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Potent cough suppression by physiologically active substance in human plasma

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Abstract. Human plasma contains wide variety of bioactive proteins that have proved essential in therapeutic discovery. However, many human plasma proteins remain orphans with unknown biological functions. Evidences suggest that some plasma components target the respiratory system. In the present study we adapted heparin affinity chromatography to fractionate human plasma for functional bioassay. Fractions from pooled human plasma yielded particular plasma fractions with strong cough suppressing effects. Purification yielded a fraction that was finally identified as an activated blood coagulation factor XIa (fXIa) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF-MS). The fraction almost completely suppressed coughs induced by either chemical or mechanical stimulation applied to larynx or bifurcation of guinea-pig trachea. Cough suppressing effect of the fraction and commercially available fXIa were one million times stronger than codeine and codeine only partially suppressed the mechanically triggered coughing in animal model. Recent reviews highlighted prominent shortcomings of current available antitussives, including narcotic opioids such as codeine and their unpleasant or intolerable side effects. Therefore, safer and more effective cough suppressants would be welcome, and present findings indicate that fXIa in human plasma as a very promising, new therapeutic candidate for effective antitussive action.

Key words: Human plasma — Cough suppressing factor — Factor XIa

Abbreviations: 2-DE, two-dimensional electrophoresis; D-PBS, Dulbecco's-PBS; DTT, dithiothreitol; fXI(a), factor XI(a); IPG, immobilized pH gradient; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI/TOF-MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; OD, optical density; PB, phosphate buffer; RARs, rapidly adapting stretch receptors; SARs, slowly adapting stretch receptors; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis**;** SS, serum selenium; SP, substance P.

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Introduction

It is well documented that human plasma contains wide variety of bioactive proteins. Protein products fractionated from the human plasma have been in essential class of therapeutic use. The leading plasma product is now the intravenous immunoglobulin G, which has replaced serum albumin in this role (Burnouf 2007). However, many human plasma proteins remain orphans with unknown biological functions. Evidence suggests that some plasma components target the respiratory system. For example, descriptive studies suggested that low levels of serum selenium (SS) are associated with an increased prevalence and severity of asthma (Qujeq et al. 2003; Kocyigit et al. 2004; Rubin et al. 2004). SS concentrates predominantly in the plasma as selenoprotein P. Selenoprotein P accounts for more than 50% of the total plasma SS (Mostert et al. 1998). To date, however, the 25 identified human selenoproteins have not been associated with a function (Kryukov et al. 2003). We recently discovered that selenoprotein P directly inhibits the twitch-like contraction of bronchial smooth muscle evoked by indirect muscle stimulation (Ogawa-Kitamura et al. 2013).

In the present study we adapted a heparin affinity chromatography to fractionate human plasma for bioassay of function. Many physiologically active substances in human plasma bind to heparin (Burnouf and Radosevich 2001). Fractions from pooled human plasma yielded particular plasma fractions with strong cough suppressing effects. To segregate the responsible protein(s), we used LC-MS/MS and MALDI/TOF-MS. Here we report that activated human coagulation factor XIa (fXIa) strongly suppresses cough and may point to a novel candidate and promising avenue for therapeutic drug development.

Materials and Methods

Preparation of the heparin-absorbed fraction from normal human plasma

The entire purification process was performed under aseptic conditions in a cold room. We used ammonium sulfate to precipitate blood plasma samples pooled from more than 10,000 Japanese donors to a final concentration up to 31% by weight. About 25 g precipitate, which did not essentially contain antithrombin, was dissolved in 50 ml of 20 mM phosphate buffer (PB; pH 7.0), centrifuged (3000 rpm, 15 min) and filtered with a porous filter (0.2 μm) to remove undissolved materials. The filtered result was diluted with PB until reaching optical density $(OD)_{280} = 20$. About a 5000 OD (250 ml) was applied to the heparin column (100 ml of Heparin Superflow™ Plus; Sterogen, Carlsbad, CA) and eluted by 10 column volumes of the salt gradient from PB to PB+2 M NaCl after washing with 3 column volumes of PB. All experimental protocols or use of human blood was approved by the institutional review and conformed to the guiding principles for the care and use of animals approved by the Council of The Physiological Society of Japan. Blood donation is accepted by Japanese Red Cross Society (JRCS), which is the sole licensed blood establishment in Japan, and basic principles of the law on securing a stable supply of safe blood products was established in 2003. According to the law, JRCS seeks donor's approval of the memorandum of understanding, concerning the adverse action of blood donation, handling of personal information, examinations of the donated blood, and effective utilization of the blood for the research and development for diagnosis, medical treatments and promotion of the health of citizens. JRCS is securing required quantities of blood products and delivering them to medical institutions across the country. We used the donated blood supplied by JRCS, so that the donors could understand about the effective use of the blood in the laboratory.

The inclusion criteria for the donors were healthy Japanese of both sexes (18–69 years old, and body weight of more than 40 or 45 kg for female or male, respectively), free from diseases specified by the Japanese government as being worrisome, having no known treatment and unknown causes, drug application and immunization for the specific period of the donation.

To evaluate potential effects of heparin absorbed fractions on the cough reflex, we prepared 8 assay pools by use of heparin column chromatography. The eluted fractions were separated and pooled according to conductivities into 8 assay pools (pool-1 < 16.0, pool-2 < 26.0, pool-3 < 34.0, pool-4 < 45.0, pool-5 < 52.0, pool-6 < 58.0, pool-7 < 68.0, pool-8 < 83.0 mS/cm, respectively). We collected sub-fractions numbered 7–17, 18–23, 24–28, 29–35, 36–40, 41–45, 46–51, 52–61 from pools-1 to 8, respectively (Fig. 1). Each pool was concentrated to 1.5 OD using CentriPrep™ (YM-3; Millipore), then dialyzed against Dulbecco's-PBS (D-PBS). We confirmed that endotoxins were not present in any pools (< 0.05 endotoxin units/ml).

Size exclusion chromatography

Size exclusion chromatography was performed using a Superdex Hiload 200 pg column (GE Healthcare Science, Piscataway, NJ), equilibrated with D-PBS and monitored at OD_{280}.

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was carried out in an electrophoresis system for the first-dimensional isoelectric focusing using immobilized pH gradient (IPG) Ready Strip gel (17 cm, linear gradient between pH 3–10; Bio-Rad

Laboratories, Hercules, CA) and for the second-dimension in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a gradient gel (19 × 17 cm, 10–16%). Each sample was precipitated using Ready Prep 2-D Cleanup Kit (Bio-Rad Laboratories) according to the manufacturer's instruction. Equivalent samples (120 μg) were resuspended in 7 mol/l urea, 2 mol/l thiourea, 20 mmol/l dithiothreitol (DTT), 2 mmol/l Tris-(2-cyanoethyl) phosphate, 2% CHAPS, 0.2% (v/v) Bio Lyte 3–10 and applied to each gel. The first dimensional immobilized pH-gradient formation on the IPG gel was carried out according to the manufacturer's instruction. After the first dimension, the IPG gel was equilibrated with equilibrium buffer A consisting of 50 mmol/l Tris-HCl (pH 8.5), 6 mol/l urea, 30% glycerol, 2% SDS and 1% DTT and 0.005% bromophenol blue for 15 min and further equilibrated with equilibrium buffer B consisting of 50 mmol/l Tris-HCl (pH 8.5), 6 M urea, 30% glycerol, 1% SDS, 4.5% iodoacetamide and 0.005% bromophenol blue. The IPG gel was subjected to second-dimensional SDS-PAGE. The gels were stained by SYPRO Ruby (Invitrogen, Carlsbad, CA), and images were captured by scanning the SYPRO Ruby stained gels using Molecular Imager FX (Bio-Rad, Hercules, CA).

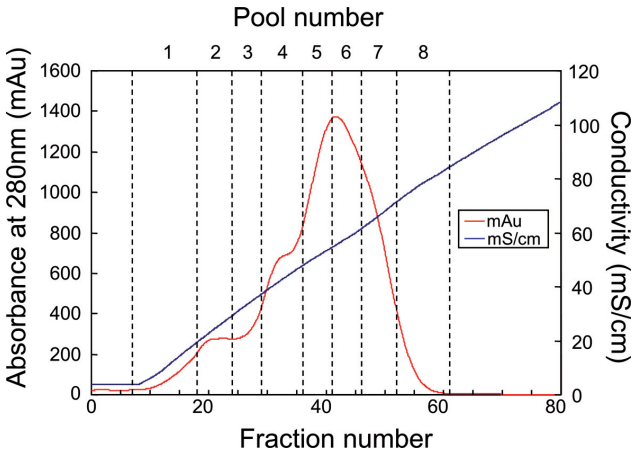

Figure 1. Elution pattern of heparin-absorbed fraction on the heparin-Super flow column. To evaluate potential effects of heparin absorbed fractions on the cough reflex, we prepared 8 assay pools by use of heparin column chromatography. The eluted fractions were separated and pooled according to conductivities (pool-1 < 16.0, pool-2 < 26.0, pool-3 < 34.0, pool-4 < 45.0, pool-5 < 52.0, pool-6 < 58.0, pool-7 < 68.0, pool-8 < 83.0 mS/cm, respectively). Pools (pools-1 to 8) collected sub-fractions numbered 7–17, 18–23, 24–28, 29–35, 36–40, 41–45, 46–51, 52–61, respectively.

Protein identification by mass spectrometry

After 2-DE and staining the gel with SYPRO Ruby, the bands of proteins were excised and treated with trypsin. The tryptic digest was concentrated and desalted using C18 Zip Tips (Millipore, Billerica, MA) and analyzed by MALDI/TOF-MS using Voyger-DE STR (Applied Biosystems, Foster City, CA). The tryptic digest of a smear band was directly analyzed by Paradigm MS2 (Michrom BioResources Inc.

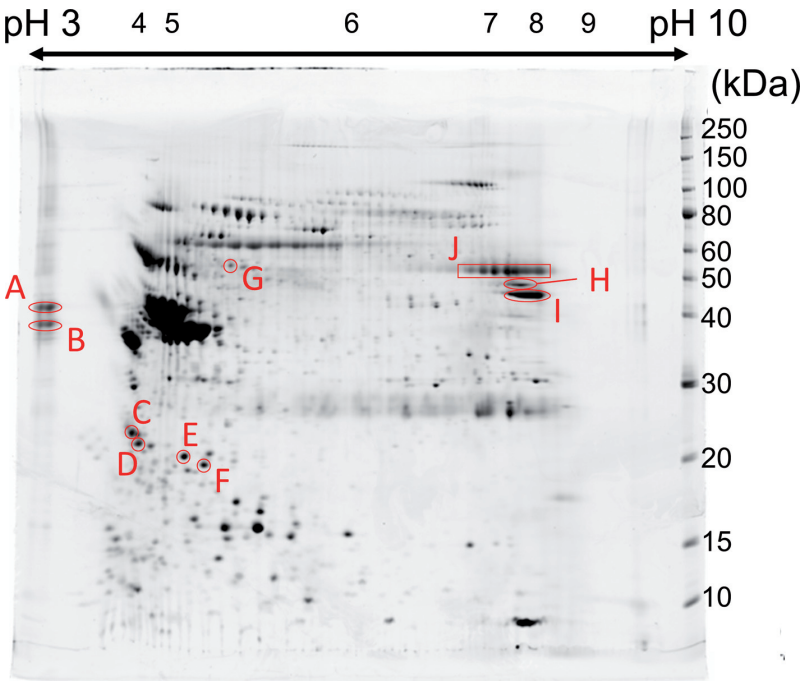

Figure 2. Separation of the heparin-absorbed fraction G1 by 2-dimensional gels. Each equivalent amount (120 μg) of the samples G1, pool-6 and pool-5 was separated on two dimensional electrophoresis. Ten spots (A–H), which represented higher intensity on pool-6 than that of pool-5 and G1, were identified by MALDI/TOF-MS (except J) or LC-MS/MS (J). In addition, tandem mass spectra were analyzed using Mascot, which allows for the correlation of experimental data with theoretical spectra generated from known protein sequences. The analysis of the target protein bands by MALDI/TOF-MS or LC-MS/MS indicated that the bands with strong antitussive effects corresponds to blood coagulation factors, factor XI (fXI), factor XIa (fXIa) and kallikrein (Kal) by use of a compiled protein data base that was composed of a public protein database.

Auburn, CA) on a L-column ODS (0.1 × 50 mm; Chemicals Evaluation Research Institute, Tokyo, JP) coupled to a tandem mass spectrometer (Q-Tof2; Waters Micromass, London, UK) equipped with a nanoelectrospray ionization source. Tandem mass spectra were analyzed using Mascot, which allows for the correlation of experimental data with theoretical spectra generated from known protein sequences. The data were used to search a compiled protein database that was composed of a public protein database (http://www.ncbi.nlm.nih.gov/). 2-DE electrophoresis and analyses of the target protein bands by MALDI/TOF-MS or LC-MS/MS were done at APRO life Science Institute (Tokushima, JP) (Ishida et al. 2006; Alonso-Faustea et al. 2012). Each equivalent amount (120 μg) of the samples G1, pool-6 and pool-5 was separated on 2-DE. We targeted ten spots in G1 (high molecular weight, A to I in Fig. 2), which represented stronger intensities in the order of G1 > pool-6 > pool-5, were identified by MALDI/TOF-MS(except J) or LC-MS/MS(J).

Animals

We used 120 guinea pigs (Male Hartley, 5–7 weeks old). The animals were purchased from Kyudo Pharm (Kumamoto, Japan) and housed in the animal house of the Life Science Division, Kumamoto Health Science University, at a room temperature of 25 ± 2°C. All experimental protocols were approved institutional review and conformed to the guiding principles for the care and use of animals approved by the ethics committee of Kumamoto Health Science University and the Council of The Physiological Society of Japan.

Cough experiments

Guinea pigs were anaesthetized with pentobarbital-Na (30 mg/kg, i.p.) to a level sufficient to prevent nociceptive reflexes but which preserved cough reflexes. The animals were placed on their backs, and the limbs were fixed to a restraint board. The anterior skin of the neck was shaved and then cut open in a circle (diameter about 8 mm), and the musculature under the skin deflected to expose the trachea along with the midline. A portion of the anterior wall of the trachea about 3.5 cm caudal from the cricoid cartilage was removed in a rectangle (1 × 1 mm) (Takahama et al. 1997). The procedure minimized blood loss from both musculature and tracheal wall. The small opening in the trachea was tightly closed with a small piece of saline saturated cotton to maintain airway conditions and exposed only during mechanical stimulation of the trachea. Coughing was monitored and recorded on a electronic recorder (R-02e, Rikadenki, Tokyo, Japan) through a flowmeter (TV-241T, Nihon Kohden, Tokyo, Japan) *via* a differential pressure transducer (TP-602T, Nihon Kohden, Tokyo, Japan) as changes in air flow within a tube connected to a pneumograph placed on to the caudal end of the chest. A ventilation volume amplifier (AR-601G, Nihon Kohden, Tokyo, Japan), connected to the flow meter measured the amplitude of the cough response.

A rabbit whisker with the tip 70 μm in diameter was used to mechanically stimulate both sites of the larynx and the bifurcation of the trachea. The whisker stimulated both sites by inserting it through a small hole to stimulate the both sites. After the whisker tip reached the site for stimulation, the stimulator was left for 1 s and carefully withdrawn. Paired stimuli at 5 min intervals were applied to the larynx and bifurcation. Five min after the administration of the fractions or codeine, paired stimuli were applied at 10 min intervals. The mean amplitude of cough response was taken as a pre-administration control value (control). The value at the most effective time was considered as the post-administration value and normalized within animals as% of the pre-administration value. To evaluate cough suppressing activity, the mean% of the amplitude was compared for the fractions and vehicle-treated groups by means of an unpaired Student's *t*-test. The difference was considered statistically significant, when $p < 0.05$. Data were expressed as means ± SEM.

For comparison, we also induced cough responses by chemical stimulation. Namely, cough responses to aerosolized capsaicin were also recorded in conscious and unstrained guinea-pigs by use of whole body plethysmography (WBP) (PUL 10M M.I.P.S Co.). Before taking readings, the box was calibrated with a rapid injection of 150 μl air into the main chamber. We measured the pressure difference between the main chamber of the WBP containing animal, and a reference chamber (box pressure signal). This box pressure signal is caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. A pneumotachograph (RECTI-HORIZ-8K, NEC) with defined resistance in the wall of main chamber acts as a low-pass filter and allows thermal compensation. Aerosolized capsaicin (10^{-7} or 10^{-6} mol/l) were prepared by nebulizer (NE-U12, Omron Co.), and applied through an inlet of the main chamber for 5 min. Cough was identified by the sounds and movement of the animals during application of capsaicin, together with the pneumotachographic recordings.

To evaluate the cough suppressing effects of fXIa or codeine treatment, we used pre-and post applications of aerosolized capsaicin (10^{-7} and 10^{-6} mol/l, respectively, to avoid desensitization, if any) before and after the application of fXIa (4–400 ng/kg) or codeine (10 mg/kg). First, we applied 10^{-7} mol/l aerosolized capsaicin and counted the number of the coughs during and after the treatment (5 min each). To obtain full recovery, the animals were exposed to fresh air

for 1 hour. Then, 20 min before capsaicin tests (10^{-6} mol/l), fXIa (4–400 ng/kg) or codeine were administered into the hind limb peripheral vein, and the number of the coughs measured during and after the application of capsaicin (5 min each). The ratio of the number of coughs induced by pre- and post application of capsaicin (%, post/pre × 100) or the inhibitory ratio (%, (the ratio of coughs in control) – (the ratio of coughs after fXIa or codeine injection)/(the ratio of coughs in control) × 100) were obtained.

Drugs

Human coagulation factor XI, activated factor XI and anti factor XI mono- and poly-clonal antibodies were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Human plasma kallikrein was purchased from GenWay Biotech, Inc. (San Diego, CA). All the plasma proteins were diluted using D-PBS with 0.1% human serum albumin for the animal experiments. Codeine was from Sankyo (Tokyo, Japan).

Results

Cough triggered by mechanical stimulation – effects of codeine

Mechanical stimuli applied to the bifurcation of the trachea or to the larynx evoked cough responses consisting of a large inspiration followed by expiration (Fig. 3A). Response amplitudes from either region were similar and quite stable over 60 min after saline injection (Fig. 3B) (Takahama et al. 1997).

Intravenous codeine injection (3 mg/kg) completely abolished the cough responses to the stimulation of larynx, while the response to the stimulation of the bifurcation of the trachea was reduced but persisted (55. 9 ± 10.8% of control, $n = 9$) (Fig. 3C and D).

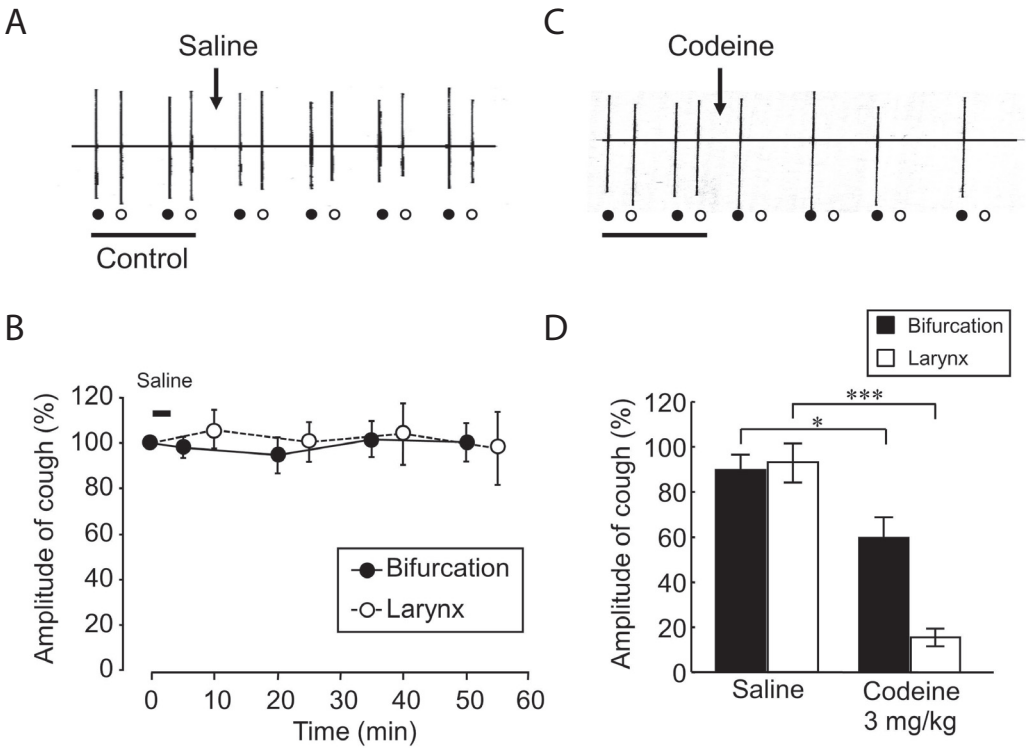

Figure 3. Typical recordings of the cough responses induced by mechanical stimulation applied to the bifurcation and larynx regions of the trachea in guinea-pigs, and effect of codeine on the cough responses. **A.** The typical recording of the cough responses induced by mechanical stimulation applied to the bifurcation of trachea (●) or larynx (○) regions of the guinea pigs airway. **B.** The time course of the effects of intravenous injection of saline on the amplitude of the cough responses. The cough responses evoked by mechanical stimulations were stable after saline injection with consistent amplitudes of the cough responses. Saline was injected after 2 mechanical stimulations as control. Upward and downward deflections in each recording represent expiration and inspiration. **C.** Effect of codeine on the amplitude of the coughs induced by mechanical stimulation applied to the bifurcation (●) and larynx (○) of trachea. **D.** Codeine suppressed much selectively the cough responses evoked in larynx region. Each bars is the average ± SEM ($n = 9$). * $p < 0.05$, *** $p < 0.001$.

Selected heparin-absorbed fractions of human plasma diminish cough responses

Ammonium sulfate precipitated human blood plasma followed by fractionation by heparin column chromatography. The eluted fractions were separated and pooled according to conductivities into eight assay pools (see Materials and Methods) (Fig. 1). These fractionated pools 1–8 were tested on laryngeal and tracheal stimulated cough responses. pools-2 and pool-6 suppressed most effectively the cough responses of both the trachea or larynx sites (Fig. 4A). Pool-6 showed the strongest antitussive effect and the most balanced and effective cough suppression regardless of sites stimulated (to 29.4 ± 11.3% and 32.3 ± 9.7% of control, respectively). Figure 4B indicates that the cough inhibitory effects of pool-6 peaked at approximately 30–40 min after the injection and persisted regardless of the site of stimulation.

Characterization of pool-6

To further characterize the most effective pool fraction, we heated pool-6 to inactivate proteins in the sample. Heating pool-6 (100°C, 5 min) substantially reduced the efficacy for cough inhibition of either the bifurcation or the larynx (Fig. 4C). In addition, we separated three fractions from pool-6

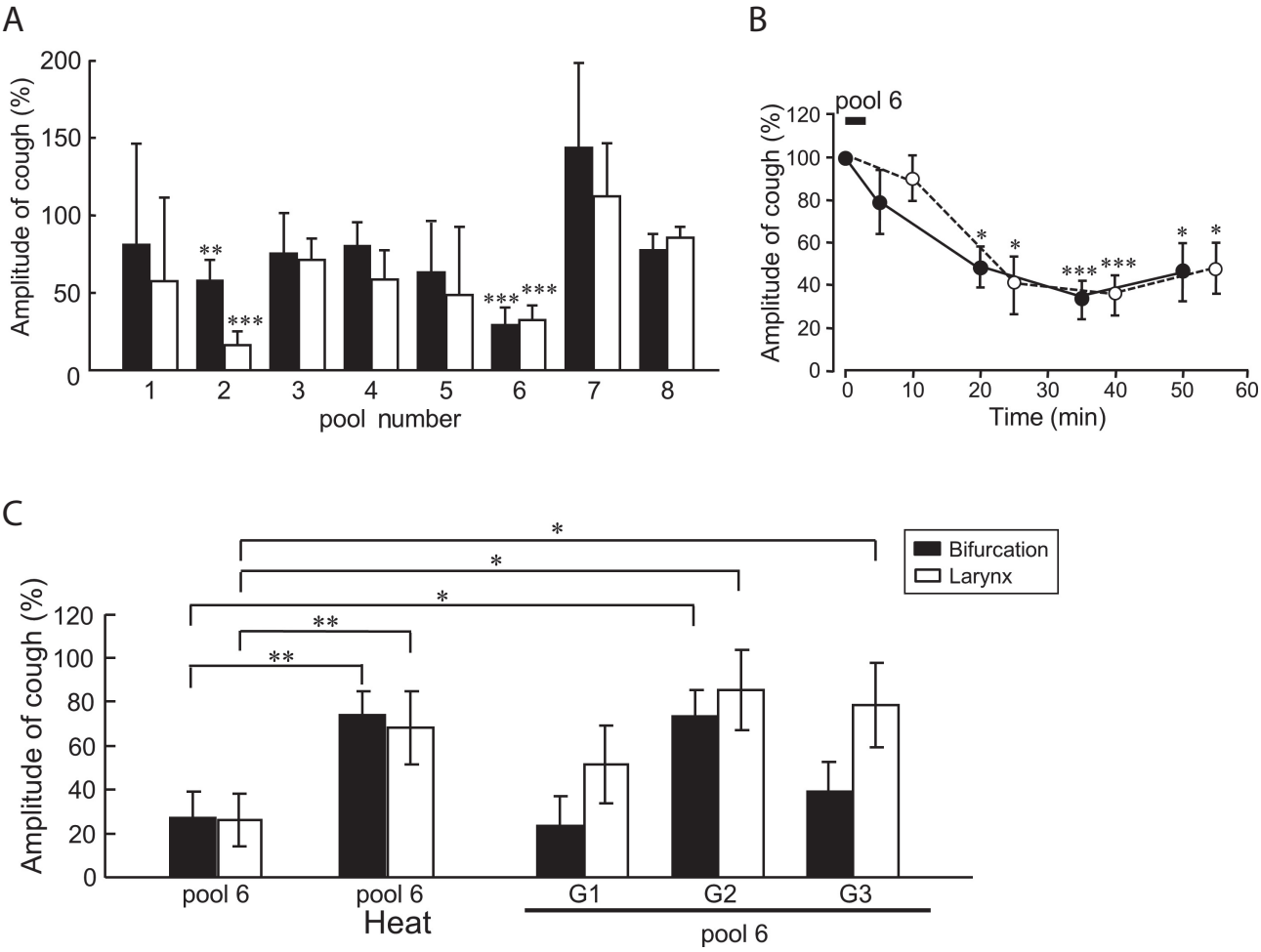

Figure 4. Effects of heparin-absorbed fractions (pool 1–8) on the cough response. **A.** Effects of heparin-absorbed fraction (pool 1–8) on cough response. The concentration of pool 1–8 was adjusted to OD_{280} = 1, and 10 μl of each fraction was injected through brachial vein. pool-2 and pool-6 showed strong antitussive effects. Each bars is the average ± SEM (n = 4–7). ** $p < 0.01$, *** $p < 0.001$. **B.** The time course of the effects of pool-6 on cough response induced by mechanical stimulation to the bifurcation (●) and larynx (○) regions of trachea (n = 7). * $p < 0.05$, *** $p < 0.001$. **C.** Effect of heated or gel filtered pool-6 on the cough responses. pool-6 was heated for 5 min at 100°C, or separated into 3 samples depending on the size of molecule (G1; high molecular weight, G2; middle molecular weight, G3; low molecular weight) and the effects of each molecules on the amplitudes of the coughs were observed. Among the three, G1 showed the strongest antitussive effect. Each bars is the average ± SEM (n = 4–9). * $p < 0.05$, ** $p < 0.01$.

(G1, G2 and G3) by the size exclusion chromatography. Sub-fraction G1 which contained the heaviest molecule, showed the strongest inhibitory effects on the cough responses at both sites (Fig. 4C). The thermal inactivation is consistent with a protein as the active element for cough inhibition in pool-6.

Identification of antitussive activity of pool-6

We used fraction G1 to better identify the protein(s) in pool-6, since G1 is the sub-fraction prepared from pool-6 by the size extrusion chromatography and shows the strongest cough suppressing effects as mentioned above (Fig. 4). Equivalent samples (120 μg) of G1, pool-5 and -6 were applied to the two-dimensional electrophoresis and selected 10 target spots (A to J) in fraction G1 according to the intensities in the order of G1 > pool-6 > pool-5 (Fig. 2). Namely we used pool-5, which showed no significant cough suppressing effect (Fig. 4), as a negative control to identify the active proteins.

The protein spots from "A" to "H" in Fig. 2 were analyzed by MALDI/TOF-MS, whereas "J" by LC-MS/MS since it was mixed with several proteins. As a result, most of the spots (A to G) were identified as "histidine-rich glycoprotein precursor". Spots H, I and J contained "coagulation factor XI precursor, plasma kallikrein, and mixture of ATP synthase, H^+ transporting mitochondrial F1 complex, alpha subunit, cardiac muscle and plasma kallikrein" respectively, thereby indicating that these proteins are the candidates for cough suppressing factor in the human plasma (Table 1).

Effect of the blood coagulation factors on cough responses

To confirm the biological activity of these identified candidate constituents of G1, we purchased the above mentioned molecules except ATP synthase, H^+ transporting mitochondrial F1 complex, alpha subunit, cardiac muscle and tested them in the same manner as our plasma fraction on the cough suppression. Among the candidates, fXIa but not factor XI (fXI) strongly suppressed coughing with a potency that corresponded to that of G1. However, plasma kallikrein, which was also contained in spot I and J, did not alter the cough responses (Fig. 5A). The histidine-rich glycoproteins in spots A to J did affect the cough responses, but were less effective compared to fXIa. Mono- and poly- clonal antibodies against fXIa prevented the cough suppressing effects of fXIa (Fig. 5B).

Effect of codeine and fXIa on cough responses evoked by capsaicin

Table 2 shows the effects of fXIa (4 to 400 ng/kg) and codeine (10 mg/kg) on the coughs evoked in response to pre-and post- inhalation of aerosolized capsaicin (10^{-7} or 10^{-6} mol/l) observed in conscious and unrestrained guinea-pigs. Extremely low doses of fXIa (> 4 ng/kg) effectively suppressed the number of the coughs, or the ratio of coughing before and after the application of fXIa (the ratio of post/pre application), making it nearly a million times more effective than codeine on a weight basis. Furthermore, the inhibitory ratio of fXIa was also dose-dependent. Codeine required a dose of 10 mg/kg to produce inhibition approaching that of 40 ng/kg fXIa.

Table 1. Proteins identified by MALDI/TOF-MS or LC-MS/MS in 10 spots (A–J) in G1 fraction

	MW (nominal)	Sequence coverage (score)	Identified protein
A	59541	17% (94)	histidine-rich glycoprotein precursor
B	59541	17% (87)	histidine-rich glycoprotein precursor
C	59541	10% (75)	histidine-rich glycoprotein precursor
D	59541	13% (66)	histidine-rich glycoprotein precursor
E	59541	12% (52)	histidine-rich glycoprotein precursor
F	59541	8% (42)	histidine-rich glycoprotein precursor
G	59541	20% (105)	histidine-rich glycoprotein precursor
H	70064	29% (71)	coagulation factor XI precursor
I	71323	21% (65)	plasma kallikrein
J	(59671)	9% (293)	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
	(71295)	4% (58)	plasma kallikrein

To identify the proteins in spots A to J in G1fraction, which represented stronger intensities in the order of G1 > pool-6 > pool-5, we used MALD1/TOF-MS (A to I) and LC-MS/MS (J), respectively. A to J in the Table 1 are identical to spots A to J in Fig. 2. MW, molecular weight.

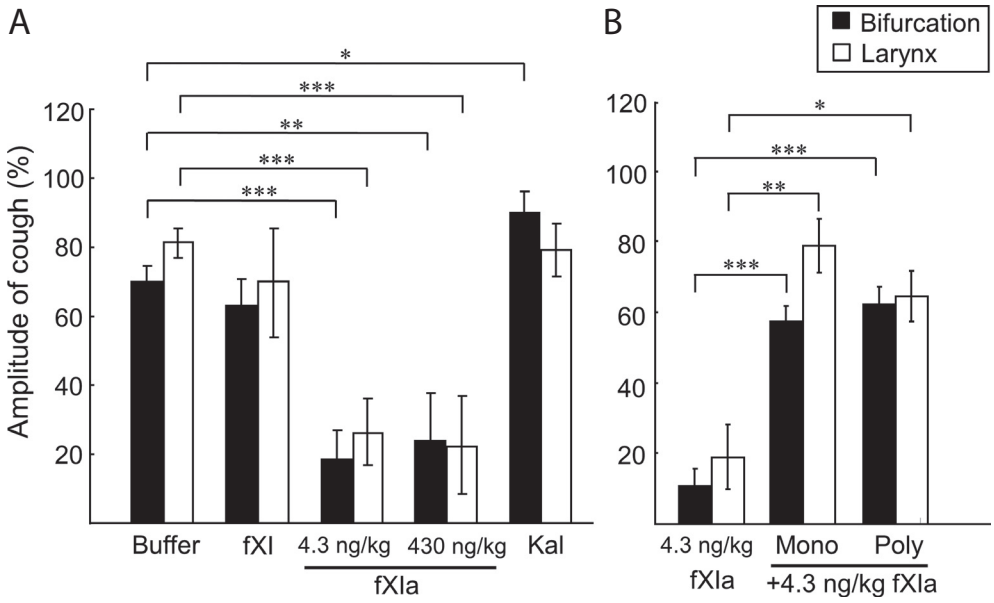

Figure 5. Effects of purchased factor XIa (fXIa), kallikrein and antibodies against fXIa on cough responses. **A.** We purchased each blood coagulation factors, factor XI (fXI), factor XIa (fXIa) and kallikrein (Kal) from Haematologic Technologies, Inc. and observed the effects of these factors on cough responses induced by mechanical stimulation to the bifurcation (filled column) and larynx (open column) regions of the trachea. The extremely low dose of factor XIa (fXIa) but not kallikrein showed strong antitussive effects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **B.** The effects of mono- and poly-clonal antibodies raised against fXIa on the antitussive action of fXIa. Mono-and poly-clonal antibodies abolished the antitussive effects of fXIa. Each bars is the average ± SEM ($n = 4–9$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

In the present study, we discovered fraction within human plasma containing a new, very strong cough suppressing protein. Heparin column chromatography separated fractions that contained a protein identified as blood coagulation factor fXIa using bioassay and LC-MS/MS and MALDI-TOF/MS. Intravenous injection of the activated coagulation factor fXIa at doses of 0.15–15 μg/ml × 10 μl/guinea-pigs produced long lasting and remarkably effective antitussive effects. These doses of fXIa correspond to 4.3–430 ng/kg, when the body weight of guinea pig was assumed as 350 g. In these same assays, a similarly effective dose of codeine in laryngeal evoked coughing (3 mg/kg), was not effective for the tracheal irritation (Takahama et al. 1997). Therefore, fXIa was more potent than codeine (ten-thousandth to millionth of the dose of codeine) and better suppressed the cough evoked by mechanical stimulation in the guinea-pig.

Afferent nerve fibers arising bilaterally in the vagus nerves play an essential role in regulating the cough reflex. Recent studies identified Aδ afferent neurons as essential in cough in the anesthetized guinea-pig, and the term "cough receptors" (Widdicombe 1954a, 1954b) was re-introduced (Canning et al. 2004; Mazzone et al. 2005, 2009) for the following reasons. (i) Conduction velocity of "cough receptors" is much slower than that of rapidly adapting stretch receptors (RARs) or slowly adapting stretch receptors (SARs) (Bergren and Sampson 1982; Ho et al. 2001; Canning et al. 2004; Undem et al. 2004), but much faster than that of C-fibers (Riccio et al. 1996; Ho et al. 2001). These "cough receptors" can also be differentiated from intrapulmonary RARs and SARs (Sano et al. 1992; Schelegle and Green 2003; Widdicombe 2003), by their insensitivity to smooth muscle contractions and/or lung distension (Fox et al. 1993; Canning et al. 2004). (ii) The cough receptors are primarily localized to the extra pulmonary airways. In contrast RARs and SARs in guinea-pigs are localized to the intrapulmonary airways and lungs, thereby indicating that tracheal and laryngeal afferent neurons regulating cough are polymodal Aδ fibers (Bergren and Sampson 1982; Keller et al. 1989; Riccio et al. 1996).

In addition, it is also known that C-fiber selective stimulants such as capsaicin and bradykinin are effective at evoking cough (Karlsson and Fuller 1999). Furthermore, repeated treatment with capsaicin-caused degeneration of C-and Aδ-nociceptors (Jancso et al. 1977; Lundberg and Saria 1983; Jancso 1992), and enhanced the effect of codeine in suppressing the mechanically-induced cough (Lundberg and Saria 1983; Takahama et al. 1997). Angiotensin-converting enzyme inhibitor (ACEI) is reported to

Table 2. Effects of fXIa and codeine on the coughs evoked by inhalation of aerosolized capsaicin (10^{-7} and 10^{-6} mol/l) in guinea-pigs

	Dose ($n = 8$)	Number of coughs		C_{pre}/C_{post}	Inhibitory ratio
		Pre	Post		
Control	0 ng/kg	9.3 ± 0.6	12.4 ± 1.2	132.6 ± 0.6	–
fXIa	4 ng/kg	9.4 ± 0.6	8.6 ± 0.9	92.7 ± 9.5**	30.1
fXIa	40 ng/kg	9.5 ± 0.6	6.4 ± 0.5*	67.2 ± 4.5**	49.3
fXIa	400 ng/kg	9.4 ± 0.8	4.9 ± 0.2**	54.3 ± 4.5**	59.0
Codeine	10 mg/kg	9.3 ± 0.6	6.0 ± 0.4##	65.3 ± 3.9##	50.8

To evaluate the cough suppressing effects of fXIa or codeine treatment, we used pre- and post-applications of aerosolized capsaicin (10^{-7} and 10^{-6} mol/l, respectively, to avoid desensitization, if any) before and after the application of fXIa (4–400 ng/kg) or codeine (10 mg/kg). First, we applied 10^{-7} mol/l aerosolized capsaicin and counted the number of the coughs during and after the treatment (5 min each). To obtain full recovery, the animals were exposed to fresh air for 1 hour. Then, 20 min before capsaicin tests (10^{-6} mol/l), fXIA (4–400 ng/kg) or codeine (10 mg/kg) were administered into the hind limb peripheral vein, and the number of the coughs measured during and after the application of capsaicin (5 min each). The ratio of the number of coughs induced by pre- and post-application of capsaicin (%, post/pre × 100) or the inhibitory ratio (%, (the ratio of coughs in control) − (the ratio of coughs after fXIa or codeine injection)/(the ratio of coughs in control) × 100) were obtained. Each value is the mean ± SEM ($n = 8$). C_{pre}/C_{post}, the ratio of coughs induced by pre- and post-application of capsaicin. * $p < 0.05$; ** and ## $p < 0.01$. Statistical analysies (*, ** and ##) were made by Dunnett or Steel-Dwass multiple comparison and Student's t or Aspin-Welch testing.

sensitize nociceptive fibers and induces codeine-resistant chronic cough in conscious guinea-pigs (Fox et al. 1996). These observations taken together led to the hypothesis that coughs mediated by nociceptive fibers are resistant to codeine (Takahama and Shirasaki 2007). Indeed, inhaled neurokinin-A caused codeine-insensitive cough in guinea-pigs, and it is well documented that substance P (SP) is involved in the pathogenesis of cough in humans (Otuka et al. 2011). The density of SP-like immunoreactive nerves is significantly higher in the patients with cough-variant asthma than in normal subjects and patients with classical asthma (Lee et al. 2003). Moreover, co-administration of codeine and neurokinin 2 (NK2) receptor antagonist almost abolished the citric acid-induced coughs in conscious guinea-pigs. High doses of codeine alone failed to fully inhibit citric acid-induced coughs (Takahama and Shirasaki 2007). Thus, it seems that coughs triggered by neurokinin-containing nociceptive fiberes may be resistant to codeine. Recent findings support this view that transient receptor potential vanilloid-1 (TRPV-1) is increased in the airway nerves in the patients with chronic cough (Groneberg et al. 2004) and acid sensing ion channels (ASICs) are localized in Aδ fibers in guinea-pigs (Gu and Lee 2006). Therefore our observations suggest that fXIa suppresses the cough induced by "cough receptors", RARs and nociceptor Aδ and c-fibers that are insensitive to codeine.

Recent reviews highlighted the prominent shortcomings of current available antitussives, including narcotic opioids such as codeine and their unpleasant or intolerable side effects (Dicpinigaitis 2009; Bosler 2010). Furthermore in a double-blind, placebo-controlled, crossover study, 60 mg of codeine in coughing subjects with chronic obstructive pulmonary disease (COPD), experienced no benefit with codeine over placebo in response to citric acid in measures of objective cough frequency or subjectively assessed cough and cough reflex sensitivity (Smith et al. 2006). Therefore safer and more effective cough suppressants would be welcome.

The mechanism of action of fXIa is unknown. However, the cough induced by the electrical stimulation of superior laryngeal nerve was not suppressed by fXIa (unpublished observation by Takahama), a finding consistent with a peripheral action of fXIa.

fXI deficiency occurs predominantly in ethnic Jewish populations of Ashkenazi descendents (Rosenthal et al. 1953; Asakai et al. 1989, 1991). In mice, fXI deficiency protects from carotid artery occlusion induced by high concentration of ferric chloride ($FeCl_3$) (Rosen et al. 2002). However, the relationships between fXI deficiency and cough in man and in fXI-deficient mice (Rosen et al. 2002; Wang et al. 2005, 2006) need to be clarified.

Clinical management of chronic cough seeks to treat the underlying cause(s). However, certain situations including idiopathic cough would requires effective cough suppression for symptomatic relief. Our findings suggest that fXIa offers a promising new direction for drug development which has the potential to be far more efficacious than current therapies.

In conclusion, the cough suppressing effect of the fraction and fXIa prepared from human plasma were nearly one million times stronger than codeine on a weight basis and codeine only partially suppressed the mechanically triggered coughing. These observations indicate that fXIa in human plasma is a very promising, new therapeutic candidate for effective antitussive action.

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