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ANGIOTENSIN-(1-9), THE PRODUCT OF ANGIOTENSIN I CONVERSION IN PLATELETS, ENHANCES ARTERIAL THROMBOSIS IN RATS

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Angiotensin (Ang) (1-9) is the renin-angiotensin-system peptide found in the plasma of healthy volunteers and after angiotensin-converting-enzyme inhibitors therapy. *In vitro* experiments proved that Ang-(1-9) may be produced from Ang I. In our study, we tried to expand the poor data about the *in vivo* properties of Ang-(1-9). We revealed that Ang-(1-9) enhanced electrically stimulated arterial thrombosis in the carotid artery of Wistar rats. Losartan, a selective blocker of AT_1 receptor for Ang II, abolished the prothrombotic activity of Ang-(1-9). This peptide increased plasma level of fibrinogen, augments fibrin generation, and similarly to Ang II, potentiated collagen induced platelet aggregation. Using HPLC, we found that after incubation of Ang-(1-9) with platelet homogenates or after intravenous administration this peptide is converted to Ang II. We concluded that Ang-(1-9) exerts an Ang II-like prothrombotic effect due to the conversion to Ang II in the circulatory system of rats and that platelets are involved in this process.

Key words: *angiotensin 1-9, arterial thrombosis, platelets, renin-angiotensin system, fibrin, fibrinogen, angiotensin-converting enzyme inhibitors*

INTRODUCTION

There is a number of studies, including ours, indicating that peptides of renin-angiotensin-system (RAS) can modulate thrombosis by affecting various elements of haemostasis (1-7). Large clinical trials demonstrated that angiotensin-converting-enzyme inhibitors (ACE-Is) and AT_1 receptor antagonists (AT_1-As) reduce the risk of mortality due to cardiovascular causes after myocardial infarction (MI) or stroke (8-14). The main pathogenic factor responsible for those urgent clinical events is intravascular arterial thrombosis. Our and other experimental studies provided evidence for the antithrombotic action of ACE-Is and AT_1-As in arterial thrombosis models (15-17). All these evidences clearly indicate that RAS may affect the development of arterial thrombosis.

Ang-(1-9) is the RAS peptide found in the plasma of healthy volunteers and in patients treated with ACE-Is (18). It was also demonstrated that the concentration of Ang-(1-9) increases in plasma and heart tissue of rats after MI (19). It can be produced from Ang I by CPA or a CPA-like enzyme (20-22) or by the recently recognized ACE-2 (23-25). Campbell *et al*. in *in vitro* studies found a higher than Ang II concentration of Ang-(1-9) in the kidney homogenates, where it reaches about 50% of Ang I level (26). Moreover, the main products of Ang I degradation in human heart tissue are both Ang-(1-9) and Ang II generated by heart chymase, ACE or carboxypeptidase A (21). Interestingly, Snyder *et al*. showed that the main product of Ang I conversion by human platelet enzymes is not Ang II but Ang-(1-9) (27, 28). All those evidences indicate the importance of the Ang-(1-9) in the pathways of RAS component formation, especially in the heart, kidney and platelets.

The data about the properties of Ang-(1-9) are poor and divergent. Ang-(1-9) was shown to be a strong competitive inhibitor of ACE (at multiple-fold lesser concentration than Ang I) both in human heart tissue and platelets (21, 27). Moreover, Ang-(1-9), like Ang-(1-7) and ACE-Is, increases nitric oxide and arachidonic acid release due to enhanced bradykinin action on its B_2 receptor (29), while the linkages among vasodilator systems: NO, prostaglandins and bradykinin are well known (30). In contrast, there is one *in vivo* study indicating that Ang-(1-9) is a potent dipsogen when injected into the cerebroventricles of rats. It was suggested that the drinking response probably requires a second hydrolysis to Ang II (22). This thesis is supported by *in vitro* experiments showing that Ang-(1-9) is a source of Ang II in rat kidneys (28, 31).

We have previously shown the prothrombotic effect of Ang II (2, 3) and the anthitrombotic action of Ang-(1-7) (1) in renovascular hypertensive rats. Thus the question arises if Ang-(1-9) effects are similar to the properties of Ang II or maybe properties of Ang-(1-7) in *in vivo* conditions. Therefore, in our present study we tried to determine if Ang-(1-9) is the main metabolite of Ang I also in rat platelets and in which manner this peptide affects platelet function. Since we have previously shown that various RAS peptides may modulate progress of thrombotic process and platelets activity (1-3), we tried to investigate if Ang-(1-9) may affect the arterial thrombotic process in *in vivo* conditions.

MATERIALS AND METHODS

Animals and ethics

Male Wistar rats (200-300 g) were used in the experiments. They were housed in a room with a 12 hours light/dark cycle, in group cages as appropriate, were given tap water and fed a standard rat chow. Rats were anesthetized with pentobarbital (40 mg/kg, i.p.).

The procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research (32).

Chemicals and drugs

Angiotensins (Bachem, UK), losartan (Dup 753, DuPont Merck Pharmaceutical Co., USA), heparin (Heparinum, Polfa, Poland), DX-600 (Phoenix Pharmaceuticals, INC. USA), benzylsuccinate, universal protease inhibitors cocktail, tris buffer (Sigma Chemical Co., USA), acetonitrile, trifluoroacetic acid, EDTA and methanol (Merck, Germany), pentobarbital (Vetbutal, Biovet, Poland) were used in the study. Reagents to prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (Fg) level measurements were bought in HemosIL, Instrumentation Laboratory (USA).

Angiotensin 1-9 and losartan administration

VEH (n=11) or Ang-(1-9) were infused into the right femoral vein of Wistar rats in doses of 400 (n=6), 800 (n=8) and 1600 pmol/kg/min (n=10) 10 min before the induction of arterial thrombosis. The infusions were continued for 55 min, and were given in a volume of 2 ml/kg/h to maintain euvolemia. Doses of Ang-(1-9) were chosen based on our previous studies, where Ang II or Ang-(1-7) exerted their effects after infusion into renovascular hypertensive rats in doses of 400 or 10 pmol/kg/min, respectively. Since we have previously observed much smaller activity of angiotensins in normotensive animals, we administered Ang-(1-9) in higher doses. Moreover, doses were chosen based on other studies with RAS peptides (33-35). Losartan (LOS), a selective AT_1 antagonist, was administered into the left femoral vein in a dose of 30 mg/kg 5 minutes before Ang-(1-9) or VEH infusions to investigate the involvement of AT_1 receptors in the prothrombotic action of Ang-(1-9).

Induction of arterial thrombosis

Male Wistar rats were fixed in the supine position on an operation table and the right femoral vein was cannulated to administer the drug. We induced arterial thrombosis by electrical stimulation of the common carotid artery according to the method previously described by Schumacher *et al*. (28) and by us (3, 36). Briefly, the anode, a stainless steel L-shaped wire, was inserted under the artery and connected to a circuit with a constant current generator. The cathode was a subcutaneous metal needle attached to the hindlimb. The stimulation (1 mA) took 10 min. A Doppler flow probe was placed in contact with the carotid artery downstream from the site of electrical stimulation. It was connected to the flowmeter (Hugo Sachs Elektronik, Germany) and carotid blood flow (CBF) was continuously monitored before, during and after electrical stimulation. 45 min after stimulation, the segment of the common carotid artery with the formed thrombus was dissected, opened lengthwise and the thrombus was completely removed, air-dried at 37°C and weighed 24 hours after the end of experiment.

Coagulation parameters and fibrin generation

PT and APTT were automatically determined by optical method (Coag-Chrom 3003; Bio-ksel, Poland) adding routine laboratory reagents to the collected rat plasma. The fibrinogen plasma level (Fg) was evaluated according to the Clauss method. We used the previously described method (37, 38), modified and adapted to use on laboratory animals (39) to measure fibrin generation. A fibrin generation curve was created by adding $CaCl_2$ (36 mM) to the Tris buffer (66 mM Tris and 130 mM NaCl, pH=7.4) and mixed with the rat plasma sample. Optical density was measured *via* the microplate reader (Dynex Tech., USA) in 1 min intervals for 15 minutes and expressed as area under the curve (AUC).

Ang-(1-9) degradation in rat circulatory system

The rats were anesthetized and the femoral vein was isolated and canulated for the administration of compounds. Then the right carotid artery was isolated and cannulated, as described previously, for blood collection (40). Ang-(1-9) was administered introvenously (i.v.) in a dose of 200 µmol/kg. We administered this nonapeptide in the high dose of 200 µmol/kg to omit endogenous levels of the measured peptides. The control group received 0.9% NaCl solution (2 ml/kg). An universal protease inhibitor cocktail solution in 3.8% EDTA in a volume ratio of 1:10 was used to collect blood samples in volume of 1 ml every 20 seconds after a single IV administration. We measured 4-7 times peptide plasma concentration at each time interval, but no more than 4 ml was collected from each rat. The samples were centrifuged (15 min, 2000g, 4°C), and then platelet poor plasma (PPP) was frozen for further peptide analysis.

Ang I and Ang-(1-9) degradation by platelet homogenates

Citrated rat blood was used for platelet isolation. The platelets were washed according to Snyder *at al*. (28). The platelet rich plasma (PRP) was centrifuged and the platelets were suspended in phosphate buffer saline containing glucose and trisodium citrate. Then the mixture was centrifuged and suspended in the same buffer again. Next, it was centrifuged and suspended in acetate buffer, pH=7.0 to gain 10^4 platelets per 1 µl of buffer. After that, the platelets were freeze-thawed and sonificated twice to obtain buffered platelet homogenate (BPH).

According to Snyder work the BPH was incubated with Ang I, Ang-(1-9), Ang-(1-7) or Ang II (in a concentration of 20 µmol/l) for 0, 15 or 30 minutes at 37°C, pH=7.0. Ang-(1-9) was also incubated with BPH and DX-600 (10 nmol/l) and benzylsuccinate (10 µmol/l). The reaction was stopped by cooling (4°C) and adding a cold universal cocktail of protease inhibitors. Angiotensin peptides concentration was determined by HPLC.

The measurement of angiotensins concentration in rat plasma and platelet homogenates by HPLC

The specimens were extracted using the SEP technique (Waters, Sep-Pak Vac 1cc C18), filtered (0.45 µm) and analyzed by high performance liquid chromatography (HPLC) using the HP-1200/1050 device with UV detection (λ=214 nm). In brief, peptide separation was achieved on a Waters µBondpack C18 column, 10 µm, 125 Å using a mobile phase of 0.05% trifluoroacetic acid and acetonitrile, in time of 70 minutes as described by others (41). Retention time and recovery (after preparation from plasma mixed with protease inhibitors cocktail) of angiotensin peptides are shown in *Table 1*.

Platelet aggregation measurement

Platelet aggregation was studied in *ex vivo* and *in vitro* conditions. Whole blood platelet aggregation was monitored by measuring electrical impedance in the Chronolog aggregometer (Chrono-log Corp., USA) according to the method described by Cardinal *et al*. (29). Blood samples were collected from animals receiving a 2-hour infusion of VEH (0.9% NaCl) (n=6) and Ang-(1-9) (n=6) or Ang II (n=5) in doses of 1600 or 400 pmol/kg/min, respectively, into 3.13% trisodium citrate in a volume ratio of 10:1. After 15 minutes of incubation at 37°C with 0.9% NaCl, collagen (5 µg/ml) was added. Then, changes in resistance were registered for 6 minutes. The maximal extension of the aggregation curve at the 6th minute was expressed in Ohms (Ω). In addition, in the *in vitro* study, blood samples from untreated rats were mixed with Ang-(1-9) in concentrations of 10^{-12} M (n=6), 10^{-9} M (n=8) and 10^{-6} M (n=10) or VEH solutions (n=15) before incubation, and then collagen (10 µg/ml) was added.

Table 1. Retention times and recovery of angiotensin peptides from rat plasma.

Sample	Shortcut	Retention time [min.]	% recovery
angiotensin-(1-7)	Ang-(1-7)	9.10	53.01±2.83
angiotensin-(1-5)	Ang-(1-5)	11.90	61.13±3.40
angiotensin-(1-9)	Ang-(1-9)	16.84	63.40±2.10
angiotensin III	Ang III	26.50	47.50±4.80
angiotensin II	Ang II	27.78	51.67±8.67
angiotensin IV	Ang IV	28.25	50.70±8.83
angiotensin-(2-10)	Ang-(2-10)	40.03	48.30±2.27
angiotensin I	Ang I	40.94	47.87±1.45

Data for percentage of peptides recovery in respect to standards dissolved in rat plasma inactivated with protease inhibitor coctail are presented as mean ±SEM; n=5. Retention times are stated for a representative peptide separation by reversed phase HPLC in time of 70 minutes. Minimum detectable level for all the peptides was approximately 10^{-8} M.

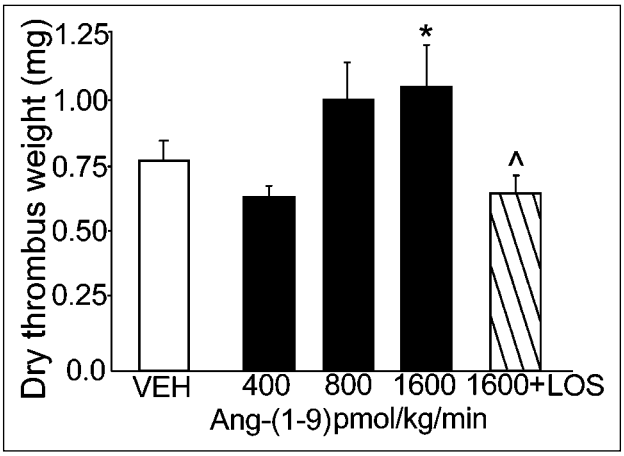

Fig. 1. The columns represent the thrombus weight in Wistar rats treated with: 0.9% NaCl (VEH) (white column), Ang-(1-9) (400, 800 and 1600 pmol/kg/min) (black columns), losartan (LOS) - selective AT_1 receptor antagonist (30 mg/kg) followed by infusion of Ang-(1-9) (1600 pmol/kg/min) (diagonal lined column); * p<0.05 *vs*. VEH; ^ p<0.05 *vs*. Ang-(1-9) (1600 pmol/kg/min). Data are expressed as mean ±SEM.

RESULTS

The effect of Ang-(1-9) on the arterial thrombus formation

Ang-(1-9) infusion resulted in a dose-dependent rise of arterial thrombus weight (0.62±0.05, 1.03±0.12 and 1.08±0.10 mg, for doses of 400, 800 and 1600 pmol/kg/min, respectively, *vs*. 0.77±0.08 mg in the VEH treated group; not significant (ns), ns, p<0.05, *Fig. 1*). LOS administered with Ang-(1-9) abolished the increase in thrombus weight caused by this peptide (0.65±0.05 for Ang-(1-9) with LOS *vs*. Ang-(1-9) administered alone in a dose of 1600 pmol/kg/min; p<0.05).

The effect of Ang-(1-9) on fibrin generation and coagulation parameters

Ang-(1-9) dose dependently increased both the fibrinogen plasma concentration (*Fig. 2*) and enhanced fibrin generation (*Fig. 2* and *Fig. 3*) when compared to VEH treated group. Ang-(1-9) failed to influence APTT and PT, although some tendency (on the border of statistical significance) to shortening the APTT time could be observed (*Table 2*).

The effect of Ang-(1-9) on platelet aggregation

Ang-(1-9) or Ang II infused into animals in a dose of 1600 or 400 pmol/kg/min, respectively, increased collagen induced platelet aggregation (1.45±0.15 for Ang-(1-9) and 1.59±0.16 for Ang II *vs*. 0.77±0.08 Ω in VEH treated group; p<0.05, p<0.01) (*Fig. 4*).

Ang-(1-9) slightly but significantly increased platelet aggregation in *in vitro* conditions (1.81±0.19; 1.87±0.13 and 2.05±0.10 Ω in concentrations of 10^{-12} M, 10^{-9} M or 10^{-6} M, respectively *vs*. 1.79±0.07 Ω in VEH treated group; ns, ns, p<0.05) (*Fig. 5*).

Ang I and Ang-(1-9) processing system in rat platelets

The concentration of Ang I after incubation with BPH (*Fig. 6A*) decreased from 20.00±0.32 µmol/l (time "0") to 14.30±2.52 µmol/l in the 15th minute and to 11.12±2.34 µmol/l in the 30th minute. In the same specimens, we observed an increase of Ang-(1-9) concentration from undetectable concentration at the beginning of incubation to 4.60±2.88 µmol/l in the 15th minute and to 7.00±0.80 µmol/l in the 30th minute. We also detected Ang

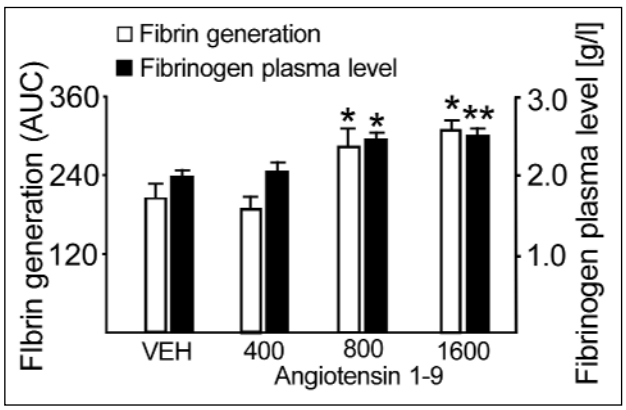

Fig. 2. The columns represent values of fibrin generation (AUC) (white columns) and fibrinogen levels (g/l) (black columns) in plasma of Wistar rats developing arterial thrombosis treated with: 0.9% NaCl (VEH) or Ang-(1-9) (400, 800 and 1600 pmol/kg/min); * p<0.05 *vs*. VEH. Data are expressed as mean ±SEM.

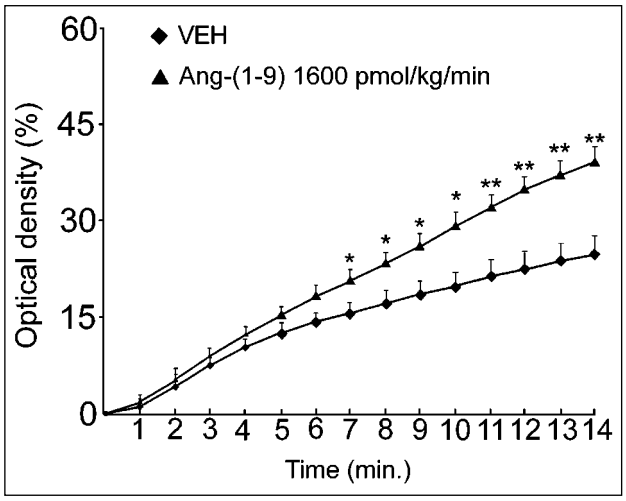

Fig. 3. The curves represent optical density (%) changes during fibrin generation in plasma of rats with induced arterial thrombosis treated with 0.9% NaCl (VEH) or Ang-(1-9) (1600 pmol/kg/min); * p<0.05 *vs*. VEH. Data are expressed as mean ±SEM.

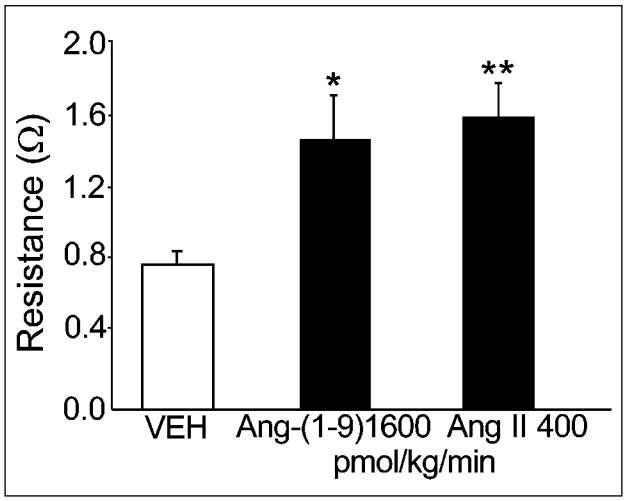

Fig. 4. The columns represent platelet aggregation induced by collagen expressed as resistance between electrodes in whole blood of Wistar rats treated with: 0.9% NaCl (VEH) (white column), Ang-(1-9) (1600 pmol/kg/min) or Ang II (400 pmol/kg/min) (black columns); * p<0.05, ** p<0.01 *vs*. VEH. Data are expressed as mean ±SEM.

Table 2. PT and APTT values from Wistar rat plasma.

Parameter	VEH	Ang-(1-9) 400	Ang-(1-9) 800	Ang-(1-9) 1600
n	10	8	9	7
PT [sec.]	19.52 ± 0.65	22.62 ± 1.38	21.11 ± 0.81	21.44 ± 0.59
APTT [sec.]	29.70 ± 2.11	33.20 ± 0.71	30.35 ± 1.47	25.95 ± 1.87

Prothrombin time (PT) and activated partial thromboplastin time (APTT) values measured in plasma of Wistar rats treated intravenously (*iv*) with 0.9% NaCl or Ang-(1-9) in dose of 400, 800 and 1600 pmol/kg/min. Data are presented as mean ±SEM;

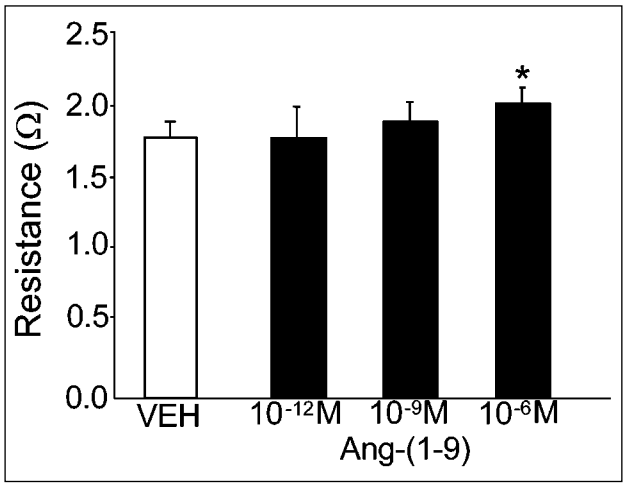

Fig. 5. The columns represent platelet aggregation induced by collagen expressed as resistance between electrodes in whole blood of Wistar rats incubated with: 0.9% NaCl (VEH) (white column), Ang-(1-9) (10^{-12}, 10^{-9} and 10^{-6} M) (black columns);* p<0.05 *vs*. VEH. Data are expressed as mean ±SEM.

II in the 15th minute (0.36±0.36 µmol/l) and in the 30th minute (0.75±0.03 µmol/l).

After incubation of Ang-(1-9) with BPH (*Fig. 6B*), the concentration of this peptide decreased from 20.00±0.11 µmol/l (time "0") to 18.31±1.20 µmol/l in the 15th minute and to 14.37±1.11 µmol/l in the 30th minute. In the same experiment, we detected Ang II in the 15th minute (1.29±0.45 µmol/l) and in the 30th minute (1.83±0.23 µmol/l).

Also we investigated the degradation of Ang-(1-7) and Ang II by BPH in the same conditions. The concentration of Ang II decreased fast from a value of 20.00±0.26 µmol/l (time "0") to 1.45±0.45 µmol/l in the 30th minute, whereas the concentration of Ang-(1-7) almost did not change (3% decreased during 30 minutes of incubation) (*Fig. 6C*). Moreover neither DX-600 nor benzylsuccinate did not inhibit conversion of Ang-(1-9) into Ang II (*Fig. 6D*).

Plasma levels of Ang II and other peptides after Ang-(1-9) single i.v. injection

After a single i.v. administration of Ang-(1-9), we detected this peptide immediately after injection (0.016±0.011 µmol/l), and its concentration was increasing until the 50th second (0.127±0.090 µmol/l), then decreased to 0.006±0.006 µmol/l at 70th second and it was undetectable later (*Fig. 7*). Ang II, similarly to Ang-(1-9), appeared immediately after injection 0.077±0.189 µmol/l, then its concentration increased until 70th second (0.451±0.552 µmol/l). Between 70th and 110th second concentration of Ang II decreased to 0.123±0.247 µmol/l and it was undetectable later. We also found Ang-(1-7) in the concentration increasing immediately after administration of Ang-(1-9) to 0.648±0.270 µmol/l. After that the concentration of this peptide decreased to 0.470±0.330 µmol/l at 50th second, increased once again to 0.625±0.400 µmol/l at 70th second, then it decreased and was undetectable after 110th second. Ang-(1-7)/Ang II ratio vary depending on the time interval from 1,37 to 4,4. We also found smaller peaks of some shorter fragments such as Ang-(1-5) or Ang IV (data not shown).

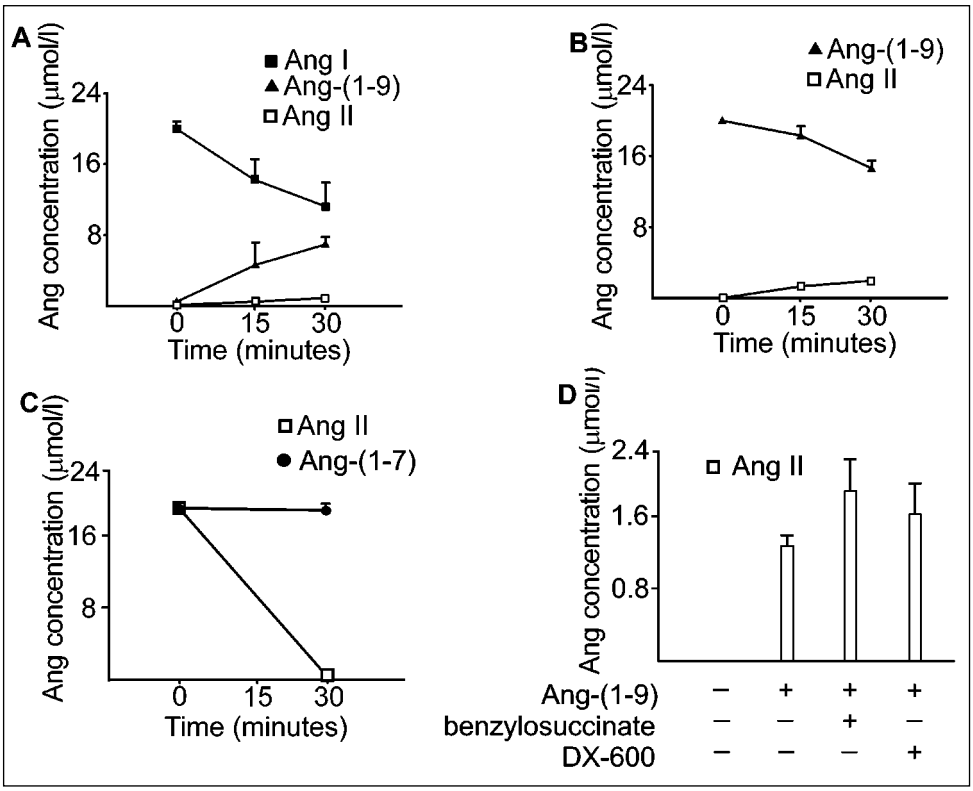

Fig. 6. Time-course changes (μmol/l) of Ang I (black squares) Ang-(1-9) (black triangles) and Ang II (white squares) during incubation of 20 μmol/l of Ang I (graph A), Ang-(1-9) (graph B), Ang II or separately Ang-(1-7) (graph C) and Ang-(1-9) with benzylosuccinate or DX-600 (graph D) with buffered platelet homogenates (BPH). Data are expressed as a mean ±SEM for time zero (the beginning of incubation), after 15 or 30 minutes from the beginning (end of the experiment).

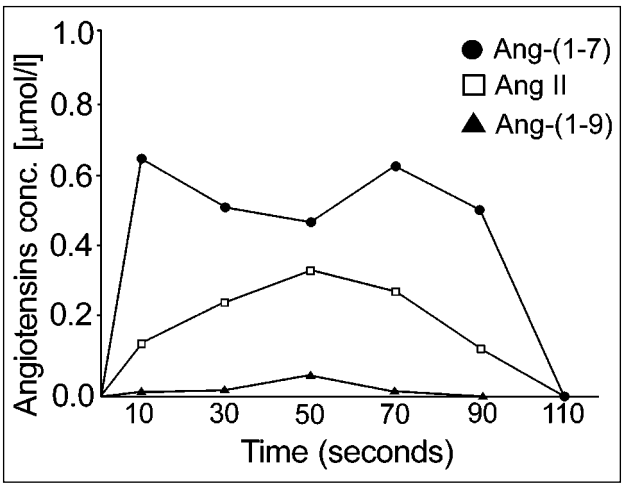

Fig. 7. Time-course changes of plasma levels (μmol/l) of Ang-(1-9) (black triangles), Ang II (white squares) or Ang-(1-7) (black circles) after intravenous injection of Ang-(1-9) (0.2 μmol/kg). Time zero = start of injection. Data are expressed as mean value for each time interval.

DISCUSSION

In our study, we found that Ang-(1-9) enhances electrically stimulated thrombosis in rats and that this effect is abolished by losartan - an antagonist of the AT_1 receptor. The prothrombotic activity of Ang-(1-9) was accompanied by the enhancement of *ex vivo* platelet aggregation. In our *in vitro* experiments Ang-(1-9) also increased platelet aggregation. Moreover, both in *in vivo* and *in vitro* conditions Ang-(1-9) was converted into Ang II.

We have previously shown that the main RAS peptide - Ang II enhances venous (2) and arterial (3) thrombosis. In the current study, we chose the same model of electrically induced arterial thrombosis in the carotid artery of rat (42). This method was found to be successful in investigating compounds that influence arterial thrombosis including aspirin (43, 44). Although thrombus formation was initiated by electrical stimulation producing arterial injury that is unrelated to a clinical situation, the thrombus morphology suggests that the growth of platelet rich intravascular thrombotic material in response to electrolytic injury is physiologically relevant (45). For the first time in the present study we have provided *in vivo* evidence that Ang-(1-9) is an arterial thrombosis promoter. This effect is similar, although is much weaker, to the prothrombotic action of Ang II, showed previously by us (2, 3). We have also clearly showed the opposite action of Ang-(1-9) to Ang-(1-7), which we found to be antithrombotic agent in the past (1). In the line with our results is the second *in vivo* study with Ang-(1-9) demonstrating it is, like Ang II, a potent dipsogen (22).

In the next step we tried to investigate the mechanisms in which Ang-(1-9) promotes arterial thrombosis. We found that Ang-(1-9) dose dependently increased the plasma concentration of fibrinogen (*Fig. 2*). Since the increase in fibrinogen levels can lead to a cardiovascular event, especially an atherosclerotic event, including infiltration of the vessel wall by fibrinogen, rheological effects due to increase blood viscosity, increased platelet aggregation and thrombus formation, and increased fibrin formation (46), it can be also a reason of enhanced thrombus formation demonstrated in our study. Additionally similarly to our previous study with Ang II, the fibrin generation was also enhanced by Ang-(1-9) confirming these both peptides can be risk factors for coronary heart disease (*Fig. 2* and *Fig. 3*). A number of data supports the concept that Ang II enhances platelet activation induced by various factors, which is expressed as the potentiation of aggregation, releasing process, expression of P-selectin and shape change and causes exaggeration of the cardiovascular responses (5, 7, 47-51), but there is no any data concerning Ang-(1-9) effect on platelets. The studies of Snyder

et al. (27, 28) support the concept, that Ang-(1-9) could be an important regulator of platelet function. They showed that the main product of Ang I conversion by human platelet enzymes is not Ang II or Ang-(1-7) but Ang-(1-9), in contrast to *e.g*. stomach wall in which predominantly Ang-(1-7) is formed (52) and in rat aorta in which Ang I is converted in Ang II by ACE (53). We observed a similar direction in Ang I metabolism in rat platelets homogenates in the same optimal experimental conditions as Snyder group (*Fig. 6A*). It was interesting if Ang-(1-9) may affect platelets, since they play a crucial role in arterial thrombosis development. We measured platelet response in whole blood collected from the animals i.v. infused with the highest dose of Ang-(1-9), and we found a large, 88% increase when compared to saline treated rats in platelet aggregation induced by collagen (*Fig. 4*). Once again our result is the first *in vivo* data demonstrating that Ang-(1-9) may activate platelets. Similarly to prothrombotic effect, when compared with Ang II, the effect of Ang-(1-9) on platelets was much weaker (*Fig. 4*). To check whether Ang-(1-9) itself may affect platelets we incubated Ang-(1-9) with rat whole blood and found a slight (15%) but statistically significant increase in platelet aggregation (*Fig. 5*). The difference (73%) in the strength of the *in vitro* and *ex vivo* effect of Ang-(1-9) on platelets suggests that its action is also mediated by other factor.

The similar properties of Ang-(1-9) and Ang II found in our experiments led us to conclusion that the biological effects of Ang-(1-9) and Ang II are similar or the effect of Ang-(1-9) could be mediated by Ang II. *In vitro* studies demonstrated that Ang-(1-9) may be metabolized by ACE into Ang-(1-7), and further into Ang-(1-5) and Ang-(1-4) (24). Nevertheless, Drummer *et al*. (31) showed that in the kidney, the main product (71%) of Ang-(1-9) conversion is Ang II, due to ACE - independent aminopeptidase and N-like carboxypeptidase, accompanied by small amounts of Ang III and Ang-(2-9). Furthermore, in 2005, Singh *et al*. confirmed that Ang II and Ang-(1-7) may be formed from Ang-(1-9) in glomeruli of streptozotocin-induced diabetes mellitus rats (41). Unfortunately there is no data about the conversion of Ang-(1-9) into Ang II in platelets, since Snyder group did not continued their studies. To check whether Ang-(1-9) may be locally converted by platelet enzymes, we incubated this peptide with platelet homogenates. Indeed, the peptide was further degraded and only Ang II was formed (*Fig. 6B*). The direction of angiotensins conversion depends mostly on the presence and the activity of different RAS enzymes. The possible reason of lack of Ang-(1-7) formation in platelets could be significantly higher activity of monocarboxypeptidases, such as ACE-2 and the lower activity of dicarboxipeptidases such as ACE in platelets. However inhibitor of carboxypeptidases A-D - benzylsuccinate and selective ACE-2 inhibitor - DX 600 did not inhibited the formation of Ang II from Ang-(1-9). Thus the role of these enzymes should be excluded (*Fig. 6D*). The concentration of Ang II was unproportionally low compared with the substrate concentration, however it must be taken into consideration that Ang II was intensively cleaved to shorter peptides throughout the whole experiment, whereas Ang-(1-7) was not (*Fig. 6C*). Since platelets are playing crucial role in the arterial thrombosis development, the experiments with platelets confirmed their involvement in the mechanism of prothrombotic effect of Ang-(1-9) at least in the experimental arterial thrombosis.

It is well established that the AT_1 receptor mediates the vasoconstricting and prothrombotic effects of Ang II and some of its metabolites (2-4, 54), while there is no data concerning angiotensin receptors involved in Ang-(1-9) action. Our finding that losartan abolished the prothrombotic effect of Ang-(1-9), similar to the previous study with Ang II (2), indicates that Ang-(1-9) may possess a high affinity to the AT_1 binding site and exert its effects *via* this receptor. On the other hand, described above *in vitro* data showing that the conversion of Ang-(1-9) to Ang II is possible and the results of our experiments with platelets and platelets homogenates suggest that Ang II could mediate the observed prothrombotic effect of Ang-(1-9) *via* the AT_1 receptor. The authors of the *in vivo* study demonstrating that Ang-(1-9) is a potent dipsogen also suggested that the drinking response probably requires a second hydrolysis to Ang II (22). Indeed, after a single intravenous administration of Ang-(1-9), we found Ang II and even more amount of Ang-(1-7) in rat blood (*Fig. 7*). Nevertheless the enhanced thrombus formation, increased fibrinogen plasma level, fibrin generation and platelet aggregation observed during Ang-(1-9) infusion suggests that this small quantity of generated Ang II, a very potent peptide, overcomes the potential antithrombotic activity of Ang-(1-7). This also proves that there are enzymes able to convert Ang-(1-9) to Ang II in rat blood and that all the effects observed in our study could be mediated by Ang II. Unfortunately, we were not able to completely confirm the involvement of Ang II, because the angiotensin receptor mediating selectively effects of Ang-(1-9) has not been described untill now and it is not known which enzyme is responsible for its conversion to Ang II. Thus, we cannot use *in vivo* a specific inhibitors to block the prothrombotic effect of Ang-(1-9).

Summing up, we have shown for the first time that Ang-(1-9) infused into rats enhances the thrombotic process. The mechanism of the prothrombotic effect of Ang-(1-9) seems to be very complex, and it may depend on the increasing of fibrin formation, activation of platelets and AT_1 receptor or more likely the action of Ang-(1-9) could be mediated by the Ang II formed from Ang-(1-9) by the enzymes present in the blood as well as those contained and released from platelets.

We have previously shown that Ang II accelerates venous (2) and arterial thrombosis (3) whereas Ang-(1-7) inhibits the thrombotic process in hypertensive animals (1). One study indicated the possible role of Ang-(1-9) in MI development. The concentration of this peptide and the activity of ACE-2 increased in the plasma and heart tissue of rats after MI (19). In the present study, we found clear *in vivo* evidence for the prothrombotic effect and the activation of platelets by Ang-(1-9). Therefore Ang-(1-9) may be recognized as a promoter of intravascular arterial thrombosis.

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