolonged at NP2 concentrations above 0.04 μ g/ml without changes in the PT. In order to verify the effects of NP2 in thrombin generation in plasma, experiments were performed as described in Methods. Fig. 3 shows that NP2 (20–40 μ g/ml) inhibited thrombin formation by 50–80%, as compared to the control. Overall, data presented on Figures 1–3 and Table 1 confirm that inhibitory properties of NP2 towards rat FIX(a) are similar to that previously observed for the human counterparts (16–20).

To determine whether NP2 exerts antithrombotic action *in vivo*, we employed two distinct models of thrombosis in rats. In an arterio-venous shunt model (24), NP2 at 400 μ g/kg (*i.v.*, single bolus administered 15 min before thrombus induction) significantly reduced thrombus weight (7.73 ± 1.27 mg versus 18.67 ± 2.50 mg in controls) (Fig 4A). The extent of the antithrombotic effect of NP2 was similar to that observed with 1 mg/kg of heparin (8.28 ± 1.23 mg). In order to demonstrate that the antithrombotic action of NP2 is dependent on FIX(a) interaction, we performed control experiments employing NP1, a recombinant lipocalin which shows no anticoagulant activity despite presenting great sequence similarity to NP2 (14). No antithrombotic activity was observed (data not shown). We have also evaluated the time-dependence of the antithrombotic activity of NP2. Fig 4B shows that thrombosis inhibition produced by a single dose of NP2 (400 μ g/kg) remained for up to 48 h, returning to control values after 72 h.

Next, antithrombotic action of NP2 was tested in the rose Bengal/laser injury model of arterial thrombosis. This model is based on a photochemically-induced endothelial injury that decreases the carotid artery blood flow, attributable to the development of stable thrombus (24). Figure 5 shows that the blood flow of control (PBS injected) animals stopped in ≈ 19 minutes. In contrast, NP2 delayed thrombus formation, increasing the carotid artery occlusion time by $\approx 35\%$ (25.3 ± 5.6 min) and $\approx 155\%$ (48.6 ± 9.4 min), at doses of 8 and 80 μ g/kg body weight, respectively.

Finally, the effect of NP2 on hemostasis was evaluated in a tail-transection model (26). Fig. 6A shows that bleeding was not significantly increased in the presence of antithrombotic concentrations of NP2, as compared with rats receiving PBS. In contrast, heparin at 1 mg/kg - an effective, but not excessive antithrombotic dose (data not shown), significantly

increased blood loss. Remarkably, NP2 did not affect the aPTT *ex vivo*, as shown in Fig. 6B. Again, heparin produced a significant change in this hemostatic parameter. Considering a blood volume of ~7.5 mL/100 g, it is possible to estimate a concentration of ~250 nM NP2 in the blood of 200 g animals that received 400 µg/kg doses. According to Fig. 2B, this may explain why such dose does not alter aPTT *ex-vivo* nor increase tail bleeding.

Discussion

NP2 is a member of the nitrophorin (NP) family of heme-binding lipocalins which consists of at least four related molecules found in *R. prolixus* salivary gland. These molecules display distinct functions related to blood feeding such as carrying nitric oxide which serves as a vasodilator in the host, or binding histamine and attenuating anti-inflammatory events (14,21,27,28). NP2 is the unique member of the NP group displaying potent anticoagulant activity which has been characterized as a FIX and FIXa-binding protein (16,18–20). Accordingly, NP2 decreases FX activation by FIXa bound to either the phospholipid or activated platelet surface or to the cofactor FVIII, possibly by interfering with the assembly of FX-activating complex (18). In fact, kinetic analyses have demonstrated that NP2 decreases both K_m and V_{max} of FX activation by the intrinsic Xase complex. Moreover, complex formation between NP2 and FIX decreases the rate of zymogen activation by FVIIa/TF complex (19). Further, NP2 binding to FIX strongly inhibits FIXa generation by FXIa (19). Therefore, NP2 can be a unique tool to dissect the contribution of Factor IX(a) in coagulation *in vitro*, and thrombosis *in vivo*.

The aim of this study was to investigate how the blockade of FIX(a) affects thrombosis in rats. SPR experiments confirmed that NP2 binds to rat FIX(a) with an affinity comparable to human FIX(a) ($K_D \sim 34$ nM for FIX and 16 nM for FIXa) (20). In addition, NP2 prolonged aPTT without affecting PT. These results indicated that NP2 is a suitable molecule to be tested as an anticoagulant in rats. Accordingly, our experiments demonstrate that NP2 decreases thrombus formation induced by extracorporeal arterio-venous shunt and also by laser-induced arterial injury. In both models, coagulation cascade has been implicated as a major player leading to vascular occlusion (24,29). However, NP2 was less effective in the arterio-venous model possibly because the relative contribution of FVIIa/TF-induced FIX activation or the intrinsic Xase as a thrombotic stimulus differ. At this point, it is not clear whether the effects of NP2 occur because it prevents activation of FIX by TF/VIIa, or attenuates productive assembly of the intrinsic Xase, or because it interfered with FXIa-induced FIX activation *in vivo*. Likewise, it has been demonstrated that a sulfated galactan with antithrombotic properties, inhibits arterial thrombosis, whereas it is inactive in the arterio-venous shunt in rats (30). It is conceivable that NP2 effects are results of synergistic and redundant inhibition of distinct and sequential steps of coagulation cascade both in the extrinsic and intrinsic pathways (i.e., targeting FIX and FIXa directly, or FVIIa/TF complex and FXIa indirectly). This contention is supported by the prominent antithrombotic effects of NP2 *in vivo*. Most remarkably, antithrombotic doses of NP2 showed minor effects on hemostasis as assessed by tail-transection and aPTT measurements, while administration of a therapeutic dose of heparin was associated with bleeding and significant increase in aPTT.

Our results using a selective FIX(a) inhibitor are in line with results obtained with inhibition/genetic deficiency of IX(a) and other components of the intrinsic pathway in the preclinical animal models of thrombosis. Accordingly, an anti-FIX antibody display antithrombotic properties in a rat model of FeCl₃-induced arterial thrombosis without affecting bleeding time (31). Likewise, inhibition of recurrent arterial thrombosis by active site-blocked FIXa has been effectively tested in a guinea pig model without impaired hemostasis (32). Further, the inhibition of the intrinsic coagulation cascade with catalytically inactive FIXa limits cerebral injury in stroke without increasing intracerebral hemorrhage

(33). Finally, a tick salivary protein (Ir-CPI) which targets contact phase factors (FXIIa, FXIa, and kallikrein) inhibits thrombus formation without an increase in blood loss (34).

Clinical data in humans and genetically altered mice also suggest that components of the intrinsic pathway of coagulation may play distinct roles in thrombosis and hemostasis (35). FXI deficiency is often associated with mild to moderate bleeding in humans, while bleeding does not occur in FXII deficiency (33). In genetically modified mice, FXI and FXII-knockout are less susceptible to thrombus formation with negligible impact on hemostasis (36,37). On the other hand, FIX deficiency is protective against experimental thrombosis but is associated with bleeding in humans and in mice (4,36,38). It is plausible that NP2 doses administered to rats in our study did not produce bleeding because it did not reach plasma levels to saturate FIX since its concentration is relatively high (90 nM) in the blood (4). This contention is consistent with no prolongation of aPTT *ex vivo* (Figure 6B). It is conceivable that tailoring FIXa activity or perhaps tailoring the intrinsic Xase assembly (through different mechanism) is the most appropriate approach when attempting to prevent thrombosis without impairing hemostasis.

Analysis of the time-dependence of the antithrombotic action of NP2 indicates a prolonged half-life which remained effective for up to 48h after a single *i.v.* dose. The long-lasting antithrombotic action of NP2 may derive from a stable complex formation with FIX in plasma. This is in accordance with the pharmacokinetic profile observed for an anti-FIX monoclonal antibody, which has a half-life of about 90 hours in primates (39). In addition, prolonged half-lives have been reported for NAPc2 and Ixolaris which are high-affinity ligands of FX (40,41). In this context, future measurements of the pharmacokinetic profile of NP2 will be essential to understand its long-lasting antithrombotic effect. Finally, exogenous inhibitors, targeting multiple steps of coagulation, have been identified. Their activity towards zymogen is particularly interesting because it may represent an evolutionary strategy to effectively block coagulation at multiple points. For example, Ixolaris from tick saliva uses FX(a) as scaffolds for the inhibition of FVIIa-tissue factor complex (42) and at appropriate concentrations it decreases prothrombin activation by FXa (43) and FX activation by the intrinsic Xase complex (44). Bothrojaracin from snake venom is a high-affinity ligand of pro(thrombin) that decreases both the enzyme activity and zymogen activation (45,46).

In conclusion, NP2 displays potent antithrombotic effect *in vivo*, with prolonged half-life and no major bleeding. The absence of major hemostatic changes at effective antithrombotic concentrations of NP2 may indicate an advantage in terms of therapeutic window when compared to antibodies against FIX and catalytic site blocked FIXa, which have been evaluated experimentally or in clinical trials (11,31,32). Overall, NP2 appears to be a promising anticoagulant, or as a prototype to develop anticoagulants for the prevention and treatment of thrombosis or other pathologies associated with dysregulated coagulation (47,48).

What is known about this topic?

- Factor IXa plays a central role in thrombin generation during initiation and amplification of the coagulation cascade.
- Genetic disruption of components of the intrinsic pathway of coagulation (factor XII and factor XI) protects against thrombosis with minor effects on hemostasis.

- Nitrophorin 2 has been characterized *in vitro* as an inhibitor of the intrinsic pathway of coagulation upon binding to factor IX(a).

What does this paper add?

- Nitrophorin 2 is a potent antithrombotic agent *in vivo*, as demonstrated in rat models of thrombosis.
- Effective antithrombotic doses of nitrophorin 2 do not cause excessive bleeding in the rat tail-transection model.
- Factor IX(a) might be a potential target for the treatment of thrombosis with minor hemorrhagic effects.
- Nitrophorin 2 is a useful tool to understand the contributions of FIX(a) in hemostasis or thrombosis.

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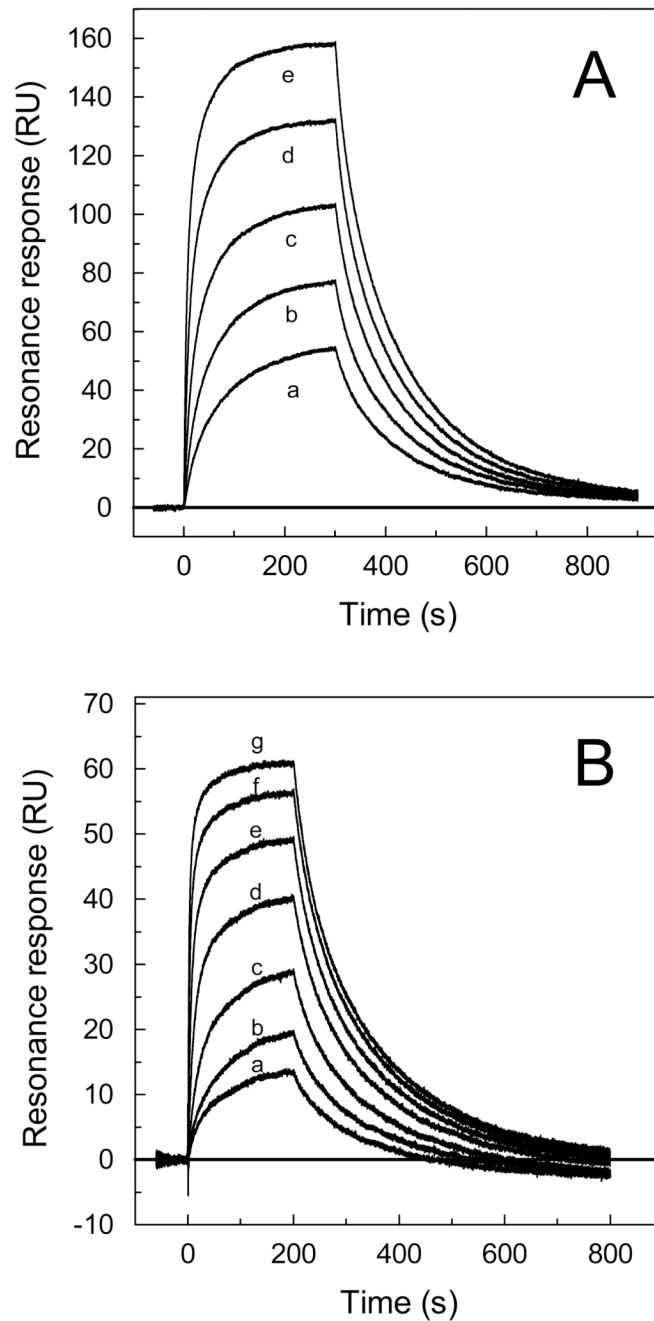


Figure 1. NP2 binds to rat Factor IX and Factor IXa

Typical sensorgrams of NP2 interaction with Factor IXa (A) and Factor IX (B). Different concentrations of FIXa (in nM: a, 20; b, 40; c, 80; d, 160; and e, 320) or FIX (in nM: a, 10; b, 20; c, 40; d, 80; e, 160; f, 320; g, 640) were injected over immobilized NP2 for 180 s. Dissociation of NP2-FIXa and NP2-FIX complex was monitored for 900 s and 800 s, respectively, and a global two-state binding model was used to calculate kinetic parameters. Representative sensorgrams are shown.

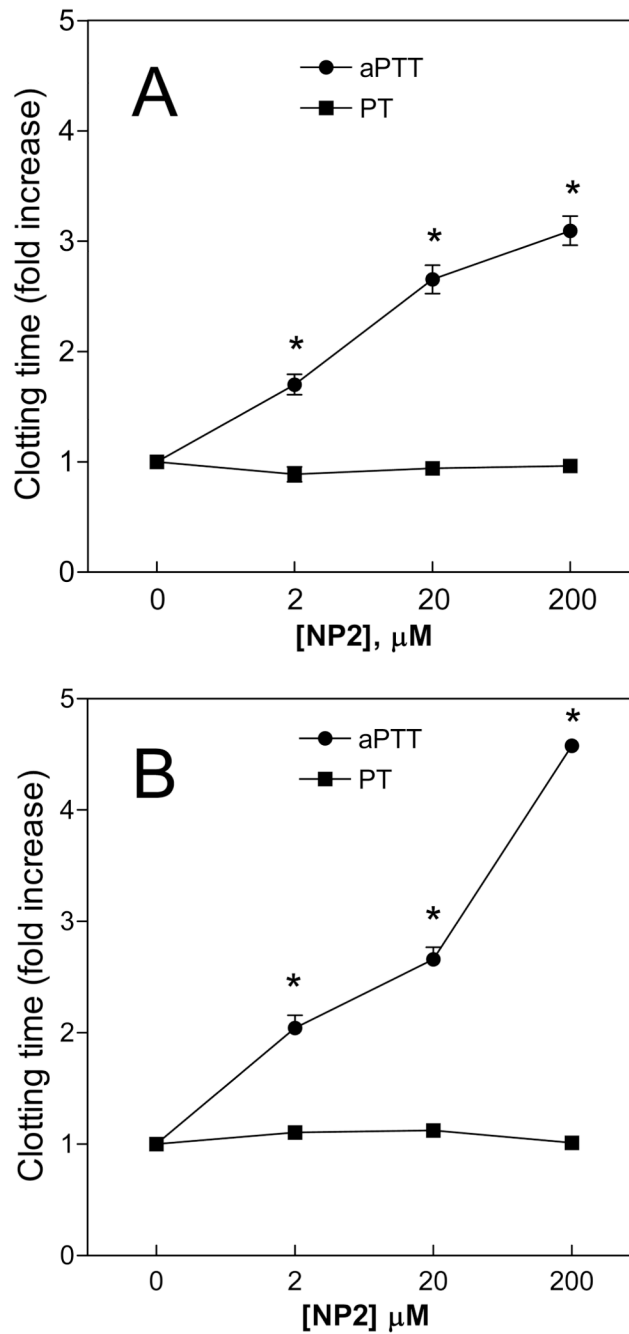


Figure 2. *In vitro* anticoagulant activity of NP2

NP2 at the indicated concentrations was incubated with human (A) or rat (B) citrated plasma for 2 min. Coagulation aPTT (●) and PT (■) were determined as described in Materials and methods. Each point represents mean \pm SE of 5 independent determinations. Asterisk, $P < 0.01$, as compared to values observed in the absence of NP2.

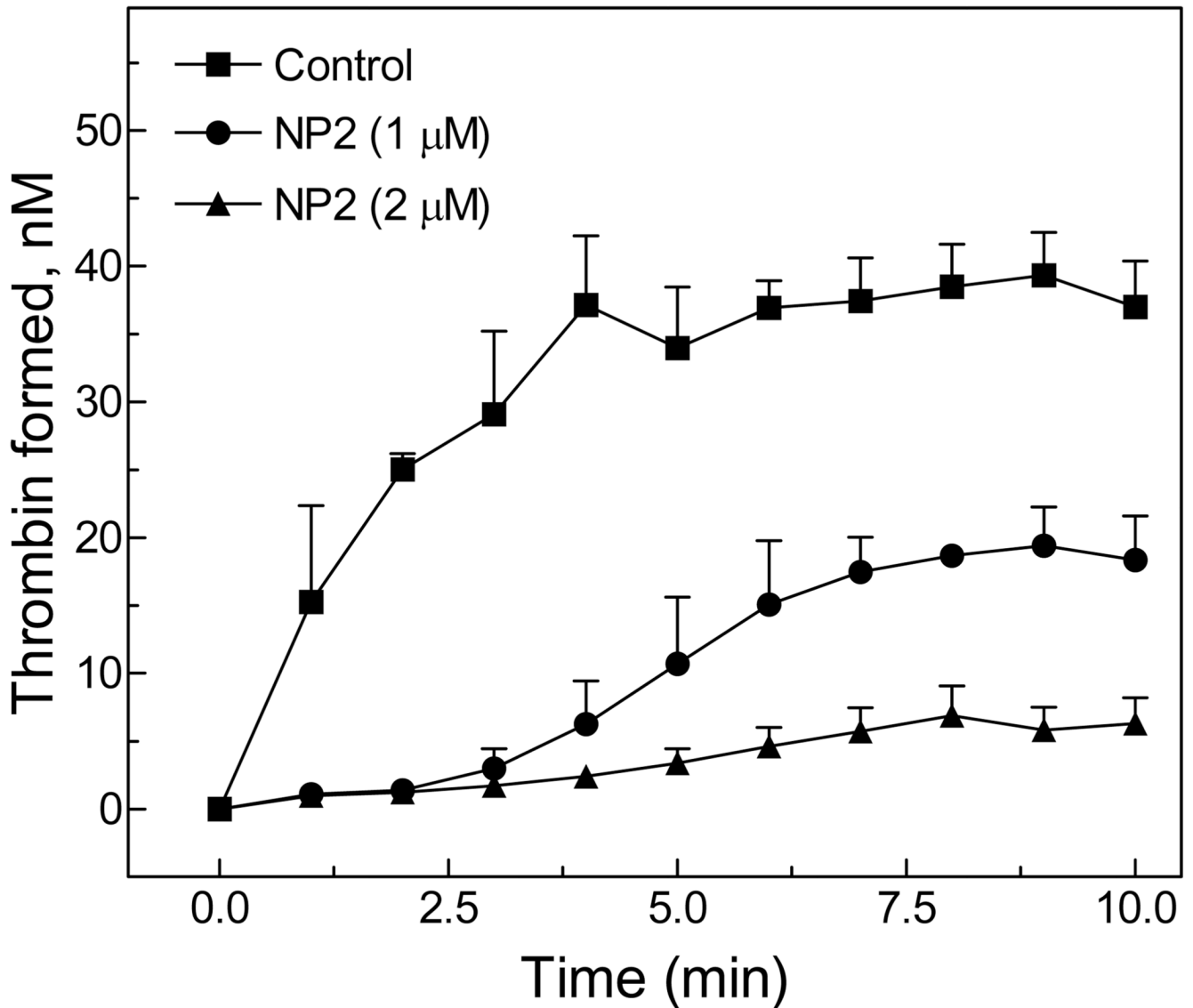


Figure 3. Effect of NP2 on thrombin generation in plasma

Thrombin generation in human plasma was followed after initiation of the intrinsic pathway. Curves show thrombin formed in plasma in the absence or in the presence of NP2 at the indicated concentrations. Assay conditions are described in the Materials and methods section. Each point represents mean \pm SE of 5 independent determinations. Asterisk, $P < 0.01$, as compared to values observed in the absence of NP2.

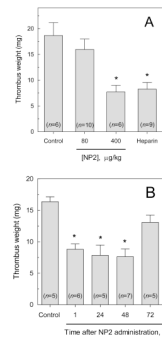


Figure 4. Antithrombotic activity of NP2 in an arterio-venous shunt model

The antithrombotic activity of NP2 was investigated using an arterio-venous shunt model, as described in Materials and methods. (A) NP2 at indicated doses or PBS (control) were injected intravenously. Asterisk, $P < 0.01$, as compared to values observed in the absence of NP2. (B) NP2 (400 $\mu\text{g}/\text{kg}$) was administered i.v. to animals, and thrombosis was induced after the indicated times. Each bar represents mean \pm SE, and the number of animals tested for each condition is shown in the Figure. Asterisk, $P < 0.01$, as compared to values observed in the control.

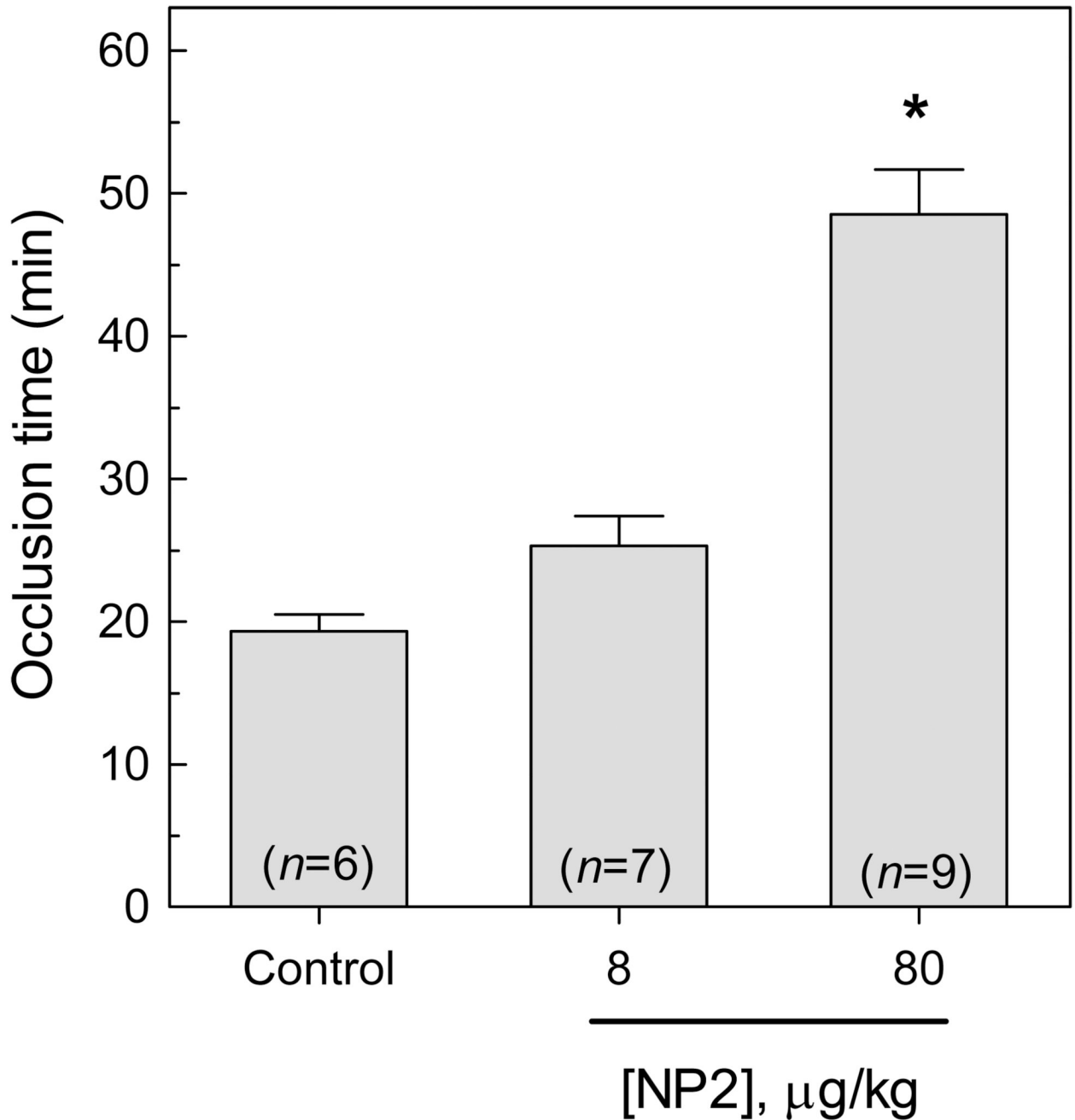


Figure 5. NP2 prevents thrombus formation in a rose bengal/laser induced model

NP2 (8 or 80 $\mu\text{g}/\text{kg}$) or PBS (control) was injected in the cava vein of rats and thrombosis was induced by slow injection (over 2 min) of 90 mg/kg body weight of rose bengal dye into the cava vein at a concentration of 60 mg/mL. Before injection, green light laser was applied to the desired site of injury from a distance of 3 cm and remained on until stable occlusion occurred. Each bar represents mean \pm SE, and the number of animals tested for each condition is shown in the Figure. Asterisk, $P < 0.01$, as compared to values observed in control.

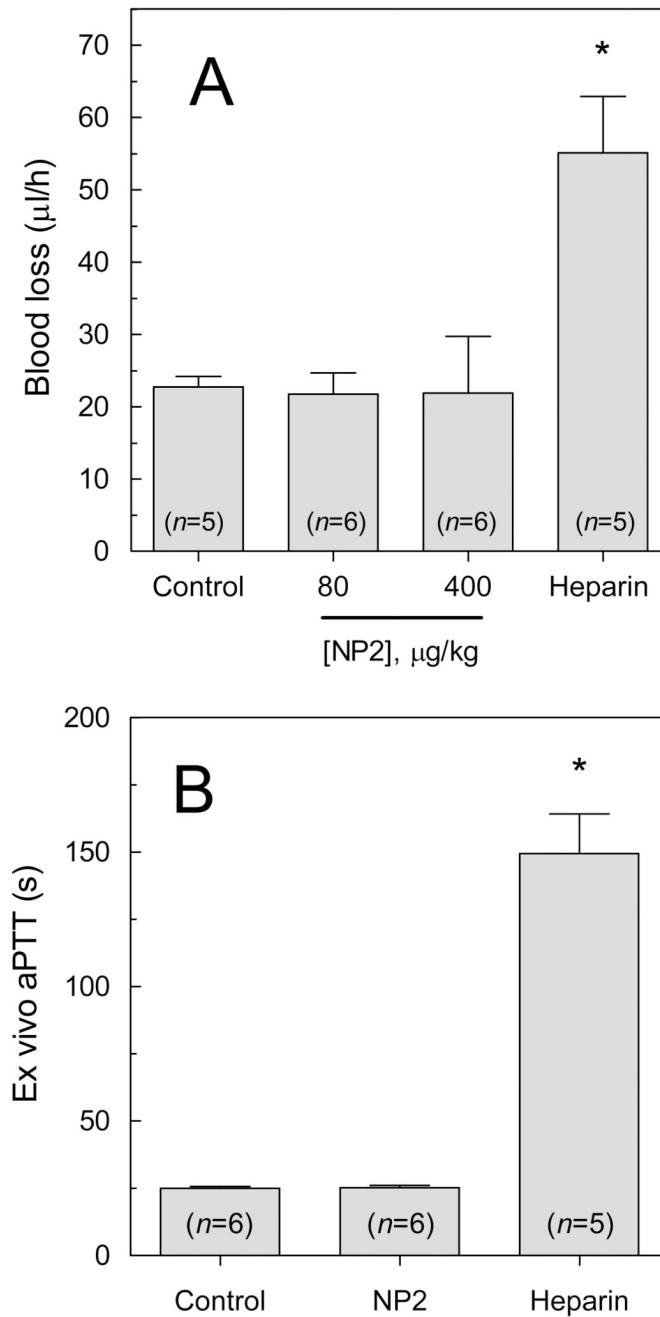


Figure 6. Hemostatic parameters are not changed by NP2

(A) Bleeding effect by transection model. PBS (control), NP2 (80 or 400 µg/kg) or heparin (1 mg/kg) was administered i.v. and allowed to circulate for 15 min. Blood loss was determined as a function of hemoglobin in water (A540 nm), as described in the material and methods section. Data are reported as mean ± SE. Asterisk, $P < 0.01$, as compared to values observed in control group. (B) Ex vivo aPTT. PBS (control), NP2 (400 µg/kg) or heparin (1 mg/kg) was administered i.v. and allowed to circulate for 30 min. Blood collection, plasma preparation and aPTT measurement were performed as described in the Material and methods section.

Table 1

Kinetics of NP2 interaction with rat FIX and FIXa estimated by SPR.*

	K_{a1} ($M^{-1}s^{-1} \times 10^5$)	K_{d1} (s^{-1})	K_{a2} (s^{-1})	K_{d2} (s^{-1})	K_D (nM)
FIX	5.70	0.069	0.015	0.009	43.0
FIXa	2.86	0.026	0.075	0.080	47.0

* Responses were obtained by injecting FIX or FIXa over immobilized NP2 for 180 s at a flow rate of 30 μ l/min.