



A phospholipase A1 platelet activator from the wasp venom of *Vespa magnifica* (Smith)[☆]

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Abstract

Wasp is an important venomous animal that can induce human fatalities. Aortic thrombosis and cerebral infarction are major clinical symptoms after massive wasp stings but the reason leading to the envenomation manifestation is still not known. In this paper, a toxin protein is purified and characterized by Sephadex G-75 gel filtration, CM-Sephadex C-25 cationic exchange and fast protein liquid chromatography (FPLC) from the venom of the wasp, *Vespa magnifica* (Smith). This protein, named magnifin, contains phospholipase-like activity and induces platelet aggregation. The cDNA encoding magnifin is cloned from the venom sac cDNA library of the wasp. The predicted protein was deduced from the cDNA with a sequence composed of 337 amino acid residues. Magnifin is very similar to other phospholipase A₁ (PLA₁), especially to other wasp allergen PLA₁. Magnifin can activate platelet aggregation and induce thrombosis in vivo. The current results proved that PLA₁ in wasp venom could be contributable to aortic thrombosis after massive wasp stings.

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1. Introduction

The Vespidae family includes hornets (genera *Vespa* and *Dolichovespula*), yellow jackets (genus *Vespula*) and paper wasps (genus *Polistes*). They possess highly toxic venom, which is rich in toxins, enzymes and biologically active peptides (Habermann, 1972; Nakajima, 1984). They are important venomous animals endangering human life, causing fatalities in serious cases. Vespidae venoms have similar venom compositions. The clinical symptoms induced by wasp stings include local reactions (such as pain, wheal,

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edema and swelling), immunological reactions usually leading to anaphylaxis with subsequent anaphylactic shock, and systemic toxic reactions caused by large doses of venom, resulting in hemolysis, coagulopathy, rhabdomyolysis, acute renal failure and hepatotoxicity (Chao and Lee, 1999; Chen et al., 2004; Evans and Summers, 1986; Korman et al., 1990; Sakhuja et al., 1988; Watemberg et al., 1995). Aortic thrombosis and cerebral infarction is also one of the clinical symptoms after massive wasp stings (Chen et al., 2004). Local reactions may result from biologically active peptides such as bradykinin-like peptides, chemotactic peptides and mastoparans. Allergic reactions induced by wasp venoms have been extensively focused on and studied. The major wasp venom allergens are phospholipase A₁ (PLA₁), hyaluronidase, antigen 5 and serine proteases (Habermann, 1972; Nakajima, 1984). Quite a few literatures have been reported on the reasons causing systemic toxic reactions of wasp envenomation. Aortic thrombosis and cerebral infarction are uncommon systemic toxic reactions following wasp stings but they are dangerous complications. The reason causing aortic thrombosis and cerebral infarction is still not known, although several cases have been reported.

PLA₁ enzymes hydrolyze the sn-1 fatty acids to produce 1-acyl-lysophosphatidic acid (LPA) from phospholipids (Kini, 1997). LPA including 1- and 2-LPA is a lipid mediator with multiple biological functions. These include inducing of platelet aggregation, smooth muscle contraction and stimulation of cell proliferation (Aoki, 2004; Baldini et al., 2005; Goetzl and An, 1998). PLA₁ and PLA₂ are involved in LPA production. A large number of documentation on venomous PLA₂ involved in platelet functions has been reported (Fuly et al., 2004; Gao et al., 1999; Takagi et al., 1988), but no record of venomous PLA₁ involved in platelet functions has ever been reported. Several PLA₁s have been identified as major allergens from wasp venoms but no investigation of their effects on platelets and thrombosis has been carried on (Hoffman, 1986; King et al., 1996; Lu et al., 1995; Sanchez et al., 1994; Soldatova et al., 1993).

2. Materials and methods

2.1. Wasp venom

The wasps *V. magnifica* were collected in Yunan, China. The collected wasps were stimulated by

alternative current (6 V) lasting for 6–10 s. The wasp venom was secreted onto a clean glass plate (50 × 50 cm), immediately collected and stored at –20 °C.

2.2. Protein purification

Lyophilized wasp venom samples of *V. magnifica* (0.3 g) were dissolved in 10 ml 0.1 M phosphate buffer (PBS), pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-75 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M PBS, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. PLA activities of fractions were determined as indicated below. The protein peak containing PLA activity was pooled (45 ml), lyophilized and re-suspended in 5 ml 0.05 M PBS, pH 6.5, and further dialyzed against 0.05 M PBS (pH 6.5). The product was consequently subject to the cation-exchange CM-Sephadex C-25 column (Amersham Biosciences, 1.6 × 30 cm). The elution was achieved with a linear NaCl gradient in 0.05 M PBS, pH 6.5, at a flow rate of 1 ml/min. The peak with PLA activity was collected and finally purified on an AKTA Mono S (1 ml volume, Amersham Biosciences) cationic exchange column equilibrated with 20 mM phosphate buffer solution, pH 6.5, using fast protein liquid chromatography (FPLC, Amersham Biosciences), equilibrated with 0.02 M PBS, pH 6.5. Elution was performed with a linear NaCl gradient in 0.02 M PBS, pH 6.5, at a flow rate of 1 ml/min as illustrated in Fig. 1C.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

About 5 µg of purified protein from FPLC was mixed with 10 µl of SDS sample buffer and boiled for 5 min before being loaded onto a 15% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue and destained to allow visualization of the protein (Laemmli, 1970).

2.4. Protein quantification

The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA) with BSA as standard.

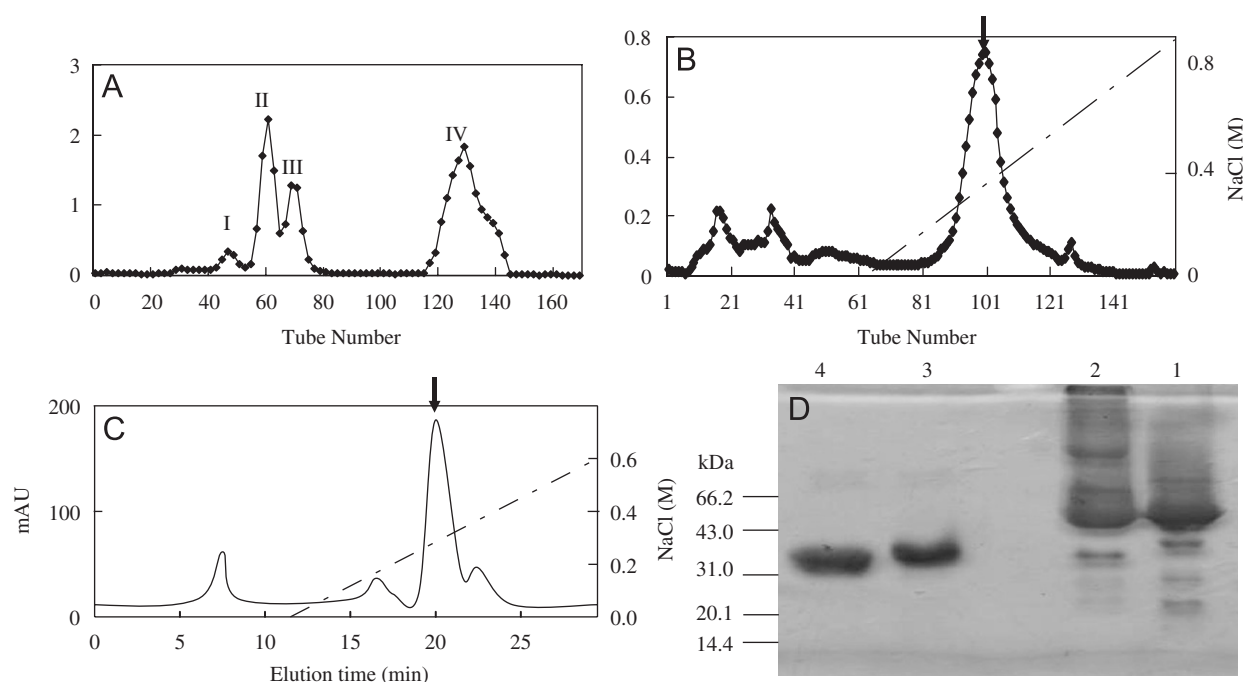


Fig. 1. Purification of magnifin from *V. magnifica* venoms: (A) shows fractionation of *V. magnifica* venoms applied to a Sephadex G-75 (Superfine, Amersham Biosciences, 2.6×100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml (A). Peak II with PLA activity from Sephadex G-75 was further purified, subject to the cation-exchange CM-Sephadex C-25 column (Amersham Biosciences, 1.6×30 cm) equilibrated with 0.05 M PBS, pH 6.5. The elution was performed at a flow rate of 1 ml/min with the indicated NaCl gradient (B). The peak with PLA activity (indicated by an arrow) from CM-Sephadex C-25 was applied to Mono S (1 ml volume, Amersham Biosciences) cationic exchange column equilibrated with 20 mM phosphate buffer solution, pH 6.5, using fast protein liquid chromatography (FPLC, Amersham Biosciences), equilibrated with 0.02 M PBS, pH 6.5. The elution was performed with a liner NaCl gradient in 0.02 M PBS, pH 6.5, at a flow rate of 1 ml/min as illustrated in (C). (D) SDS-PAGE analysis of magnifin. Lane 1, crude venom (non-reduction); lane 2, crude venom (reduction); lane 3, purified magnifin (non-reduction); lane 4, purified magnifin (reduction).

2.5. Protein sequencing

The amino acid sequences of the N-terminus and partial interior amino acid fragments recovered from the trypsin hydrolysis were determined by the automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491.

2.6. Phospholipase activity assay

The phospholipase activity assay upon egg yolk emulsion was performed using the potentiometric titration method as described by De Haas et al. (1968).

2.7. cDNA library construction and clone screening

The total RNA of fresh *V. magnifica* venomous glands was extracted using the TRIzol method (Life Technologies). A SMARTTM PCR cDNA

synthesis kit (Clontech) was used to synthesize the cDNA. The first strand was synthesized with the SMARTTM IV Oligonucleotide, 5'-AAGCAG TGGTATCAACGCAGAGTGGCCATTACGGC CGGG-3', and the CDS III/3' PCR primer 5'-AT TCTAGAGGCCGAGGCGGCCGACATG- d(T) 30N₋₁N-3' (N = A, G, C or T; N₋₁ = A, G or C). The second strand was amplified by using Advantage polymerase and 5' PCR primer IIA, 5'-AAGCAGTGGTATCAACGCAGAGT-3'.

Two primers (a degenerate primer, 5'-GGIYT-NATHCCIAARTGYAARYT-3' (N = A, G, C or T; Y = C or T; H = A, C or T; R = A or G; I = hypoxanthine) designed according to the sequence determined by Edman degradation and a reverse primer 5'-ATTCTAGAGG CCGAGGCG GCCGACATG-3' based on the adopter sequence in the cDNA library) were used to screen the magnifin-encoding sequence. The PCR conditions were 2 min at 95 °C, followed by 35 cycles of 10 s at 94 °C, 30 s

at 59 °C and 40 s at 72 °C. The cDNA products with about 700 bp in length were reclaimed from 1% agarose gel and purified before being ligated into the pMD18-T vector.

2.8. Preparation of washed platelets and platelet aggregation study

ADP was from Trinity Biotech (Wicklow, Ireland). Human platelet-rich plasma, from the Kunming Blood Center, was centrifuged at 500g, for 10 min, at room temperature. The pellets were suspended in Tyrode's buffer A (137 mM NaCl, 2 mM KCl, 0.3 mM NaH₂PO₄, 12 mM NaHCO₃, 5.5 mM glucose, 0.35% BSA, 1 mM MgCl₂ and 0.2 mM EGTA, pH 6.5). After successive washes and centrifugation, the pelleted platelets were resuspended in Tyrode's buffer B (137 mM NaCl, 2 mM KCl, 0.3 mM NaH₂PO₄, 12 mM NaHCO₃, 5.5 mM glucose, 0.35% BSA and 2 mM CaCl₂, pH 7.4) at the concentration of 5×10^8 platelets/ml counted with a thrombocounter. Platelet aggregation was monitored by light transmission in an aggregometer (Plisen, Beijing) with continuous stirring at 37 °C. For aggregation study, the tested sample was added and the aggregation rate was recorded.

2.9. In vivo magnetic resonance imaging study protocol

Rabbits were obtained from the experimental animal center of Kunming Medical College. Rabbits were anesthetized with intramuscular ketamine (30 mg/kg) and xylazine (5 mg/kg). After anesthesia, they were injected with the tested samples resolved in 0.5 ml 0.9% salt water (0.5 mg/kg body weight) through auricular veins. The same volume of 0.9% salt water was used as control. The animals were placed prone in the magnetic resonance imaging (MRI) scanner table under respiratory gating. All the experiments were approved by Kunming Institute of Zoology, Chinese Academy of Sciences. MRI was performed on a 1.5 T superconductive MR scanner equipped with a body coil (Siemens Medical System, Magnetom Sonata Maestro Class, Germany). MR images of cut sections were obtained using a T1-weighted spin-echo (SE) MRI sequence (time of repeat, TR/time of echo, TE = 520 ms/14 ms) and a T2-weighted turbo SE MRI sequence (TR/TE = 3710 ms/94 ms). Time of flight magnetic resonance angiography was used

clinically to diagnose the arteries. The sequence was acquired in a 0.8 mm slice thickness, with TR of 34 ms, TE of 4.45 ms, average of one, flip angle of 25°, field of view (FOV) of 200 mm, FOV of phase of 75% and base resolution of 384.

3. Results

3.1. Purification of PLA₁

The wasp venom was divided into four fractions by Sephadex G-75 as illustrated in Fig. 1A. The peak II (tubes 54–64) was found to exert PLA and platelet aggregation activities, and was applied to the CM-Sephadex C-25, eluting with 0.3 M NaCl as indicated in Fig. 1B. The fraction from CM-Sephadex C-25 cationic exchange chromatography containing both PLA and platelet aggregation activities (marked by an arrow in Fig. 1B) was collected and purified by AKTA Mono S cationic exchange column under the conditions indicated in Fig. 1C, and the purified PLA was indicated by an arrow.

3.2. SDS-PAGE analysis and partial amino acid sequencing

Purified PLA was named 'magnifin'. Fig. 1D shows the SDS-PAGE analysis of the purified native magnifin. SDS-PAGE was performed under non-reducing conditions and reducing conditions (5% β-mercaptoethanol). It appeared as a homogeneous protein in both conditions. The purified magnifin appeared as a single band in both reduced and unreduced conditions and, therefore, consisted of a single polypeptide chain of approx. 34 kDa. The N-terminal sequence and several interior sequences were analyzed by automated Edman degradation. Their amino sequences are illustrated in Fig. 2.

3.3. cDNA cloning

A positive clone, which contained an insert of around 1000 bp, was identified and isolated from the venom gland cDNA library of *V. magnifica*. The complete nucleotide sequence of cDNA (GenBank accession DQ868667) and deduced amino acid sequence are shown in Fig. 2. The cDNA sequence displayed the maximal identity (78%) with the PLA₁ from the bald-faced hornet, *Dolichovespula maculata* (GenBank accession P53357) by BLAST search. The cDNA-encoding protein precursor was

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atgaatttaaatacctattattattttttgttttagtgcaagtgtacattattgctat 60
M N L K Y L L L F F C L V Q V L H Y C Y 20
tcacatgggtgatccatcggttgtaaacgaattagatagaggcctgattccaaaatgtaag 120
S H G D P S L S N E L D R G L I P K C K 40
ctgggtgccggagcaaatctcatttgttctctctactagggaaaaccagaatgggtgttttt 180
L V P E Q I S F V L S T R E N Q N G V F 60
cttacgctagataatttaagcaaaggcggaatattaccaaaatctgatctatcatcgatt 240
L T L D N L S K G G I L P K S D L S S I 80
ccagttatatttccttatacatgggttcatttcattctgcaaataattcaaattacgtggat 300
P V I F L I H G F I S S A N N S N Y V D 100
atgacaaaagctttgttagaaaaaaatgattgtatggtaatctcgatcgattggaggagac 360
M T K A L L E K N D C M V I S I D W R D 120
ggcgcttgactcatgaatttaaattcttaagtttattggttatcctaattgccgtaaaa 420
G A C T H E F K I L K F I G Y P N A V K 140
aatacacgtgcagttggaaaatatattgctgattttaccaagttactcatgcaaaagtat 480
N T R A V G K Y I A D F T K L L M Q K Y 160
aaagtgtcactggcaaataatacgactgatcgggcatagttttaggggcacaaatagcaggt 540
K V S L A N I R L I G H S L G A Q I A G 180
tttgcagggaaagaatatcaaaagtttaagttaggaaaatatcctgaaattattgggctt 600
F A G K E Y Q K F K L G K Y P E I I G L 200
gatcctgctggggcctttgttcaagtcaaattgattgttccgagagaatctgtgagacagac 660
D P A G P L F K S N D C S E R I C E T D 220
gcacattatgttcaaattatacatatacaaaacatttaggaacagagagaactcttgggt 720
A H Y V Q I I H T S N N L G T E R T L G 240
accgtcgatttctacgtgaataacggatataatcaacctgggttgctatctttcatttctt 780
T V D F Y V N N G Y N Q P G C Y L S F L 260
ggagaagcttgcctctcatcacgagagccgtgaaatactttaccgagtggtataagacatgaa 840
G E A C S H T R A V K Y F T E C I R H E 280
tgttgttttaattgggggtccacagtcaaaaaatccgcagccagtttcgaagtgcacaaga 900
C C L I G V P Q S K N P Q P V S K C T R 300
aaggagtgcgtttgcattggattaaacgcaaaaacataatcctaaaactggctcattttat 960
K E C V C I G L N A K T Y P K T G S F Y 320
gtaccggttgaaagtaaagctccctatttgcaataacaaaggaaagaaaatttaattatat 1020
V P V E S K A P Y C N N K G K K I * 337

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Fig. 2. cDNA and deduced amino acid sequence of magnifin from *V. magnifica* venoms. The signal peptide consisting of 33 amino acid residues is shown in italics. The residues boxed were determined by automated Edman degradation. *: stop codon.

composed of 337 amino acid (aa) residues, including a predicted signal peptide of 33 aa and a mature PLA₁ of 304 aa with a predicted isoelectric point (pI) of 9.01 and a molecular weight of 33.6 kDa that matched well with the observed molecular weight from SDS-PAGE. The mature PLA₁ has 14 cysteines. The amino acid sequence of mature PLA₁ deduced from the cDNA sequence was found to

match well with the amino sequence of magnifin determined by Edman degradation.

3.4. Magnifin-induced platelet aggregation

As illustrated in Fig. 3, native purified magnifin from the wasp venom induced human platelet aggregation in a dose-dependent manner. A total

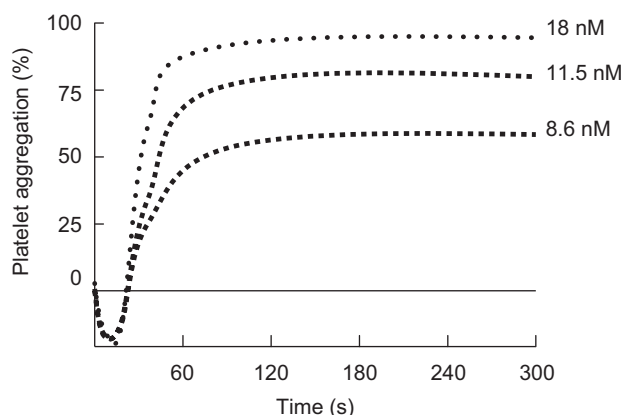


Fig. 3. Dose-dependent platelet aggregation activity of magnifin.

of 18 nM magnifin caused 85% washed platelets aggregation in 60 s at 37 °C, with 50% aggregation attained at 8.6 nM magnifin. It suggested that magnifin could activate washed platelets and act as an agonist of platelets. It has been reported that a family of bioactive peptides, mastoparans, identified from wasp venoms can activate platelets by interacting with GTP-binding proteins (Ozaki et al., 1990; Wheeler-Jones et al., 1991). No protein from wasp venoms has been reported to activate platelet aggregation.

3.5. Magnifin-induced thrombosis, abscess and narrowing of artery

By MRI, effects of magnifin on rabbit arteries were diagnosed. As illustrated in Fig. 4A and C, internal carotid arteries, common carotid arteries, subclavain arteries and vertebral arteries were clear at the beginning of magnifin administration, while all of these arteries turned vague after 1 h of treatment by magnifin (Fig. 4B and D). Magnetic resonance angiography (Fig. 4B and D) revealed the low signal intensity area suggestive of a thrombus and demonstrated artery narrowing. Furthermore, the MRIs of the cut sections as illustrated in Fig. 4G and H also demonstrated narrowing of artery induced by magnifin. Right common carotid artery and left common carotid artery were narrowed by magnifin administering in a time-dependent manner. At the beginning of magnifin treatment, right common carotid artery and left common carotid artery around the trachea were very clear (Fig. 4F). After 1 h of magnifin treatment, the radius of the common carotid arteries (Fig. 4G) became much smaller than the first one illustrated in Fig. 4F and

jugular veins were dilated (Fig. 4G). The signal that represented common carotid arteries had almost disappeared in the MRI, and jugular veins were markedly dilated (Fig. 4H) after 2 h of magnifin treatment. Artery narrowing likely resulted from thrombosis and resulted in decreased blood flow. Jugular vein dilation was suggestive of venous congestion. It was also observed that the abdominal abscess was induced by magnifin treatment as illustrated in Fig. 4E. Lastly, magnifin could induce trachea contraction and narrow the trachea as illustrated in Fig. 4F–H.

4. Discussion

A large amount of bioactive substances including amines, toxic peptides and proteins have been identified from wasp venoms. These venom components can act on the cardiovascular system, nervous system and immunological system of mammals. Some vasoactive, inflammatory and thrombogenic substances such as histamine, serotonin, dopamine, leukotrienes, thromboxanes and bradykinin have been found in wasp venoms (Levine, 1976). Serotonin and thromboxanes can induce platelet aggregation and direct vasoconstriction (McMillan et al., 1979). Effects on platelet aggregation, thrombosis and vasoconstriction of wasp PLA₁s have not been reported although several PLA₁s have been found in wasp venoms.

In the current study, a PLA₁ named magnifin was purified from wasp venoms of *V. magnifica* (Smith), a kind of big wasp with a body length up to 5 cm. Magnifin strongly activates platelet aggregation at nanomolar concentration. For example, 18 nM magnifin caused 85% washed platelets aggregation

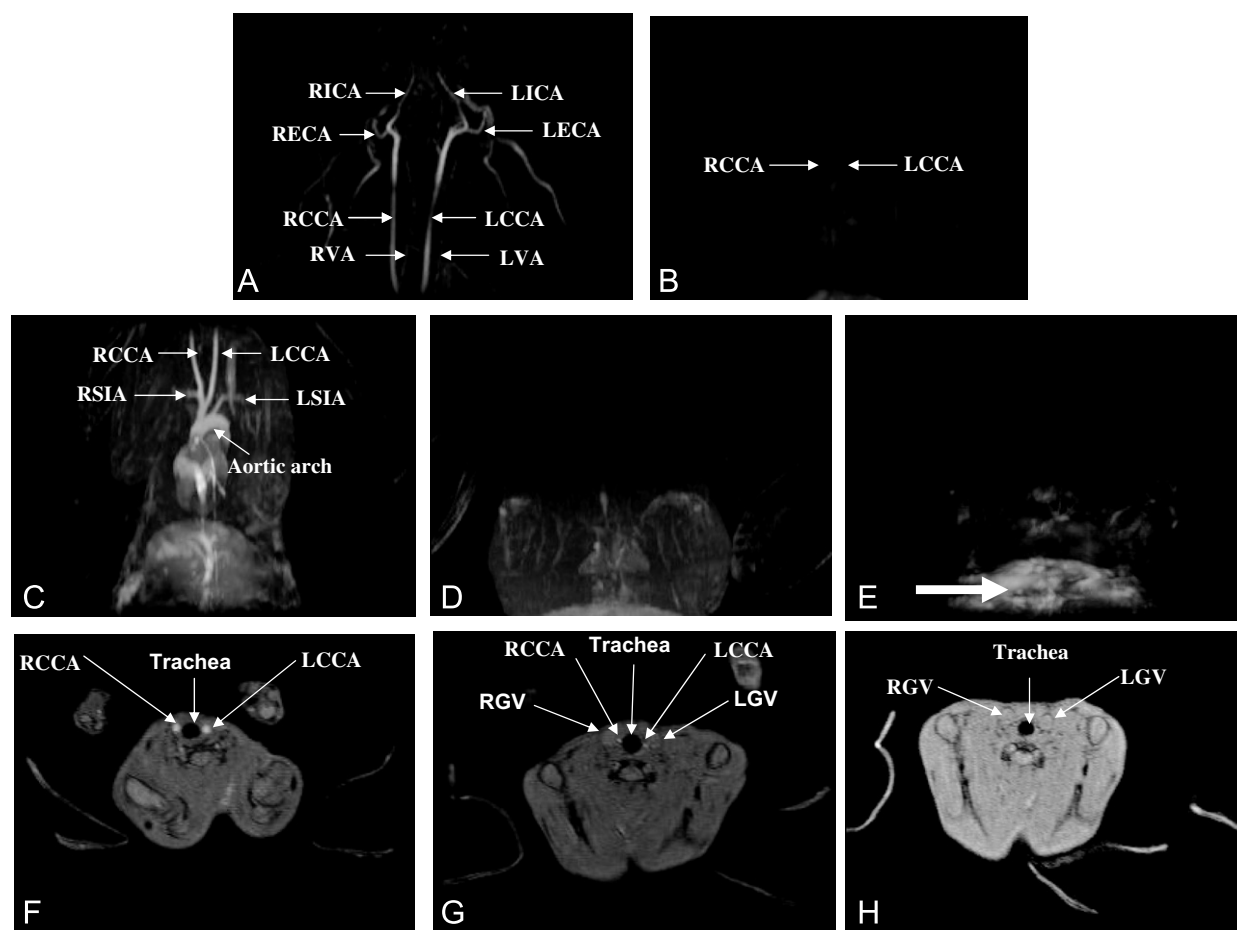


Fig. 4. The magnetic resonance angiography (MRA) and the magnetic resonance imaging of rabbit after being treated by magnifin. (A) MRA of arteries after 0 h of magnifin treatment; (B) MRA of arteries after 1 h of magnifin treatment; (C) MRA of the aortic arch after 0 h of magnifin treatment; (D) MRA of the aortic arch after 1 h of magnifin treatment; (E) abdominal abscess induced by 1 h of magnifin treatment (indicated as an arrow); (F) MRI of the cut sections after 0 h of magnifin treatment; (G) MRI of the cut sections after 1 h of magnifin treatment; (H) MRI of the cut sections after 2 h magnifin treatment. RICA: right internal carotid artery; LICA: left internal carotid artery; RECA: right external carotid artery; LECA: left external carotid artery; RCCA: right common carotid artery; LCCA: left common carotid artery; RVA: right vertebral artery; LVA: left vertebral artery; RSIA: right subclavain l artery; LSIA: left subclavain l artery; RGV: right jugular vein; LGV: left jugular vein.

in 60 s. It is the first report of PLA_1 from wasp venoms that can induce platelet aggregation. Most studies of PLA_1 s that had been reported were focused on their allergenic activities (Hoffman, 1986; King et al., 1996; Lu et al., 1995; Sanchez et al., 1994; Soldatova et al., 1993). The platelet aggregation activity may result from its enzymatic activity in hydrolyzing phospholipids to produce 1-acyl-lysophosphatidic acids that can mediate platelet aggregation. Platelet aggregation can accelerate thrombus formation. Magnifin severely induced thrombosis, narrowing of artery and jugular vein dilation as illustrated in Fig. 4. Further work is needed to prove that all these reactions induced by

magnifin are likely related with its PLA enzyme activity. Interestingly, magnifin could induce contraction of the trachea that may result from the production of some vasoactive mediators such as adrenaline induced by magnifin.

Acknowledgments

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