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# Cardioprotective effect of ornitho-kinin in an anesthetized, open-chest chicken model of acute coronary occlusion

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## Abstract

The generation of bradykinin (BK; Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) in blood and kallidin (Lys-BK) in tissues by the action of the kallikrein-kinin system has received little attention in non-mammalian vertebrates. In mammals, kallidin can be generated by the coronary endothelium and myocytes in response to ischemia, mediating cardioprotective events. The plasma of birds lacks two key components of the kallikrein-kinin system: the low molecular weight kininogen and a prekallikrein activator analogous to mammalian factor XII, but treatment with bovine plasma kallikrein generates ornitho-kinin [Thr$^6$,Leu$^8$]-BK. The possible cardioprotective effect of ornitho-kinin infusion was investigated in an anesthetized, open-chest chicken model of acute coronary occlusion. A branch of the left main coronary artery was reversibly ligated to produce ischemia followed by reperfusion, after which the degree of myocardial necrosis (infarct size as a percent of area at risk) was assessed by tetrazolium staining. The *iv* injection of a low dose of ornitho-kinin (4 µg/kg) reduced mean arterial pressure from 88 ± 12 to 42 ± 7 mmHg and increased heart rate from 335 ± 38 to 402 ± 45 bpm (N = 5). The size of the infarct was reduced by pretreatment with ornitho-kinin (500 µg/kg infused over a period of 5 min) from 35 ± 3 to 10 ± 2% of the area at risk. These results suggest that the physiological role of the kallikrein-kinin system is preserved in this animal model in spite of the absence of two key components, i.e., low molecular weight kininogen and factor XII.

Key words: Chicken; Infarct size; Kallikrein-kinin system; Ornitho-kinin; Factor XII

## Introduction

The kallikrein-kinin system (KKS) consists of a phylogenetically ancient cascade that includes substrates (kininogens), proteases (kallikreins), biologically active peptides (kinins), peptidases (kininases), and kinin receptors. In mammals, the plasma KKS is part of the so-called "contact" system, that becomes activated when blood contacts surface materials (contact activation). The contact system consists of the zymogens factor XII (FXII), factor XI (FXI), plasma prekallikrein (PK), and the nonenzymatic cofactor high molecular weight kininogen (HK). Once activated, activated factor XII (FXIIa) can activate three central substances that trigger different pathways: FXI, that triggers blood coagulation, PK, that leads to the release of bradykinin (BK; Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) from HK, and C1 esterase, that triggers the complement system (1).

Another key enzyme of the KKS is tissue kallikrein (TK), an intracellular enzyme that is released and cleaves low molecular weight kininogen (LK), releasing Lys-BK (1). A number of observations have focused on Lys-BK as a potential mediator of endogenous cardiovascular protective mechanisms. This is due to the fact that KKS components are localized in the heart and in the vascular tissues (1-4). Lys-BK is released during ischemia, causing beneficial cardiac effects and contributing to the cardioprotective effects of preconditioning (2-4). Lys-BK reduces arteriolar resistance after triggering several downstream events *via*

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activation of $B_2$ receptors, a seven-transmembrane G-protein-coupled receptor on endothelial cells, and initiating prostacyclin synthesis, with the formation of nitric oxide and smooth muscle hyperpolarization factor (1,2).

Recently, there has been renewed interest in FXII and the KKS. Data from animal models strongly suggest that these proteins play a decisive role in pathological thrombus formation in mammals. Genetically altered animals have been used strategically to study the physiological and pathological roles of several constituents of the KKS. In particular, knock-out mice genetically deficient in BK $B_2$ receptor, HK, and FXII have shown delayed thrombus formation (5-9).

The avian KKS differs significantly from the mammalian KKS. A recent study has shown that a single gene corresponding to the evolutionary predecessor of FXI and PK occurs in chickens, but the predecessor of FXII is not present in the genome (10). Accordingly, FXII activity cannot be detected in avian plasma by the classical methodology (11-14), whereas three other constituents of the KKS, i.e., HK (15), PK (16) and TK (17), have been described in this animal species. Ischemic heart damage can be reduced in birds by ischemic preconditioning (18). Whether protection against cardiac ischemic damage in birds depends on kinins is not known. Treatment of avian HK with bovine PK generates a BK-like substance, denoted ornitho-kinin [$Thr^6$,$Leu^8$]-BK (OK). Whether OK protects against ischemic damage in birds is not known.

By studying the possible cardioprotection elicited by OK in an animal model of acute ischemia-reperfusion injury, the natural deficiency of LK provides the opportunity to identify the importance of this substrate in the physiological role of the KKS. Our objective was to determine whether the OK receptor is functional in this cardiovascular system by using a Langendorff preparation and an open-chest method to assay its vasodilator and cardioprotective effects against ischemia-reperfusion injury, respectively.

## Material and Methods

### Animals and care

All procedures involving animals and their care were conducted in accordance with the Guidelines for the Use of Animals in Biochemical Research (19). This study was approved by the Comissão de Ética no Uso de Animais do Instituto Butantan (CEUAIB, protocol No. 189/04).

### Drugs

Bradykinin, captopril and 2,3,5-triphenyltetrazolium chloride were purchased from Sigma Aldrich Co. (USA), salts from Merck (Germany), sodium heparin (Liquemine) from Roche Laboratories (Brazil), and diazepam from Cristalia (Brazil). Sodium pentobarbital was a gift from Abbott Laboratories (Brazil). Ornitho-kinin ([$Thr^6$,$Leu^8$]-bradykinin) (purity >95% in high performance liquid chromatography; gradient system 5-100% in 25 min. A = 0.1% trifluoroacetic acid (TFA); B = acetonitrile with 0.1% TFA) was synthesized by the Laboratory of Applied Toxicology of Instituto Butantan (Brazil).

### Effect of *iv* ornitho-kinin on mean arterial blood pressure

Chickens were anesthetized with a mixture of sodium pentobarbital and chloral hydrate (equithesin, 2.5 mL/kg body weight), injected deeply into the pectoral musculature (20). The left brachial vein was cannulated with polyethylene (PE50) tubing and connected to a pressure transducer for drug administration. Data were recorded with a data acquisition system (Biopac Systems, Inc., USA). OK and BK were injected into the brachial vein as a bolus at doses of 1, 2, 4, 8, 16, 32, and 64 µg/kg. Captopril was then injected (0.1 mg/kg, *iv*) and the responses to OK were measured again. This dose of captopril inhibits by 50% the breakdown of BK by the angiotensin-converting enzyme in mammals (21).

### Measurement of ornitho-kinin-induced coronary vasodilation

An adaptation of the Langendorff preparation suitable for the chicken heart (22) was used. Five birds were anesthetized with equithesin and diazepam. Feathers were plucked from the skin on the medial surface of the wing, and 1000 IU sodium heparin was given through the left brachial vein to prevent blood coagulation. After thoracotomy, the heart and the brachiocephalic trunk were removed. The aorta was placed quickly on the cannula of the perfusion apparatus, and perfusion of the coronary circulation was started within 1 min after section of the carotid arteries. Perfusion pressure was measured from a port in the cannula within 10 mm of the coronary ostia. Hearts were perfused retrogradely with Krebs-Henseleit solution (6.71 g/L NaCl, 1.08 g/L KCl, 0.37 g/L $CaCl_2 \cdot H_2O$, 2.10 g/L $NaHCO_3$, 0.16 g/L $KH_2PO_4$, 0.10 g/L $MgCl_2 \cdot 6H_2O$, and 2.30 g/L glucose) with 6.25 g/L dextran. The solution was gassed with 95% $O_2$, 5% $CO_2$ and warmed to 40°C. pH was maintained between 7.38 and 7.40 by adjustment of the flow rate of $CO_2$ through the oxygenator. Coronary flow rate was adjusted to 13 mL/min, about 5% of the cardiac output for the live bird. Since pump flow was fixed, changes in perfusion pressure were indicative of alterations in coronary resistance. Changes in perfusion pressure were measured with a pressure transducer, coupled to a recording system (PowerLab/4SP, AD Instruments, USA). Ventricular pressure and rate were recorded from a fluid-filled balloon tied into the left ventricle (isovolumic heart). Increasing doses of OK (1, 2, 4, 8, 16, 32, 64, 128, 256, 512 ng) were injected as a bolus into a port of the perfusion apparatus close to the coronary ostia. Subsequent injections were made only after blood pressure returned to baseline.

### Hemodynamic changes induced by coronary artery occlusion and reperfusion

Chickens were anesthetized with equithesin. The left

brachial vein was cannulated with PE50 tubing for administration of diazepam (2.5 mg/kg). Additional doses of diazepam (1 mg/kg, *iv*) were given when noxious pinching induced withdrawal reflexes. The corneal/palpebral reflex remained present during the experiment.

The right brachial artery was cannulated with PE50 tubing for continuous mean arterial pressure and heart rate recordings. A PE50 catheter was inserted into the right external jugular vein and the tip was advanced to the right atrium for drug infusion. The animal was tracheotomized and ventilated artificially with room air (TOPO Dual mode Ventilator, Kent Scientific Corp., USA). Initial ventilation was 25 inflations/min (500-1000 mL/min). Arterial pH was maintained between 7.35 to 7.45, $P_{CO_2}$ between 20 and 40 mmHg, and $P_{O_2}$ between 90 and 135 mmHg by supplementing the inspired air with 95% $O_2$ or with 5% $CO_2$ and changing the rate of ventilation. Blood samples (5 mL) from the left brachial artery were used for blood gas analysis. Body temperature was maintained at 37° to 37.7°C. A thoracotomy was performed via an incision along the left lateral edge of the keel to the first rib, with care taken to avoid the air sacs. The pericardium was opened to expose the left ventricle. A 4-0 prolene suture was looped loosely around a marginal branch of the left main coronary artery to permit coronary artery occlusion.

After 30 min of recording, chickens were randomly allotted to one of two treatment groups of 8 animals each. Controls received a 5-min intra-atrial infusion of 0.9% NaCl solution (2 mL/min) and experimental animals received the same infusion containing OK (100 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Ten minutes after the end of infusion, 400 IU heparin was injected, and 1 min later chickens were subjected to 60 min of coronary ischemia followed by 180 min of reperfusion. This protocol for the induction of myocardial ischemic injury has been previously used in rabbits (23). Reperfusion was validated by the return of the original color. Arterial pressure and heart rate were recorded throughout the experiment.

After reperfusion, the heart was excised, the coronary artery ligature was retied, and the perfused myocardium was stained by perfusing black China ink through the aorta. The unstained region was denoted area at risk. The left ventricle with the septum was separated from surrounding tissue and cut from the apex to the base into 1-mm slices. Slices were incubated for 30 min at room temperature with 2,3,5-triphenyltetrazolium chloride (1% in 0.1 M sodium phosphate buffer, pH 7.4) in order to stain viable tissue red, leaving the infarcted region pale. The slices were photographed, and left ventricular area, area perfused by the tied artery (area at risk), and infarcted area were quantified with the ImageJ program (National Institute of Health, USA).

### Calculations and statistics

All quantitative data are reported as means ± SEM. The effect of the treatments on infarct size was compared by one-way ANOVA and *post hoc t*-tests with Bonferroni's correction. Temporal changes in hemodynamic parameters within each group were tested by one-way repeated measures ANOVA followed by paired *t*-tests with Bonferroni's correction. Since coronary flow in the Langendorff system was constant, changes in coronary vascular resistance were calculated from changes in perfusion pressure and were compared by one-way repeated measures ANOVA followed by paired *t*-tests with Bonferroni's correction. Differences were considered to be statistically significant at an error level of $P < 0.05$.

## Results

The *iv* injection of OK produced a dose-dependent and large but transient fall in arterial pressure (Figure 1) and tachycardia. The 4-µg/kg dose increased heart rate from 335 ± 38 to 402 ± 45 bpm (N = 5; $P < 0.05$). The kininase II inhibitor captopril (0.1 mg/kg, *iv*) potentiated the hypotension induced by 1 µg/kg OK (40 ± 6 *vs* 76 ± 10 mmHg, N = 5; $P < 0.05$).

The injection of OK into the coronary circulation of

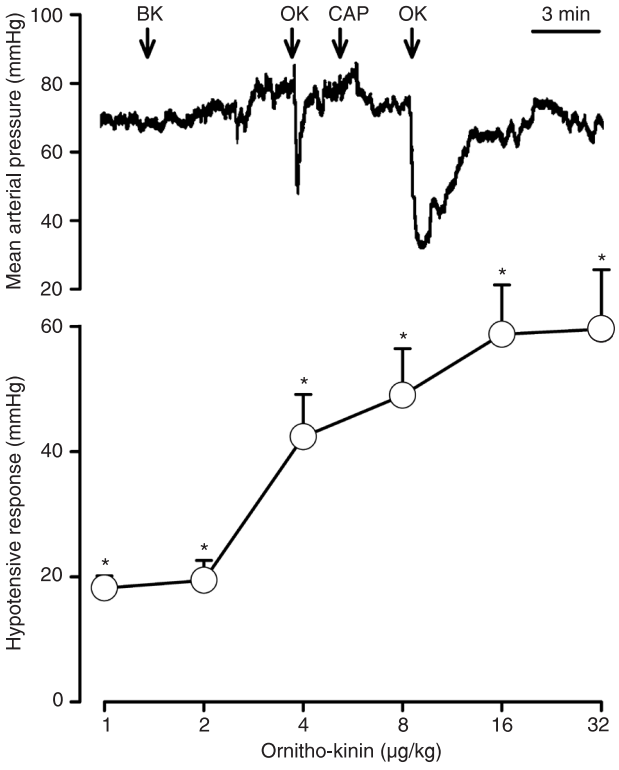


**Figure 1.** Effect of *iv* injected ornitho-kinin on arterial pressure in the anesthetized chicken. *Top*, typical example. BK = bradykinin, 20 µg/kg; OK = ornitho-kinin, 1 µg/kg; CAP = captopril, 0.1 mg/kg, *iv*. *Bottom*, group data. Data are reported as means ± SEM for 6 animals each dose. *$P < 0.05$ *vs* no ornitho-kinin (ANOVA followed by the paired *t*-test).

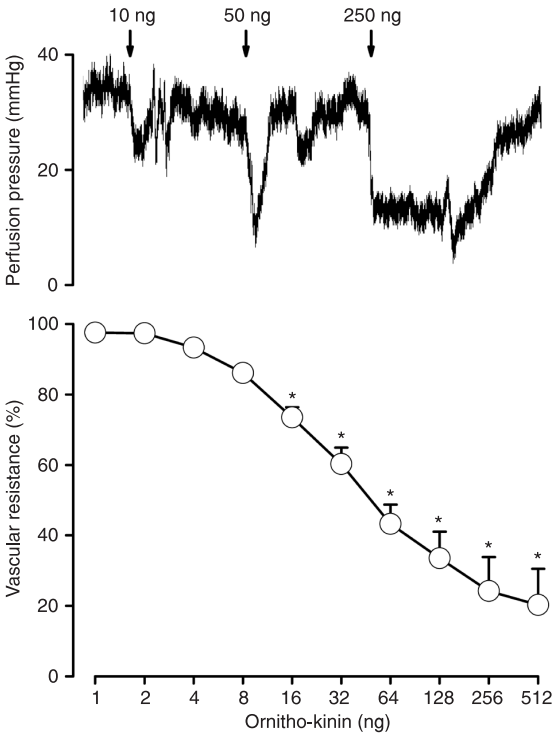


**Figure 2.** Effect of bolus injections of ornitho-kinin on coronary vascular resistance. The peptide was introduced into the perfusion medium as it entered the heart. Data are reported as means ± SEM for 6 animals each dose. *P < 0.05 *vs* no injection (ANOVA followed by the paired *t*-test).

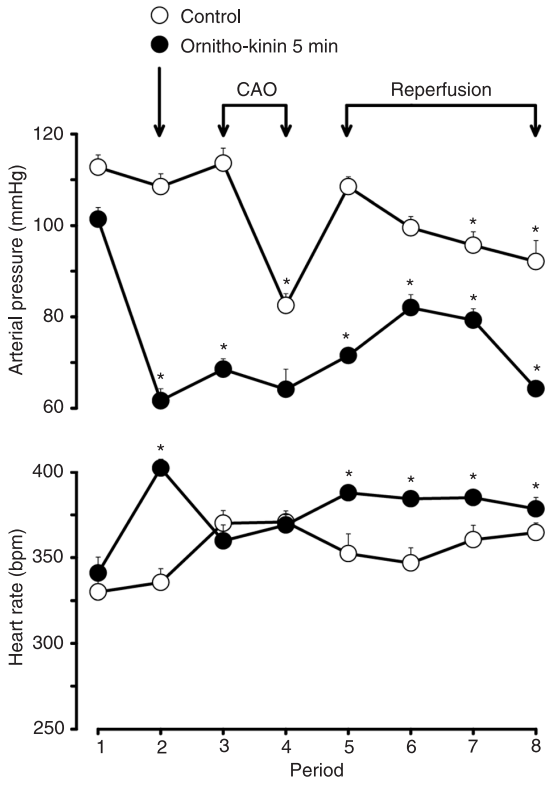


**Figure 3.** Effect of pretreatment with ornitho-kinin on changes in arterial pressure and heart rate induced by coronary artery occlusion (CAO) and reperfusion in anesthetized chickens. The long arrow indicates pretreatment (saline or ornitho-kinin, 500 µg/kg infused into the right ventricle for 5 min). Data are 1-min averages. Numbers on the X-axis indicate the following periods: 1 = just before the start of the experiment; 2 = in the middle of the period of infusion; 3 = just before CAO; 4 = at the end of 1 h of CAO; 5 = after 1 min of reperfusion, and 6-8 = after 1, 2, and 3 h of reperfusion, respectively. Data are reported as means ± SEM for 8 animals each dose. *P < 0.05 *vs* baseline (ANOVA followed by the paired *t*-test).

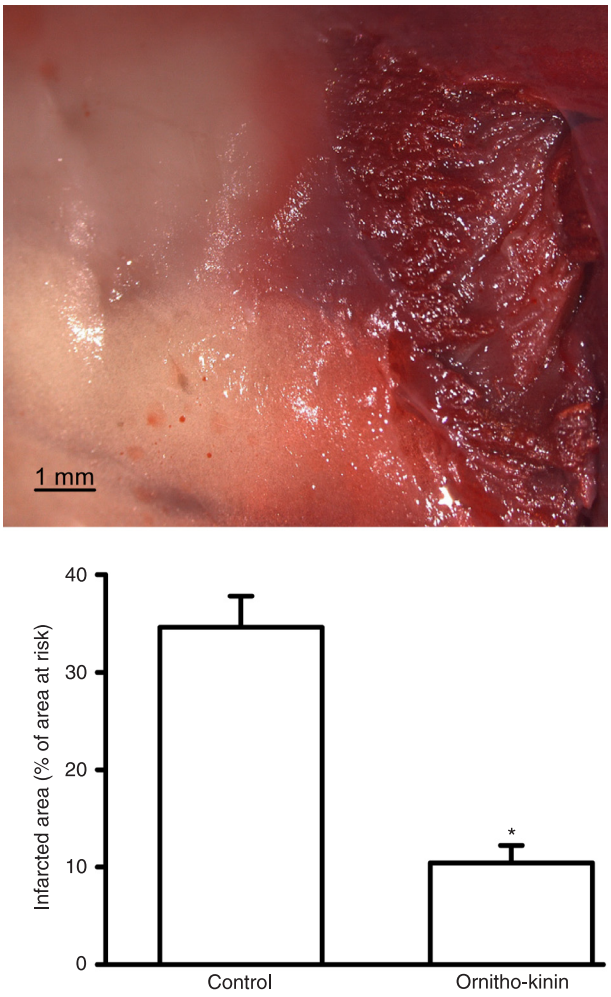


**Figure 4.** Effect of pretreatment with ornitho-kinin (500 µg/kg perfused for 5 min) on infarct size induced by coronary artery occlusion in anesthetized chickens. *Top*, Section of a triphenyltetrazolium chloride-stained wall of the left ventricle with healthy (red) and infarcted (pale) tissue. Data are reported as means ± SEM in the bar graph for 8 animals each dose. *P < 0.05 *vs* saline control (ANOVA and *post hoc t*-tests with Bonferroni's correction).

perfused hearts caused an intense and concentration-dependent vasodilation (Figure 2). OK did not significantly alter heart rate or systolic pressure (baseline: 154 ± 18 bpm, 68 ± 5 mmHg, N = 5).

Figure 3 shows the cardiovascular changes that occurred during cardiac ischemia and reperfusion. In control animals not injected with OK, occlusion of the left coronary artery significantly reduced arterial pressure. Upon release of the coronary ligature, pressure returned to pre-ischemic value, but tended to fall again during the reperfusion period, a fact possibly indicating the development of heart failure. The 5-min infusion of OK reduced arterial pressure by almost 40 mmHg. Arterial pressure fell significantly during occlusion of the coronary artery, but less than in controls, and pressure increased after release of the ligature, although it remained low throughout the rest of the experiment.

The tied coronary artery supplied blood to about half the ventricular surface in both groups (controls: 48 ± 5%, OK-pretreated: 58 ± 6%, difference not significant), indicating a constant placement of the coronary ligature. In the control group pretreated with 0.9% saline solution, ischemia followed by reperfusion caused a large myocardial infarct (35 ± 3% of the area perfused by the tied artery). Infarct size was reduced to 10 ± 2% by pretreatment with OK (P < 0.05) (Figure 4). Smaller doses of OK (25, 50, and 75 µg·kg$^{-1}$·min$^{-1}$ for 5 min, N = 4 each) failed to reduce infarct size.

## Discussion

Blockade of the left coronary artery followed by 3 h of reperfusion reliably caused an infarct in chickens. This is consistent with *in vitro* studies on a Langendorff preparation showing that ischemia can produce myocardial injury in pigeons (18). Features of the *in vivo* method used by us included little variation in the area perfused by the tied artery (area at risk of about 50% of the left ventricular wall), and an infarct size of about 30% of the area at risk, also with little variation. Arterial pressure fell during the period of ischemia, suggesting developing heart failure, similar to the changes seen in mammals.

OK caused a short-lasting vasodilation in the heart and the rest of the body, indicating that OK receptors are present in the chicken, in agreement with previous data (24). The potentiation of the effect of OK by captopril indicates that chickens contain kininases. Even large amounts of BK failed to induce hypotension in chickens, in agreement with an earlier finding that this mammalian peptide fails to contract the pigeon uterus (25). This indicates that birds may have developed their own KKS that generates a kinin structurally different from mammalian BK with appreciably different bioactivity.

In mammals, infusion of BK has been shown both to protect isolated hearts subjected to local ischemia/reperfusion and to be an important mediator of the beneficial effect of ischemic preconditioning (26). We found that OK also protected against ischemic heart damage. The dose of OK used by us (500 µg/kg in 5 min) was chosen because it reduced blood pressure without inducing cardiovascular collapse. One possible explanation for the need for these higher doses injected systemically is that the generation of Lys-BK occurs locally during episodes of acute ischemia (4) and the local concentration of Lys-BK is relatively high, improving adequate receptor binding. Whether or not this dose is the same as the level of OK at cardiac receptors during ischemic preconditioning is not known, but it is lower than the dose of BK necessary to induce cardiac preconditioning in rabbits (1250 µg/kg in 5 min) (26). Since the effect of ischemic preconditioning in rabbits was blocked with the BK receptor antagonist HOE 140 (26), it appears that this infusion resulted in levels of BK at the cardiac receptors similar to those that occur *in vivo* in ischemic preconditioning. No drugs are known to block OK receptors: the potent mammalian $B_2$ receptor antagonist, HOE 140, acts as an OK receptor agonist (24).

From these experiments, it became apparent that a functional but qualitatively different KKS (PK, HK, OK specific receptors) is present in chickens. The cardioprotective activity of OK in our acute anesthetized, open-chest chicken model of acute coronary occlusion suggests the presence of a functioning cardiac kallikrein kinin system. This finding challenges current studies on mammals connecting this cardioprotective action of the cardiac KKS to cleavage of LK by TK (1), since LK seems to be absent from the plasma of this animal species (15).

We propose the chicken as an experimental model suitable for studying several open questions concerning the physiological role of the KKS. These include: i) how PK is activated *in vivo* in the mammalian contact system. Several studies have reported the *in vivo* activation of PK either as dependent on (27) or independent of FXII (28). Since FXII is not present in avian plasma, alternative mechanisms of PK activation independent of FXII can be investigated in birds; ii) the selectivity of kallikreins for specific substrates, as seen in mammals (PK for HK and TK for LK), is not possible in the plasma of chickens since only HK was detected in this species (15). The possibility of HK cleavage by TK opens alternative routes for kininogen cleavage, and may help to understand the physiological roles of LK; iii) in mammals, the mechanism of FXI activation *in vivo* during hemostasis remains polemic, and both platelet/FXII-dependent (29,30) and FXII-independent (31) mechanisms have been proposed. Thrombocytes are homologous in function to mammalian platelets, playing a role similar to these cells with regard to aggregation and clot formation (32,33). Therefore, the activation of FXI by thrombocytes can be studied in birds in the absence of FXII, and iv) although FXII is not involved in hemostasis, there is a debate about whether it plays a key role in abnormal hemostasis or thrombosis. FXII-deficient mice fail to develop thrombosis in response to vessel injury and consequently FXII could be an ideal target for safe anticoagulation (7).

Transgenic and gene-targeting technologies allowing the generation of genetically altered animal models have greatly advanced our understanding of the function of specific genes. This is also true for the KKS, in which some, but not yet all, components have been functionally characterized using such techniques. The first genetic change in the KKS was found in a brown Norway rat that had a natural mutation that inactivated the kininogen gene. Mice deficient in or overexpressing TK, $B_1$ and $B_2$ receptors are accessible today. There is still no animal model with genetic alterations of PK, kininases I and some other degrading enzymes (34).

In conclusion, in addition to specific receptor blockers and genetically modified mouse models, this FXII-deficient animal model supplied by nature can help unravel the roles of the intertwined branches of the KKS and the physiological and pathophysiological roles of FXII, TK and LK in hemostasis, thrombosis and in the cardiovascular system.

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