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(54) Title: PEGYLATED APELIN AND USES THEREOF

(57) **Abrégé/Abstract:**

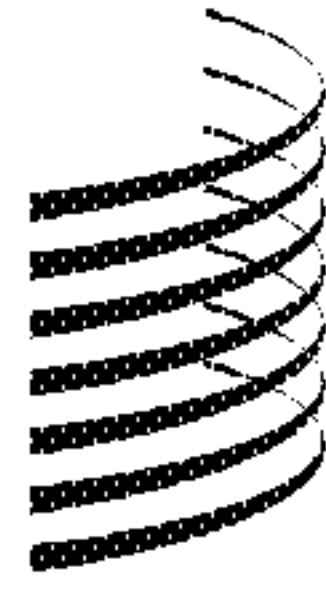
The invention provides compositions and methods for treating a disease or disorder associated with Apelin. Specifically, the invention relates to a pegylated form of Apelin to provide extended circulating life and inotropic effects, and thereby efficiently treat diseases or disorders associated with Apelin.



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(54) Title: PEGYLATED APELIN AND USES THEREOF

(57) Abstract: The invention provides compositions and methods for treating a disease or disorder associated with Apelin. Specifically, the invention relates to a pegylated form of Apelin to provide extended circulating life and inotropic effects, and thereby efficiently treat diseases or disorders associated with Apelin.

**WO 2012/125408 A8**

PEGYLATED APELIN AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Provisional Patent Application
5 61/451,623, filed March 11, 2011, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates to compositions and methods for treating a disease or disorder
associated with Apelin. Specifically, the invention relates to a pegylated form of Apelin to
provide extended circulating life and inotropic effects, and thereby efficiently treat diseases or
10 disorders associated with Apelin.

BACKGROUND OF THE INVENTION

[0003] Apelin, a peptide initially isolated from bovine stomach extracts, acts as an
endogenous ligand for G protein coupled APJ receptor. Apelin gene encodes a pre-proprotein
of 77 amino acids, with a signal peptide in the N-terminal region. After translocation into the
15 endoplasmic reticulum and cleavage of the signal peptide, the proprotein of 55 amino acids
may generate several active fragments: a 36 amino acid peptide corresponding to the
sequence 42-77 (Apelin 36), a 17 amino acid peptide corresponding to the sequence 61-77
(Apelin 17) and a 13 amino acid peptide corresponding to the sequence 65-77 (Apelin 13).

[0004] Apelin and its receptor are expressed in majority of tissues and organs, including
20 cardiovascular system. Multiple effects relevant to cardiovascular system have been reported,
including positive inotropic activities, diuretic effect, and direct myocardial protection from
ischemia reperfusion injury. As for its effect on blood pressure, the reports are conflicting.
Some of the studies showed decreased arterial pressure via a NO-dependent mechanism, but
there are also contradictory result reported with Apelin 13 increasing the arterial pressure, as
25 well as a biphasic change of mean arterial blood pressure.

[0005] Several studies have documented cardiovascular effects of Apelin, including enhanced
inotropy and vasodilation. However, these cardiovascular effects are short lived due to the
short circulating life of the Apelin peptide.

[0006] Accordingly, there exists a need for improved Apelin compositions so as to improve their circulating life, inotropic effects, and other beneficial characteristics.

SUMMARY OF THE INVENTION

[0007] The invention provides a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of an Apelin. The pegylated Apelin has a prolonged circulating life and inotropic effect, relative to a non-pegylated Apelin.

[0008] Additionally, the invention provides methods for producing the pegylated Apelin by reacting an Apelin with an activated PEG-aldehyde linker in the presence of a reducing agent to form the pegylated Apelin under conditions in which the linker is covalently attached to at least one amino acid residue in the N-terminal of said Apelin.

[0009] The invention also provides pharmaceutical compositions that comprise a therapeutically effective amount of the pegylated Apelin.

[0010] The invention further provides kits that comprise a therapeutically effective amount of the pegylated Apelin.

[0011] The invention further provides methods for treating a disease or disorder associated with an Apelin, in a subject, by administering a therapeutically effective amount of the pegylated Apelin.

[0012] Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1. The column profile of Weak Cation Exchanger chromatography of the 40kDa PEG-apelin-36.

[0014] Figure 2. N-terminal sequencing showed selective PEGylation of apelin-36 at the N-terminus. For apelin-36, the first residue L, was detected in the first cycle (the spike indicated by solid arrow in the upper panel). However, only 2% recovery of first residue L was obtained from 40kDa PEG-apelin-36 conjugate (arrow in the lower panel).

5 [0015] Figure 3. Competitive radioligand binding to APJ receptor using apelin, its PEG conjugates, and α -MSH, a negative control.

[0016] Figure 4. Attachment of an N-terminal PEG group to apelin-36 does not compromise its function as an APJ agonist. In a forskolin-mediated cAMP accumulation assay the IC₅₀ of PEG-apelin-36 was shifted 1.5-fold to the right, with no change in the maximal inhibition of
10 forskolin-mediated cAMP accumulation versus unmodified apelin-36.

[0017] Figure 5a. PEG-apelin36 resulted in prolonged inotropic effects in rats. In normal rats that received 30 nM PEG-apelin-36 (Peg36Lo) EF was increased compared to baseline (BL) and normal saline (NS) at all time points. In animals that received 30 nM apelin-36 (Ap36Lo) EF was not significantly different from BL and NS at 50 min. * $p < 0.05$ vs BL and
15 time matched NS; IV= intravenous infusion.

[0018] Figure 5b. In normal rats that received 300 nM PEG-apelin-36 (Peg36Hi) EF was increased compared to the baseline (BL) and normal saline (NS) at all time points. In animals that received 300nM apelin-36 (Ap36Hi) EF was not significantly different from BL post at 50 min, and was not significantly from NS post 60 min. * $P < 0.05$ vs BL; ^ $P < 0.05$ vs time
20 matched NS; IV = intravenous infusion.

[0019] Figure 6. PEG-apelin-36 resulted in prolonged inotropic effects in Myocardial Infarct (MI) rats. In MI rats that received PEG-apelin-36 (Peg36MI) EF was increased compared to baseline (BL) and normal saline (NSMI) at all time points. In MI rats that received apelin 36 (Ap36MI) EF was not significantly different from BL post 50 min and was not significantly
25 different from the NSMI post 60 min. * $P < 0.05$ vs BL; ^ $P < 0.05$ vs time matched NSMI; IV=intravenous infusion.

[0020] Figure 7. IV injection of PEG-apelin-36 (Peg36) or apelin-36 (Ap36) in normal rats; and apelin 36 in MI rats (Ap36MI) did not result in changes in mean aortic blood pressure (ABPmean).

[0021] Figure 8. PEG-apelin-36 (Peg36) has a longer circulating life than apelin-36 (Ap36). In the Peg36 group, the blood apelin-36 was increased compared to baseline at all time points. * $p < 0.05$ vs baseline.

DETAILED DESCRIPTION OF THE INVENTION

5 [0022] The invention relates to compositions and methods for treating a disease or disorder associated with Apelin. Specifically, the invention relates to a pegylated form of Apelin to provide extended circulating life and inotropic effects, and thereby efficiently treat diseases or disorders associated with Apelin.

10 [0023] In one embodiment, provided herein is a pegylated Apelin molecule comprising one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of an Apelin.

15 [0024] In another embodiment, provided herein is a method for producing a pegylated Apelin, comprising the step of: reacting an Apelin with an activated PEG-aldehyde linker in the presence of a reducing agent to form said pegylated Apelin under conditions in which the linker is covalently attached to at least one amino acid residue in the N-terminal of said Apelin.

20 [0025] In another embodiment, provided herein is a pharmaceutical composition comprising a therapeutically effective amount of a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of an Apelin.

25 [0026] In another embodiment, provided herein is a method for treating a disease or disorder associated with an Apelin, in a subject, the method comprising: administering to said subject a therapeutically effective amount of a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of said Apelin.

[0027] To address the limitations in Apelin peptide characteristics, a 40 KDa PEGylated Apelin was successfully produced, characterized, and evaluated *in vitro* and *in vivo*. The inventors of the instant application surprisingly and unexpectedly found that compared to

Apelin, PEGylated Apelin exhibits an extended circulating life and correspondingly, an extended inotropic effect following IV administration.

[0028] Apelin is a well known protein. The amino acid and nucleic acid sequences of Apelin are well known in the art. For example, GenBank Identification Numbers AAF25815.1 and AF179680 contain Apelin amino acid and nucleic acid sequences, respectively.

[0029] In one embodiment, Apelin comprises the amino acid sequence set forth in SEQ ID NO: 1. In another embodiment, Apelin comprises a homolog, a variant, or a functional fragment of SEQ ID NO: 1. In another embodiment, Apeline comprises an amino acid sequence that is about 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to SEQ ID NO:1. In another embodiment, Apelin comprises a fragment of the amino acid sequence of SEQ ID NO: 1. The fragment may comprise one or more functional regions. Each possibility represents a separate embodiment of the present invention.

[0030] In one embodiment, Apelin is encoded by the nucleic acid sequence set forth in SEQ ID NO: 2. In another embodiment, Apelin nucleic acid sequence comprises a homolog, a variant, or a functional fragment of SEQ ID NO: 2. In another embodiment, Apelin is encoded by a nucleic acid sequence that is about 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to SEQ ID NO:2. In another embodiment, Apelin is encoded by a fragment of a nucleic acid sequence set forth in SEQ ID NO: 2. The fragment may comprise one or more functional regions. Each possibility represents a separate embodiment of the present invention.

[0031] The term Apelin, as used herein, may refer to any functional Apelin peptide which exhibits at least 10% APJ receptor binding affinity compared to Apelin 36, preferably, at least 25%, 50%, 75%, 85%, 95% or 100%, 200%, 300%, 400%, 500%, or more binding affinity of Apelin 36. In one embodiment, Apelin is Apelin 36, which is a 36 amino acid peptide discussed above (42-77 aa of SEQ ID NO: 1). An Apelin peptide of the present invention, however, can be shorter (e.g., 12, 13, 17, 20, 30, 31, 32, 33, 34, 35 or less amino acids in length) or longer (e.g., 37, 38, 39, 40 or 50 or 60 or more, up to 77 amino acids in length).

[0032] The one or more PEG molecules may be operably linked to an Apelin peptide through any known linking method. For example, two or more PEG molecules may be operably linked through a simple covalent bond, a flexible peptide linker or a disulfide bridge. Peptide

linkers may be entirely artificial (e.g., comprising 2 to 20 amino acid residues independently selected from the group consisting of glycine, serine, asparagine, threonine and alanine) or adopted from naturally occurring proteins.

[0033] In one embodiment, an Apelin peptide is reacted with an activated PEG-aldehyde linker in the presence of a reducing agent (e.g., cyanoborohydride) to form a pegylated Apelin under conditions in which the linker is covalently attached to an amino acid residue (e.g., Leucine) in the N-terminal of Apelin.

[0034] PEGylation of the molecules can be carried out, e.g., according to the methods described in Youngster et al., Curr Pharm Des (2002), 8:2139; Grace et al., J Interferon Cytokine Res (2001), 21:1103; Pepinsky et al., J Pharmacol Exp Ther (2001), 297:1059; Pettit et al., J Biol Chem (1997), 272:2312; Goodson et al. Biotechnology NY (1990), 8:343; Katre; J Immunol (1990), 144:209).

[0035] Any kind of polyethylene glycol is suitable for the present invention provided that the PEG-polypeptide is still functionally active which can be assayed according to methods known in the art. Preferably, the polyethylene glycol of the present invention is PEG 1000, 2000, 3000, 5000, 10000, 15000, 20000, 30000 or 40000 with PEG 30000 or 40000 being particularly preferred.

[0036] In one example, the pegylated molecule comprises a monomeric Apelin. In another example, the pegylated molecule comprises is an oligomeric Apelin. In yet another example, the pegylated molecule comprises a multiarm PEG, wherein one or more monomeric Apelin are operably linked to the multiarm PEG.

[0037] The Apelin of the present invention according to one embodiment of the invention is recombinantly produced by use of a polynucleotide encoding the Apelin and vectors, preferably expression vectors containing said polynucleotides. For the production of the oligomers of the invention, the polynucleotides are obtained from existing clones, i.e., preferably encode the naturally occurring polypeptide or a part thereof. Polypeptides encoded by any polynucleotide which hybridises to the complement of the native DNA or RNA under highly stringent or moderate stringent conditions (for definitions, see Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y.) as long as that

polypeptide maintains the biological activity of the native sequence, are also useful for producing the oligomers of the present invention.

[0038] The Apelin peptides can also be prepared by solid phase synthesis according to methods well known to a person skilled in the art, see, e.g., Coligan (ed) et al., Current
5 Protocols in Protein Science, John Wiley and Sons, Inc., (2001) N.J. For example, the peptides apelin-13 and apelin-36 are prepared by solid phase synthesis using an Fmoc strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perseptive Biosystems, Cambridge, MA). Crude peptides are purified by preparative reverse phase high-performance liquid chromatography. Fractions containing
10 the appropriate peptide are pooled and lyophilized. The purity of the final product is assessed by analytical reverse phase high-performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

[0039] The recombinant vectors can be constructed according to methods well known to the
15 person skilled in the art; see, e.g., Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. A variety of expression vector/host systems may be utilized to contain and express sequences encoding the oligomers of the present invention.

[0040] Exemplary vectors include plasmids, phagemids, cosmids, viruses and phage nucleic acids or other nucleic acid molecules that are capable of replication in a prokaryotic or
20 eukaryotic host. The vectors typically contain a marker to provide a phenotypic trait for selection of transformed hosts such as conferring resistance to antibiotics such as ampicillin or neomycin

[0041] The vector may be an expression vector, wherein the nucleic acid encoding the peptide is operably linked to an expression control sequence. Typical expression vectors
25 contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the nucleic acid molecules of the invention. The vectors may also contain genetic expression cassettes containing an independent terminator sequence, sequences permitting replication of the vector in both eukaryotes and prokaryotes, *i.e.*, shuttle vectors and selection markers for both prokaryotic and eukaryotic systems.

[0042] Suitable promoters include constitutive promoters and inducible promoters. Representative expression control sequences/promoters include the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of *fd* coat protein, the glycolytic promoters of yeast, *e.g.*, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, *e.g.*, *Pho5*, the promoters of the yeast alpha mating factors, promoters derived from the human cytomegalovirus, metallothionine promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, *e.g.*, the early and late promoters of SV40.

[0043] The invention also includes non-human hosts such as cells or organisms containing a nucleic acid molecule or a vector of the invention. By "host" it is meant a non-human unicellular or multicellular organism or a "host cell", which refers to a cell or population of cells into which a nucleic acid molecule or vector of the invention is introduced. "A population of host cells" refers to a group of cultured cells into which a nucleic acid molecule or vector of the present invention can be introduced and expressed.

[0044] A host of the present invention may be prokaryotic or eukaryotic. Suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DHI, and *E. coli* MRC1, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeast and other fungi, insect cells, plant cells, human cells, and animal cells, including mammalian cells, such as hybridoma lines, COS cells, NS0 cells and CHO cells.

[0045] The invention also includes methods of producing an Apelin, the method comprising: culturing a host cell; and recovering the Apelin from said host cell.

[0046] Depending on the expression system and host selected, the molecules are produced by growing host cells transformed by an expression vector described above whereby the protein is expressed. The expressed protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the product can be purified directly from the media. If it is not secreted, it can be isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art. For example, once expressed, the product may be isolated and purified by any number of

techniques, well known in the art. A protein (e.g., Apelin) of the present invention obtained as above may be isolated from the interior or exterior (e.g., medium) of the cells or hosts, and purified as a substantially pure homogeneous protein. The method for protein isolation and purification is not limited to any specific method. In fact, any standard method may be used.

5 For instance, column chromatography, filtration, ultrafiltration, salt precipitation, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric point electrophoresis, dialysis, and recrystallization may be appropriately selected and combined to isolate and purify the protein.

[0047] For chromatography, for example, affinity chromatography, ion-exchange
10 chromatography, hydrophobic chromatography, gel filtration, reverse phase chromatography, adsorption chromatography, and such may be used (ed. Daniel R. Marshak et al. (1996) Strategies for Protein Purification and Characterization: A Laboratory Course Manual., Cold Spring Harbor Laboratory Press). These chromatographies may be performed by liquid chromatography, such as, HPLC and FPLC. Thus, the present invention provides highly
15 purified proteins produced by the above methods.

[0048] The invention also provides a pharmaceutical composition comprising the oligomer, nucleic acid, vector, or host cell of this invention and one or more pharmaceutically acceptable carriers. "Pharmaceutically acceptable carriers" include any excipient which is nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations
20 employed. The pharmaceutical composition may include one or additional therapeutic agents.

[0049] Pharmaceutically acceptable carriers include solvents, dispersion media, buffers, coatings, antibacterial and antifungal agents, wetting agents, preservatives, buffers, chelating agents, antioxidants, isotonic agents and absorption delaying agents.

[0050] Pharmaceutically acceptable carriers include water; saline; phosphate buffered saline; dextrose; glycerol; alcohols such as ethanol and isopropanol; phosphate, citrate and other
25 organic acids; ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or
30 dextrans; EDTA; salt forming counterions such as sodium; and/or nonionic surfactants such

as TWEEN, polyethylene glycol (PEG), and PLURONICS; isotonic agents such as sugars, polyalcohols such as mannitol and sorbitol, and sodium chloride; as well as combinations thereof. Antibacterial and antifungal agents include parabens, chlorobutanol, phenol, ascorbic acid, and thimerosal.

5 [0051] The pharmaceutical compositions of the invention may be formulated in a variety of ways, including for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. In some embodiments, the compositions are in the form of injectable or infusible solutions. The composition is in a form suitable for oral,
10 intravenous, intraarterial, intramuscular, subcutaneous, parenteral, transmucosal, transdermal, or topical administration. The composition may be formulated as an immediate, controlled, extended or delayed release composition.

[0052] Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene
15 glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the subject invention, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Other common parenteral vehicles include sodium phosphate
20 solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

25 [0053] More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will preferably be
30 preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of
5 dispersion and by the use of surfactants. Suitable formulations for use in the therapeutic methods disclosed herein are described in Remington's Pharmaceutical Sciences, Mack Publishing Co., 16th ed. (1980).

[0054] In some embodiments, the composition includes isotonic agents, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride. Prolonged absorption of the
10 injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0055] Sterile injectable solutions can be prepared by incorporating the molecule, by itself or in combination with other active agents, in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered
15 sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, one method of preparation is vacuum drying and freeze-drying, which yields a powder of an active ingredient plus any additional desired ingredient from a
20 previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Further, the preparations may be packaged and sold in the form of a kit such as those described in US Appl. Publ. No. 2002/0102208 A1, which is incorporated herein by reference in its entirety. Such articles of
25 manufacture will preferably have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to autoimmune or neoplastic disorders.

[0056] Effective doses of the compositions of the present invention, for treatment of conditions or diseases as described herein vary depending upon many different factors,
30 including means of administration, target site, physiological state of the patient, whether the

patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but non-human mammals including transgenic mammals can also be treated. Treatment dosages may be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

5 [0057] The pharmaceutical compositions of the invention may include a “therapeutically effective amount.” A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of a molecule may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the molecule to elicit a
10 desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the molecule are outweighed by the therapeutically beneficial effects.

[0058] The invention further provides methods of treating a disease or condition, comprising administering to a mammal in need thereof a therapeutically effective amount of a pegylated
15 Apelin molecule.

[0059] In one embodiment, provided herein is a method for treating a disease or disorder associated with an Apelin, in a subject, the method comprising: administering to said subject a therapeutically effective amount of a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the
20 N-terminal of said Apelin.

[0060] As used herein, the terms “treat” and “treatment” refer to therapeutic treatment, including prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change associated with a disease or condition. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of the extent of a disease or condition, stabilization of a disease or condition
25 (*i.e.*, where the disease or condition does not worsen), delay or slowing of the progression of a disease or condition, amelioration or palliation of the disease or condition, and remission (whether partial or total) of the disease or condition, whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not
30 receiving treatment. Those in need of treatment include those already with the disease or

condition as well as those prone to having the disease or condition or those in which the disease or condition is to be prevented.

[0061] Examples of disease or disorder caused by or otherwise associated with an Apelin, include, but are not limited to, a cardiovascular disease, an ischemia-reperfusion injury, a myocardial infarction, an acute decompensated heart failure, a chronic heart failure, a
5 cardiomyopathy, an endocrine/metabolic disorder or pulmonary hypertension.

[0062] The pegylated Apelin may be administered alone, or in combination with one or more therapeutically effective agents or treatments. The other therapeutically effective agent may be conjugated to the pegylated Apelin, incorporated into the same composition as the
10 pegylated Apelin, or may be administered as a separate composition. The other therapeutically agent or treatment may be administered prior to, during and/or after the administration of the pegylated Apelin.

[0063] The administration of the pegylated Apelin with other agents and/or treatments may occur simultaneously, or separately, via the same or different route, at the same or different
15 times. Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response).

[0064] In one example, a single bolus or infusion may be administered. In another example, several divided doses may be administered over time. In yet another example, a dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation.
20 Dosage unit form, as used herein, refers to physically discrete units suited as unitary dosages for treating mammalian subjects. Each unit may contain a predetermined quantity of active compound calculated to produce a desired therapeutic effect. In some embodiments, the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to
25 be achieved.

[0065] The composition of the invention may be administered only once, or it may be administered multiple times. For multiple dosages, the composition may be, for example, administered three times a day, twice a day, once a day, once every two days, twice a week, weekly, once every two weeks, or monthly.

[0066] It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

[0067] "Administration" to a subject is not limited to any particular delivery system and may include, without limitation, parenteral (including subcutaneous, intravenous, intramedullary, intraarticular, intramuscular, or intraperitoneal injection) rectal, topical, transdermal or oral (for example, in capsules, suspensions or tablets). Administration to a host may occur in a single dose or in repeat administrations, and in any of a variety of physiologically acceptable salt forms, and/or with an acceptable pharmaceutical carrier and/or additive as part of a pharmaceutical composition (described earlier). Once again, physiologically acceptable salt forms and standard pharmaceutical formulation techniques are well known to persons skilled in the art (see, for example, Remington's Pharmaceutical Sciences, Mack Publishing Co.).

[0068] The composition of the invention (e.g., pegylated Apelin) may be administered parenterally (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular). Further, the composition of the invention may be administered by intravenous infusion or injection. The composition of the invention may be administered by intramuscular or subcutaneous injection. In some embodiments, the composition of the invention may be administered orally. As used herein, a "composition" refers to any composition that contains a pharmaceutically effective amount of a pegylated Apelin.

[0069] The methods of treatment described herein can be used to treat any suitable mammal, including primates, such as monkeys and humans, horses, cows, cats, dogs, rabbits, and rodents such as rats and mice. In one embodiment, the mammal to be treated is human.

[0070] Any reference including patents, patent applications, or scientific publications, cited herein, are incorporated by reference in their entirety.

[0071] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

EXAMPLE 1

PEG Conjugated Apelin 36

[0072] A 40 kDa PEG conjugated apelin-36 (PEG-apelin-36) was successfully produced with
5 N-terminal conjugation, high purity (>98%) and minimum reduction of APJ receptor binding
affinity. Using an adenylate cyclase inhibition assay, comparable in vitro bioactivity was
observed between the PEG-apelin-36 and unmodified apelin-36. In vivo evaluation of the
PEG-apelin-36 was performed in normal rats and rats with myocardial infarction (MI).
Cardiac function was assessed via echocardiography before, during a 20 minutes IV infusion
10 and up to 100 minutes post peptide infusion. Similar increases in cardiac ejection fraction
(EF) were observed during the infusion of PEG-apelin-36 and apelin-36 in normal rats.
However, animals that received PEG-apelin-36 maintained significantly increased EF over
the 100 minute post infusion monitoring period compared to the animals that received
unmodified apelin-36. Interestingly, EF increases observed with PEG-apelin-36 and apelin-
15 36 were greater in the MI rats. PEG-apelin-36 had a prolonged circulating life compared to
apelin-36 in rats. There were no changes in aortic blood pressure when PEG-apelin-36 or
apelin-36 was administered.

EXAMPLE 2

Materials and methods:

20 **PEGylation of apelin**

[0073] *Conjugation of apelin-36 (Ana Spec, Fremont, CA):* The conjugation with 10kDa,
30kDa and 40kDa branched aldehyde PEGs (NOF America Corp., White Plains, NY) were
carried out at 0.2uM peptide and 0.4 uM PEG in the presence of 20mM cyanoborohydride
(Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium acetate buffer at pH 5. The conjugation was
25 conducted at 25°C overnight and quenched with 2mM Tris at pH 7.5. The conjugates were
dialyzed against water to remove the unconjugated peptide. The 40kDa PEGylated apelin-36
conjugate (PEG-apelin-36) was further purified by weak cation exchange chromatography on
a HiTrap SP FF column (G.E.Healthcare, Piscataway, NJ) to remove free PEG. Conjugate

was eluted at 1.5M salt concentration from 15 minute column run. Nanodrop A280 was used to determine the concentration of the apelin-36 conjugate.

[0074] *Determination of the site of PEGylation:* 200 pmoles of apelin-36 and PEG-apelin-36 were N-terminally sequenced on the Procise sequencer (Applied Biosystems, Foster City, CA) using the pre-programmed pulsed liquid method for 18 cycles. Apelin-36 and PEG-apelin-36 were also analyzed by MALDI-TOF mass spectrometer (Voyager-DE PRO) (Applied Biosystems, Foster City, CA) in linear mode using DHB (2,5-dihydroxybenzoic acid) as the matrix. The acceleration voltage was 25,000V.

[0075] *APJ receptor binding assay:* The binding was performed using competition with ¹²⁵I-labelled [Glp⁶⁵, Nle⁷⁵, Tyr⁷⁷]-apelin13 probe for binding to the APJ receptor in a membrane fraction from the transfected CHO cells (Perkin Elmer, Waltham, MA). Serially diluted peptide was incubated with membrane and probe for 1 hour at room temperature. The mixture was then filtered over a 1.2 um glass fiber type C filterplate (Millipore Corp., Billerica, MA), preblocked with 0.5% polyethylenimine. The plate was washed 4 times with 200ul ice cold binding buffer and then another 4 times with ice cold 50mM Tris-HCl, pH7.4, dried and counted with scintillation fluid. The data was analyzed with Graphpad Prism 4 (GraphPad Software, Inc, La Jolla, CA) using nonlinear regression.

In vitro bioactivity assay

[0076] *Generation of a cell clone with high level APJ receptor expression:* HEK293 cells (ATCC, Manassas, VA) were maintained at 37°C / 5% CO₂ in Dulbecco's-modified Eagle's Medium (DMEM) supplemented with L-Glutamine, penicillin/streptomycin, and 10% fetal bovine serum (FBS). Plasmid pCMV6-XL4 (Origene Technologies, Inc., Rockville, MD) containing a cDNA encoding human APJ was subcloned into pCMV6-Neo (Origene Technologies, Inc.) to generate pCMV6-APJ. HEK293 cells were transiently transfected with pCMV6-APJ using Superfect (Qiagen Technologies Inc., Valencia, CA), and selected in G418 (1 mg/mL) selection media. Clonal cell lines were isolated using sterile filter discs. Surface expression of APJ was detected by flow cytometry using the monoclonal APJ antibody (MAB856, R&D Systems, Minneapolis, MN). A clone expressing a high level of APJ on the cell surface (HEK293-APJ#3) was used to determine the bio-activity of apelin peptides.

[0077] *Intracellular cAMP measurement:* HEK293-APJ#3 cells were used to assess the activity of apelin peptides and pegylated apelin derivative peptides. The HEK293-APJ#3 cells were seeded into 96 well plates at 50,000 cells per well. The following day, the cells were pre-treated at 37°C for 5 minutes with assay buffer (0.5mM IBMX in DMEM containing penicillin/streptomycin and 0.5% (w/v) BSA) before addition of apelin peptides (0-300nM) and forskolin (30μM) prepared in assay buffer. After 15 minute of incubation at 37°C, the apelin / forskolin stimulation was terminated by aspiration of the assay components and addition of Lysis Buffer 1B from cAMP Enzyme Immunoassay (EIA) kit (GE Healthcare, Piscataway, NJ). Lysates (5-50 μL) were assayed for cAMP according to the instructions of the EIA kit. Intracellular cAMP data (apelin-stimulated inhibition of forskolin-mediated cAMP production) was plotted using GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA) and fit to a sigmoidal dose-response curve with variable slope.

In vivo evaluation

[0078] *Effect on heart function in normal rats:* Female Lewis rats, weighing 225-250g (Charles River Laboratories, Wilmington, MA) were anesthetized with Isoflurane (2.5%), driven by oxygen (2.5 L/min) through a nose cone. The body temperature was maintained at 36°C with a water-bath heating pad and complimented with a heating lamp controlled by a rectal probe connected to a TCAT-20F temperature controller (Physitemp Instruments, Inc., Clifton, NJ). The tail vein was cannulated with a 24G Surflo IV catheter. Animals were then randomly divided into 5 groups (n=7/grp) which received either 300nM apelin-36 (Ap36Hi), 30nM apelin-36 (Ap36Lo), 300nM PEG-apelin-36 (Peg36Hi), 30nM PEG-apelin-36 (Peg36Lo), or normal saline (NS) intravenously at a dose of 1ml/20min/250g for 20 minutes. Before infusion, and at 10, 20 (end of IV infusion), 30, 40, 50, 60, and 120 minutes post the initiation of the IV infusion, long axis 2D view echocardiography images were obtained using a 15MHz probe and GE Vivid 7 Dimension echocardiography unit (GE Healthcare Technologies, Waukesha, WI). Anesthesia was stopped between the 60 and 120 minutes time points.

[0079] *Effect on heart function in myocardial infarction (MI) rats:* MI was created by permanent ligation of left anterior descending artery (LAD) in male 250-275g Lewis rats (Charles River Laboratories, Wilmington, MA). Briefly, the animal was anesthetized by IP

injection of a cocktail of Ketamine (40-80mg/kg), Xylazine (5-10mg/kg), and diazepam (2-4mg/kg) and body temperature maintained as described in section [0080]. An 18G catheter which was inserted into the trachea and the animal was ventilated using a rodent ventilator (Model 683, Harvard Apparatus, Holliston, MA). The heart was exposed through a left thoracotomy incision at the 4th or 5th intercostal space. The LAD was ligated using a 6-0 silk at a level 1 to 2 mm distal to the edge of the left auricle. The chest was closed in layers, and the animal allowed to recover in a temperature controlled incubator. Fifty two weeks after the MI, the animals were randomly assigned to receive 30nM apelin-36 (Ap36MI; n=5), 30nM PEG-apelin-36 (Peg36MI; n=5), or normal saline (NSMI; n=5) by IV infusion. Anesthesia, temperature control, and the experiment protocol were the same as described in the section 2.3.1 except that higher levels of isoflurane (3% or to effect) and oxygen flow (3L/min) were employed since the body weight of the animals were between 600 and 650g.

[0080] *Effect on blood pressure in normal and myocardial infarction (MI) rats:* Normal male adult Sprague Dawley rats weighing 360 ± 14 g (Charles River Laboratories, Wilmington, MA) were anesthetized and body temperature maintained as described in paragraph [0088]. The left carotid artery and jugular vein were surgically exposed. The left carotid artery was cannulated with a Millar mikro-tip catheter (Millar Instruments, Houston, TX) for blood pressure monitoring and the left jugular vein was cannulated with PE-50 tubing for IV injection. The tip of the Millar catheter was placed into the descending aorta and the end of the catheter was connected to a computer with a Powerlab data requisition system (ADInstruments, Inc., Colorado Springs, CO) to monitor blood pressure. Animals were then randomly assigned to 2 groups (n=5/group) to receive either PEG-apelin-36 or apelin-36 (300nM solution, 0.4ml/kg, IV bolus). In all animals NS (0.4ml/kg, IV bolus) was first injected, followed by IV injection of the peptides. For NS injections, blood pressure was monitored before (baseline), and at 10, 20, 40, 60, 180 and 300 seconds following the NS injection. At the end of this period blood pressure returned to baseline and peptide was then injected. Blood pressure was monitored before (baseline), at 10, 20, 40, 60, 180 and 300 seconds, and at 10 and 20 minutes following the peptide injection. The blood pressure data was analyzed using Chart 5 Pro software (ADInstruments, Inc., Colorado Springs, CO). For blood pressure assessment in MI rats, myocardial infarction was performed, as described in paragraph [0079], in 9 male adult Lewis rats (329 ± 16 g; Charles River Laboratories,

Wilmington, MA). Four weeks later, blood pressure effects of apelin-36 was evaluated. The protocol for blood pressure evaluation was as described above for normal animals.

Apelin blood levels

[0081] *Administration of the peptides and blood collection:* Normal female Lewis rats, weighing 225-250g (Charles River Laboratories, Wilmington, MA) were anesthetized, with body temperature monitored and tail vein cannulated as described in the section 2.3.1. In addition, the left carotid artery was cannulated with PE-50 tubing. The animals were then randomly assigned to receive either 30nM apelin-36 (n=5) or 30nM PEG-apelin-36 (n=5) intravenously at a dose of 1ml/20min/250g for 20 minutes. Before, during (10 and 20 min) and 40 min post initiation of the IV infusion, 600 µl of whole blood was collected via the carotid artery cannula using an EDTA coated tube with aprotinin (0.6TIU/ml of blood; Phoenix Pharmaceutical Inc., Burlingame, CA) added. After the blood collection at the 40 minute time point, the PE-50 tube was withdrawn, the carotid artery ligated, and the incision closed. The animal was returned to its cage to recover from anesthesia. The 120 minute blood collection was performed by direct heart puncture after the animal was euthanized with 1ml of Euthasol (Virbac AH, Inc., Fort Worth, TX) IP and the chest opened. The blood samples were centrifuged at 1,600 x g for 15 minutes at 4°C. The plasma was then collected and kept in -80°C for future analysis.

[0082] *Plasma apelin 36 concentration:* Plasma apelin-36 concentration was analyzed using an apelin-36 enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) according to the manufacturer's protocol.

EXAMPLE 3

Results:

PEGylation of Apelin

[0083] *30kDa and 40kDa PEG-apelin-36 conjugates were successfully produced:* PEG-apelin-36 conjugates were generated by reacting the peptide with PEG aldehydes at acidic pH to favor N-terminal over lysine conjugation. For 30kDa and 40kDa PEG-apelin-36 conjugates, almost all the products were mono-PEGylated species. Significantly more di-

PEGylated products were detected in the 10kDa PEGylation reaction. The reaction mix was dialyzed to remove the trace amounts of unreacted peptides. Recovery of mono-PEGylated 30kDa and 40kDa conjugates were >75%. However, recovery of the mono-PEGylated 10kDa conjugate was much lower (<20%) presumably due to its smaller size. Unconjugated PEG
5 was removed by weak cation exchange chromatography with >80% recovery of the PEGylated peptide (Fig. 1).

[0084] *PEGylation of apelin 36 occurred at the N-terminal and the purity was > 98%:* N-terminal sequencing suggests that PEGylation of apelin-36 was highly selective for the N-terminus. When the same amounts of PEGylated and unmodified peptide were sequenced, 2
10 pmol of the first residue of apelin-36 was detected for the PEGylated peptide compared to 146 pmol of the unmodified peptide, indicating ~98% selectivity for the N-terminus (Fig. 2). To assess the level of unreacted peptide contaminant, the final material was run on a 12% Bis-Tris Gel with free peptide. With 8ug of conjugate loaded, no free peptide was observed, suggesting that the level of free peptide impurity was <2%.

15 [0085] *N-terminal PEGylation of apelin-36 had minimal impact on binding:* The 40kDa PEG conjugates retained high affinity binding with a K_i of 0.3 nM compared to a K_i of 0.05 nM for apelin-36 (Fig. 3). In contrast, di-PEGylated 10kDa PEG apelin-36 conjugate exhibited a K_i of ~1 nM. The 13 amino acid α -MSH peptide negative control showed no binding.

20 *In vitro* bioactivity (cAMP) assay

[0086] A clonal cell line (HEK293-APJ#3) expressing a high level of the APJ on the cell surface was identified by flow cytometric analysis. When challenged with apelin-36, this clone demonstrated suppression of forskolin-mediated cAMP production with an IC_{50} of 1nM. We next sought to determine whether addition of the PEG moiety to apelin-36 altered
25 its properties as a peptide agonist of APJ. As shown in Figure 4, the IC_{50} of the PEG-apelin-36 was modestly shifted to the right to 1.5nM, with no change in the maximal inhibition of forskolin-mediated cAMP accumulation versus apelin-36. These results indicate that the PEG moiety does not interfere with the agonist properties of apelin-36.

In vivo evaluation

[0087] *PEG-apelin 36 demonstrated prolonged inotropic effect in normal rats:* Rats received a 20 minute infusion of a low dose of apelin-36 or PEG-apelin-36, a high dose of apelin-36 or PEG-apelin-36, or saline and cardiac function was monitored by echocardiography at specified time points during the infusion and for 100 minutes after the end of infusion. PEG-apelin-36 demonstrated prolonged inotropic effects in normal rats. During infusion, apelin-36 and PEG-apelin-36 groups demonstrated similar significant increases in EF with peak EFs observed at the 20m time point. After the infusion ended the decline in EF was slower in the PEG-apelin-36 groups compared to the apelin-36 groups. By 30 minutes after the infusion ended (50 min time point), the EF in the apelin-36 low dose group (Ap36Lo) was not significantly different from the baseline (BL) or the normal saline (NS) group. In the PEG-apelin-36 low dose group (Peg36Lo) EFs were significantly increased compared to baseline and NS group values for the duration of the experiment (Fig. 5a). Similar changes in EF were observed in the high dose groups (Fig. 5b). Peak EFs for the high dose apelin-36 (Ap36Hi) or PEG-apelin-36 groups (PegAp36 HI) were observed at the end of IV infusion (20 min). In the PegAp36 HI group, EFs remain above BL and the NS group for the duration of the experiment. In the Ap36 HI group EF was not significantly different from baseline at 40 min after the end of IV infusion (60 min time point) and was not significantly different from the NS group at 100 min after the end of IV infusion (120 min time point)

[0088] *PEG-apelin 36 demonstrated prolonged and enhanced inotropic effect in MI rats:* As observed in normal rats, PEG-apelin-36 demonstrated prolonged inotropic effects in MI rats compared to apelin-36 (Fig. 6). In the PEG-apelin-36 group (Peg36MI) EF values were significantly increased compared to the baseline and the NS group (NSMI) for the duration of the experiment. However, in the apelin-36 group (Ap36MI) EF was not significantly different from BL at 60 min (40 min after IV infusion ended) and was not significantly different from the NS group at 120 min (100 min after the end of IV infusion).

[0089] There were no significant changes in mean aortic blood pressure (ABPmean) in normal rats following either PEG-apelin-36 or apelin-36 administration compared to baseline values, nor were there changes in ABPmean in MI rats following apelin-36 administration (Fig. 7).

Apelin blood levels

[0090] When animals received apelin-36, the plasma level of apelin-36 was significantly increased compared to baseline at the 20 min (end of IV infusion) and 40 min (20 min after the end of IV infusion) time points (2.5 and 2 fold, respectively). When animals received PEG-apelin-36, the plasma level of apelin-36 was significantly increased at 10 min (during IV infusion), 20 min (end of IV infusion), 40 min, and 120 min (end of monitoring) time points (2.5, 4, 2.7, and 2.7 fold, respectively) (Fig. 8). The kinetics of the changes in apelin-36 blood levels in animals that received either apelin-36 or PEG-apelin-36 were consistent with the observed changes in cardiac ejection fraction (EF) in these animals.

[0091] N-terminal PEGylation of apelin-36, had little effect on APJ binding where the mono 40kDa PEG conjugate had a K_i of 0.3 nM (vs K_i of 0.05 for apelin-36). The di-PEGylated 10kDa PEG apelin-36 conjugate had a K_i of ~1 nM. This 20-fold loss in binding affinity was likely due to PEGylation at one of the two internal lysines. Severe binding affinity loss observed with N-terminal PEGylation of apelin-12 and apelin-17) indicated that PEGylation at the second lysine in the sequence would probably result in a virtually inactive conjugate. The 30kDa and 40 KDa apelin-36 conjugates had similar K_i 's. As the 40kDa PEG apelin-36 was anticipated to have a longer circulating life due to its large molecular mass, this conjugate was selected for further characterization in vitro and in vivo.

[0092] The IC_{50} of the 40 KDa PEG-apelin-36 was shifted only 1.5-fold to the right, with no change in the maximal inhibition of forskolin-mediated cAMP accumulation versus apelin-36. These data indicated that attachment of an N-terminal PEG group to apelin-36 did not compromise its function as an APJ agonist, which was consistent with the receptor binding assay mentioned above and the fact that both apelin-36 and PEG-apelin-36 demonstrated similar magnitudes of EF elevation during the 20 minutes of peptide infusion in our in vivo evaluation.

[0093] The short circulating time of unmodified apelin peptides and transient inotropic effects were anticipated. Conjugating apelin-36 with 40Kda PEG, increased the duration of its inotropic effect compared to apelin-36, consistent with the anticipated increase in circulation half-life of PEG-apelin-36. PEG-apelin-36 maintained elevated inotropic effects for at least 100 minutes after the end of the IV infusion. In contrast, with the low dose of

unmodified apelin-36 EF returned to baseline and was not significantly different from the NS group at 30 min post IV infusion. In the high dose apelin-36 group EF returned to baseline at 40 min post IV infusion and was not significantly different from the NS group at 100 min after IV infusion.

5 [0094] PEG-apelin-36 IV infusion demonstrated an extended duration of increased EF in MI rats. Interestingly, the magnitude of PEGylated and unmodified apelin-36 mediated inotropic effects was much greater in MI rats than in the normal rats. Peak EF values (20 min time point for all of groups) in MI rats were 30% for the apelin-36 group (Ap36MI) and 40% for PEG-apelin-36 group (Peg36MI). EF values in normal rats that received the same dose of
10 apelin-36 (Ap36Lo) or PEG-apelin-36 (Peg36Lo) were 18% and 21%, respectively.

[0095] To elucidate the relationship between increased heart function and apelin-36 blood levels, an apelin-36 EIA was used to measure apelin-36 blood levels in animals that received either apelin-36 or PEG-apelin-36. It was noted that this assay underreported apelin-36 levels in animals that received PEG-apelin-36 possibly due to reduced binding affinity of the anti-
15 apelin antibody to the PEG-apelin-36. Due to this issue, apelin-36 levels in this study were reported as fold change compared to baseline. As anticipated, we found that the extended duration of inotropic effects with PEG-apelin-36 corresponded with an extended duration of apelin-36 blood levels above baseline.

[0096] In summary, N-terminal PEGylated apelin-36 was successfully produced with high
20 receptor binding affinity and agonist activity retained. The prolonged inotropic effects and extended circulation time of PEG-apelin-36 were demonstrated.

[0097] It will be appreciated by those skilled in the art that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments
25 disclosed, but it is intended to cover modifications that are within the spirit and scope of the invention, as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A pegylated Apelin molecule comprising one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of Apelin.

2. The molecule of claim 1, wherein said pegylated Apelin has a prolonged circulating life, relative to a non-pegylated Apelin.

3. The molecule of claim 1, wherein said pegylated Apelin has an extended inotropic effect, relative to a non-pegylated Apelin.

4. The molecule of claim 1, wherein said pegylated Apelin is a mono-pegylated Apelin.

5. The molecule of claim 1, wherein said pegylated Apelin is a di-pegylated Apelin.

6. The molecule of claim 1, wherein said one or more PEG molecules are covalently attached to said at least one amino acid residue.

7. The molecule of claim 1, wherein said one or more PEG molecules are covalently attached via linker to said at least one amino acid residue.

8. The molecule of claim 1, wherein each PEG molecule has a molecular weight ranging from about 5,000 to about 50,000 daltons.

9. The molecule of claim 1, wherein said pegylated Apelin comprises monomeric Apelin.

10. The molecule of claim 1, wherein said pegylated Apelin comprises an oligomeric Apelin, wherein one or more monomers are operably linked to each other.

11. The molecule of claim 1, wherein Apelin comprises the amino acid sequence 42-77 aa (Apelin 36) of SEQ ID NO: 1.

12. The molecule of claim 11, wherein said amino acid sequence is encoded by a nucleic acid sequence 123-234 bp of SEQ ID NO: 2.

13. A method for producing the molecule of claim 1, comprising the step of: reacting Apelin with an activated PEG-aldehyde linker in the presence of a reducing agent to form said pegylated Apelin under conditions in which the linker is covalently attached to Leucine residue in the N-terminal of Apelin.

14. The method of claim 13, wherein the reducing agent is cyanoborohydride and the reacting step is performed at a pH of 5.0 to 7.5.

15. A pharmaceutical composition comprising a therapeutically effective amount of a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of Apelin, wherein said at least one amino acid residue is Leucine.

16. A method for treating a disease or disorder associated with Apelin, in a subject, the method comprising: administering to said subject a therapeutically effective amount of the molecule of claim 1.

17. The method of claim 16, wherein said disease is a cardiovascular disease.

18. The method of claim 16, wherein said disease is an ischemia-reperfusion injury, myocardial infarction, acute decompensated heart failure, chronic heart failure, cardiomyopathy, endocrine/metabolic disorder or pulmonary hypertension.

19. A method for treating a disease or disorder associated with Apelin, in a subject, the method comprising: administering to said subject a therapeutically effective amount of a

pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of Apelin, wherein said at least one amino acid residue is Leucine.

5 20. The method of claim 19, wherein said pegylated Apelin has a prolonged circulating life, relative to a non-pegylated Apelin.

21. The method of claim 19, wherein said pegylated Apelin has an extended inotropic effect, relative to a non-pegylated Apelin.

10

22. The method of claim 19, wherein said pegylated Apelin is a mono-pegylated Apelin.

23. The method of claim 19, wherein said pegylated Apelin is a di-pegylated Apelin.

15 24. The method of claim 19, wherein said one or more PEG molecules are covalently attached to said at least one amino acid residue.

25. The method of claim 19, wherein said one or more PEG molecules are covalently attached via linker to said at least one amino acid residue.

20

26. The method of claim 19, wherein each PEG molecule has a molecular weight ranging from about 5,000 to about 50,000 daltons.

27. The method of claim 19, wherein said pegylated Apelin comprises monomeric Apelin.

25

28. The method of claim 19, wherein said pegylated Apelin comprises an oligomeric Apelin, wherein one or more monomers are operably linked to each other.

29. The method of claim 19, wherein Apelin comprises the amino acid sequence 42-77 aa
30 (Apelin 36) of SEQ ID NO: 1.

30. The method of claim 29, wherein said amino acid sequence is encoded by a nucleic acid sequence 123-234 bp of SEQ ID NO: 2.

31. The method of claim 30, wherein said disease is a cardiovascular disease.

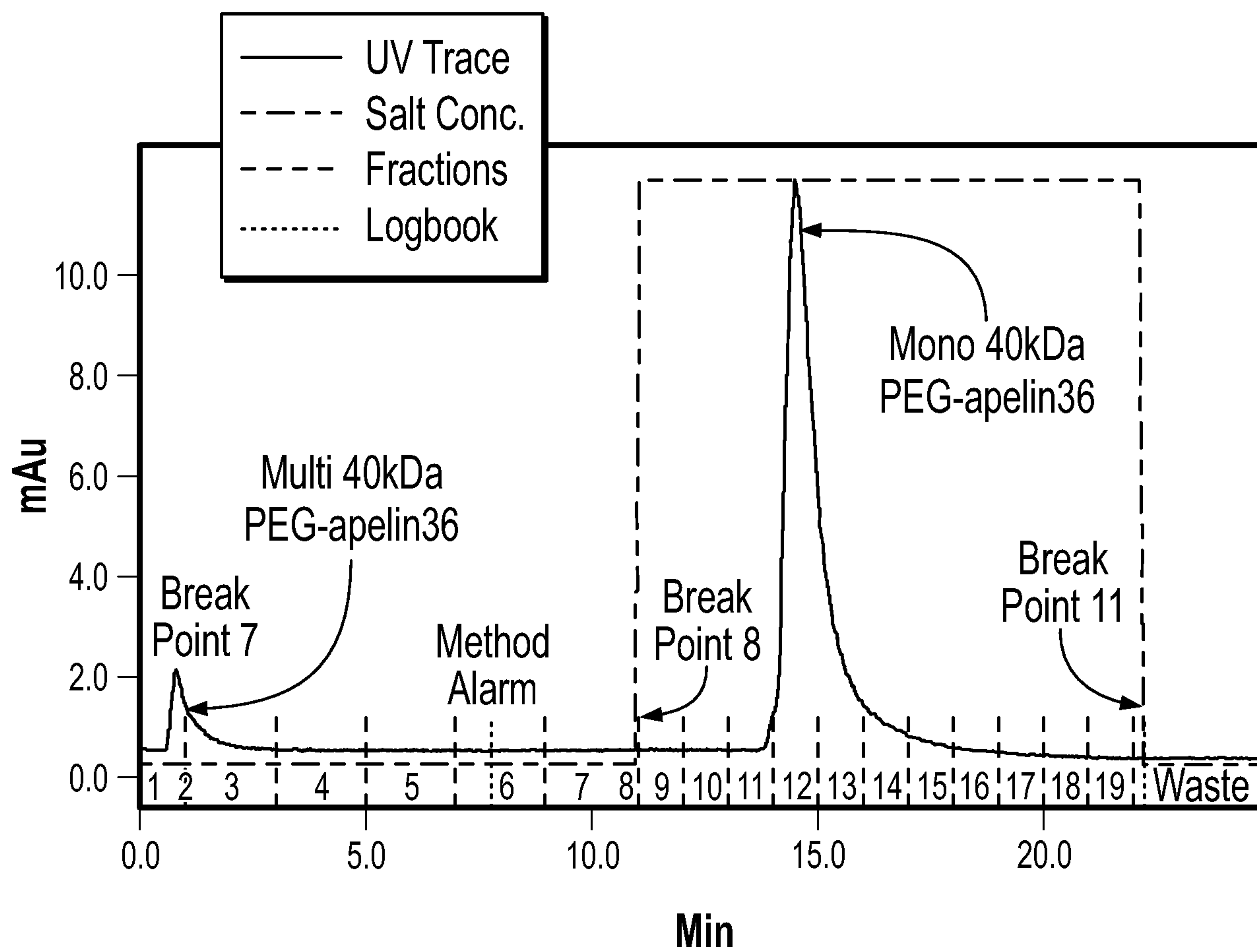
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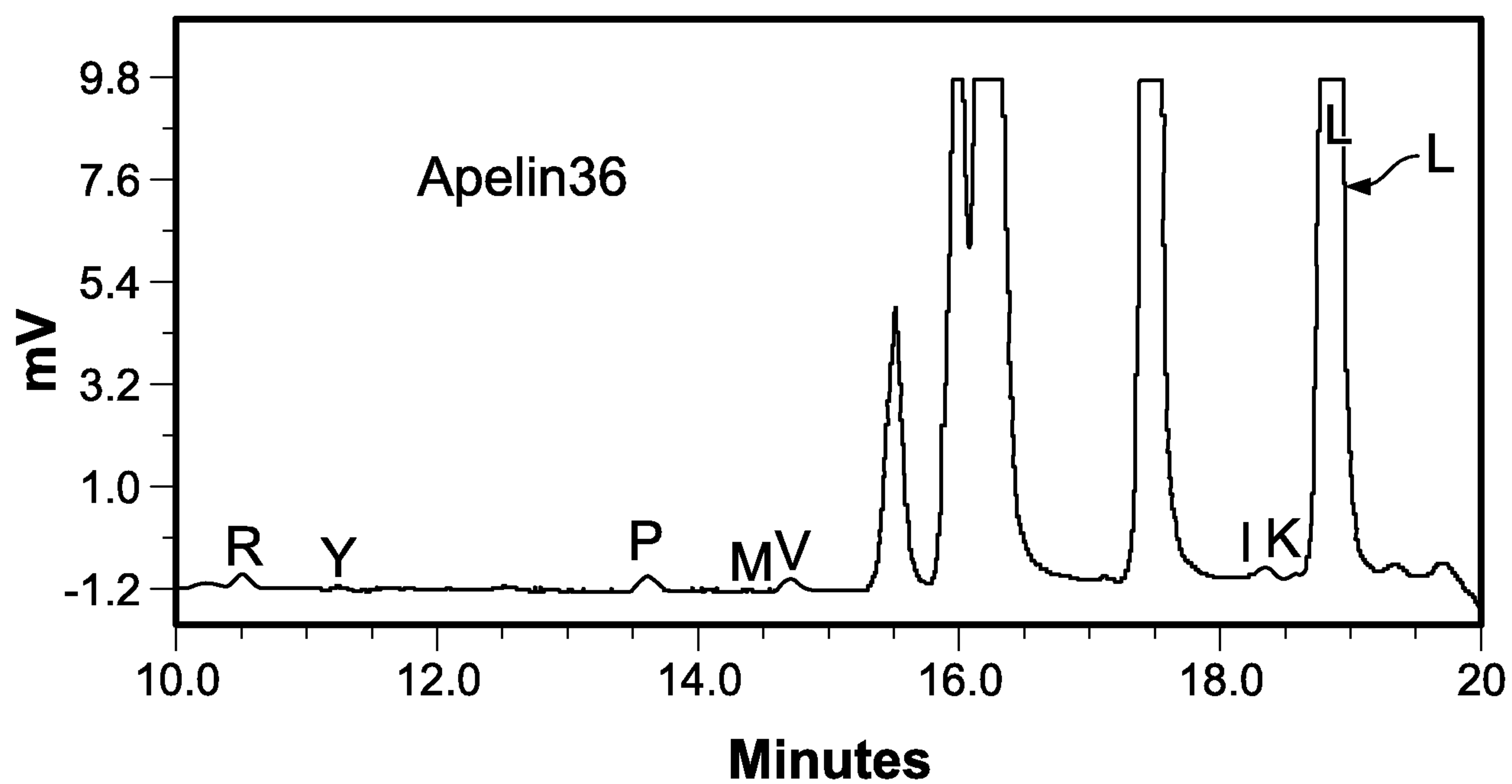
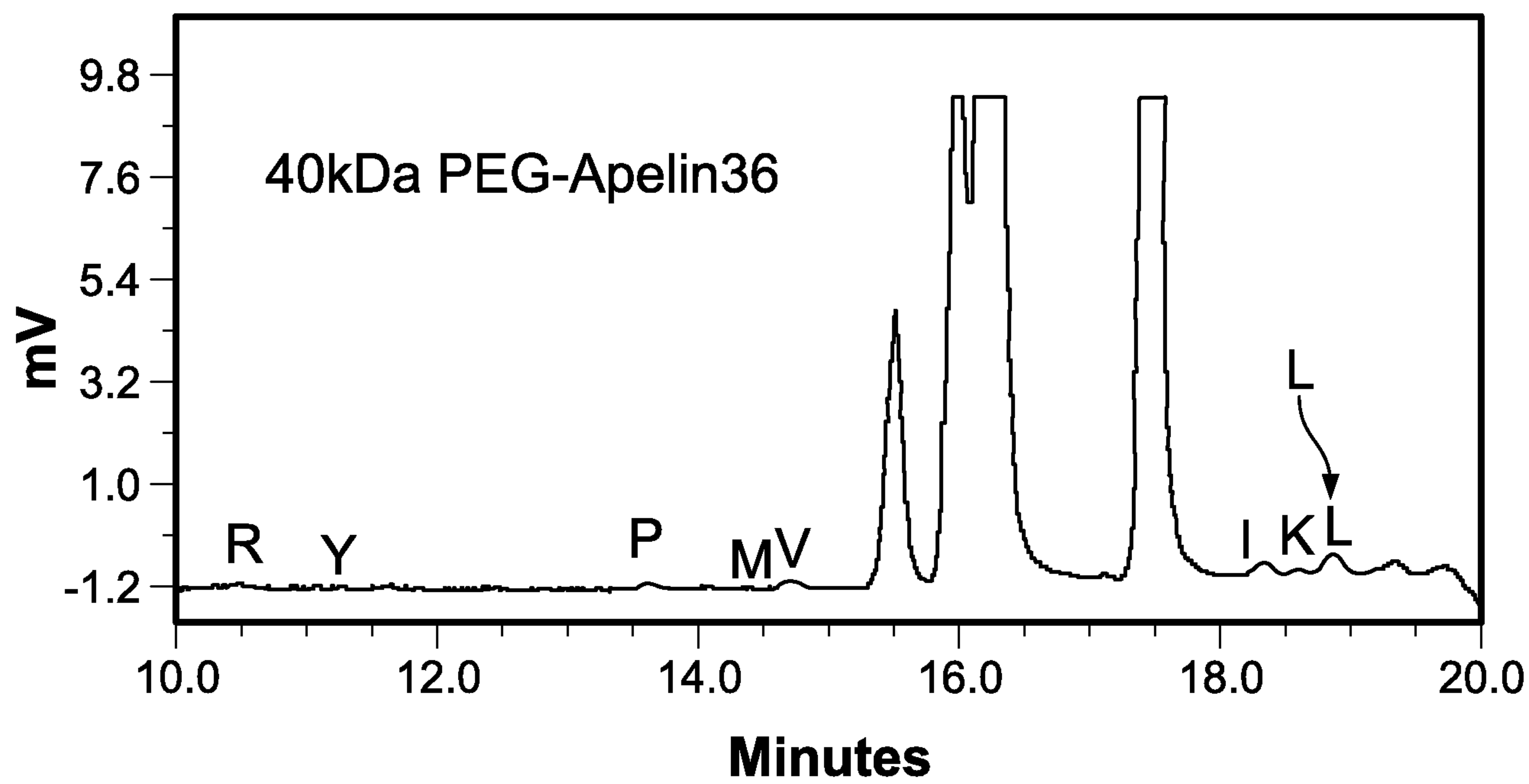
32. The method of claim 30, wherein said disease is an ischemia-reperfusion injury, myocardial infarction, acute decompensated heart failure, chronic heart failure, cardiomyopathy, endocrine/metabolic disorder or pulmonary hypertension.

10 33. A method for enhancing circulating life of Apelin to treat a disease or disorder associated with Apelin, in a subject, the method comprising: administering to said subject a therapeutically effective amount of a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of Apelin, wherein said at least one amino acid residue is Leucine.

15

34. A method for improving inotropic effect to treat a disease or disorder associated with Apelin, in a subject, the method comprising: administering to said subject a therapeutically effective amount of a pegylated Apelin that comprises one or more polyethylene glycol (PEG) molecules operably linked to at least one amino acid residue in the N-terminal of Apelin,
20 wherein said at least one amino acid residue is Leucine.

**FIGURE 1**

**FIGURE 2A****FIGURE 2B**

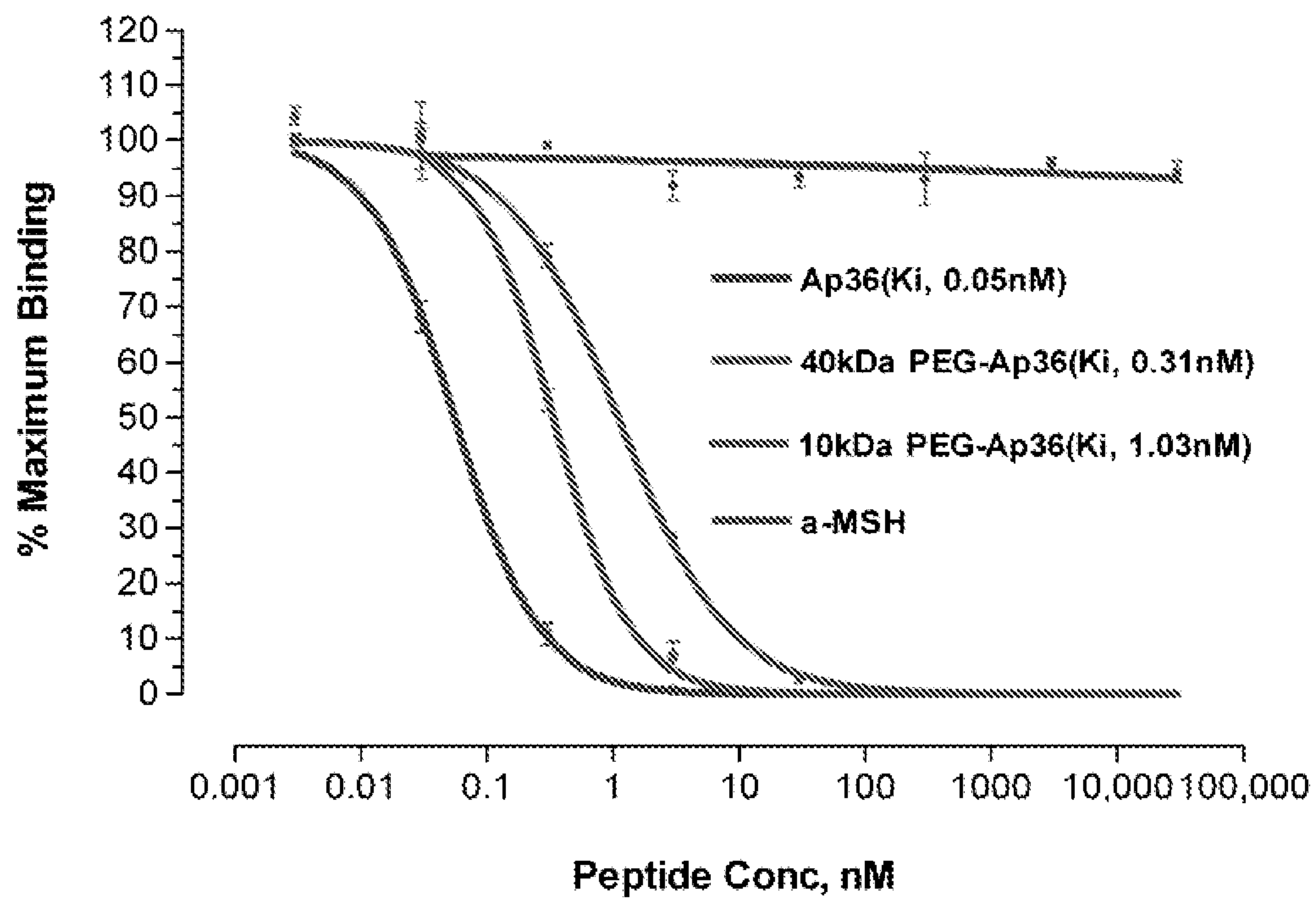


FIGURE 3

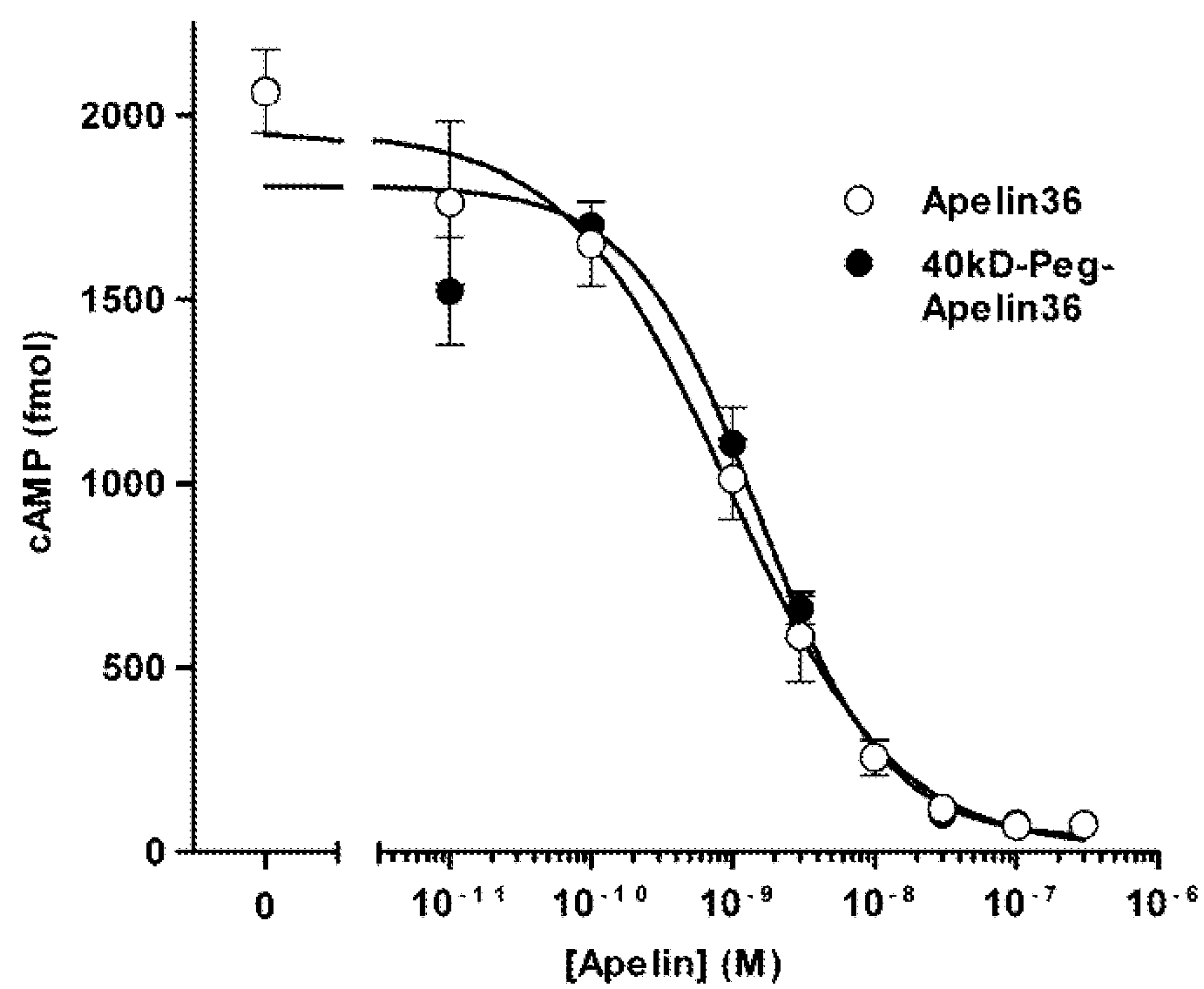


FIGURE 4

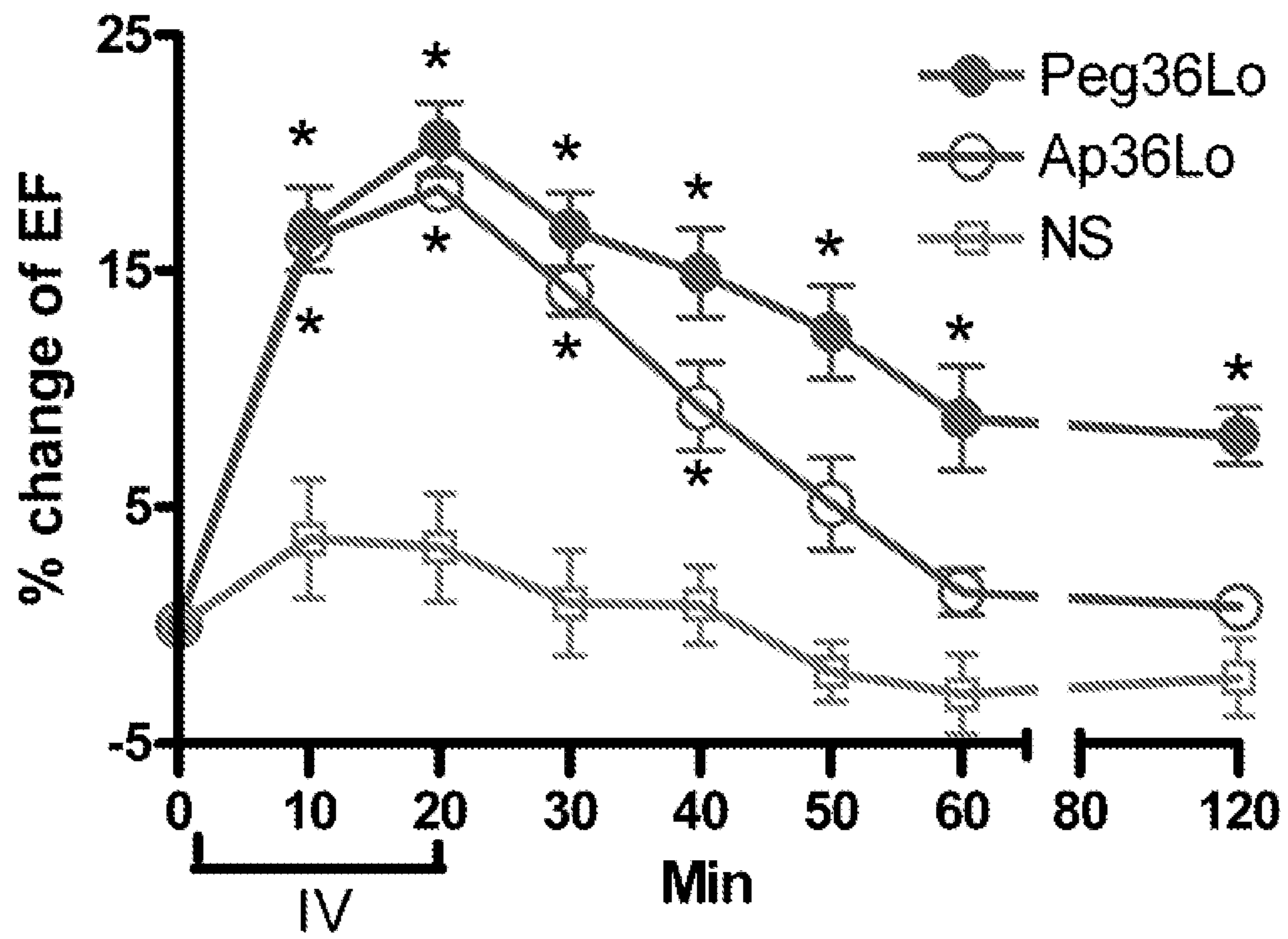


FIGURE 5A

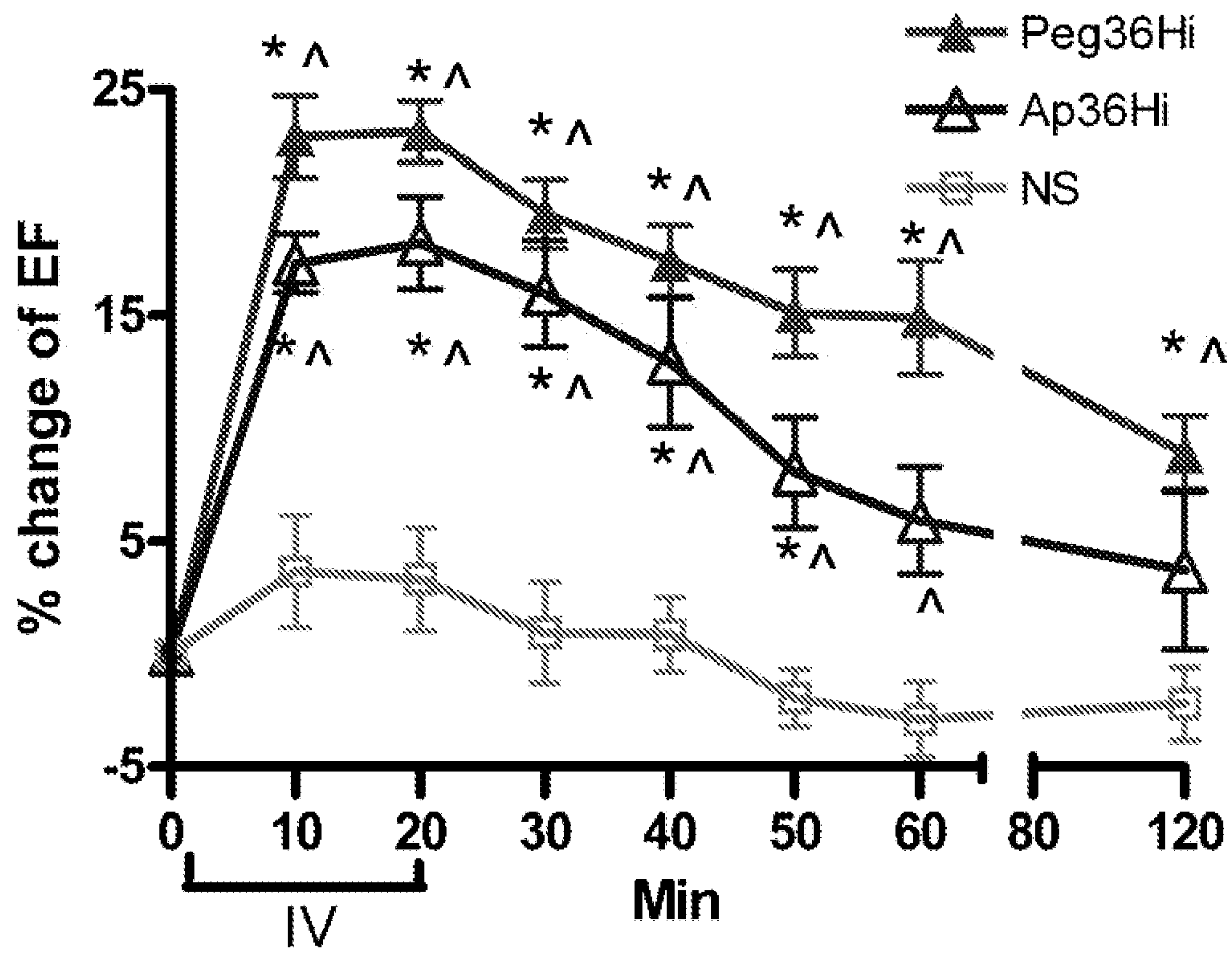


FIGURE 5B

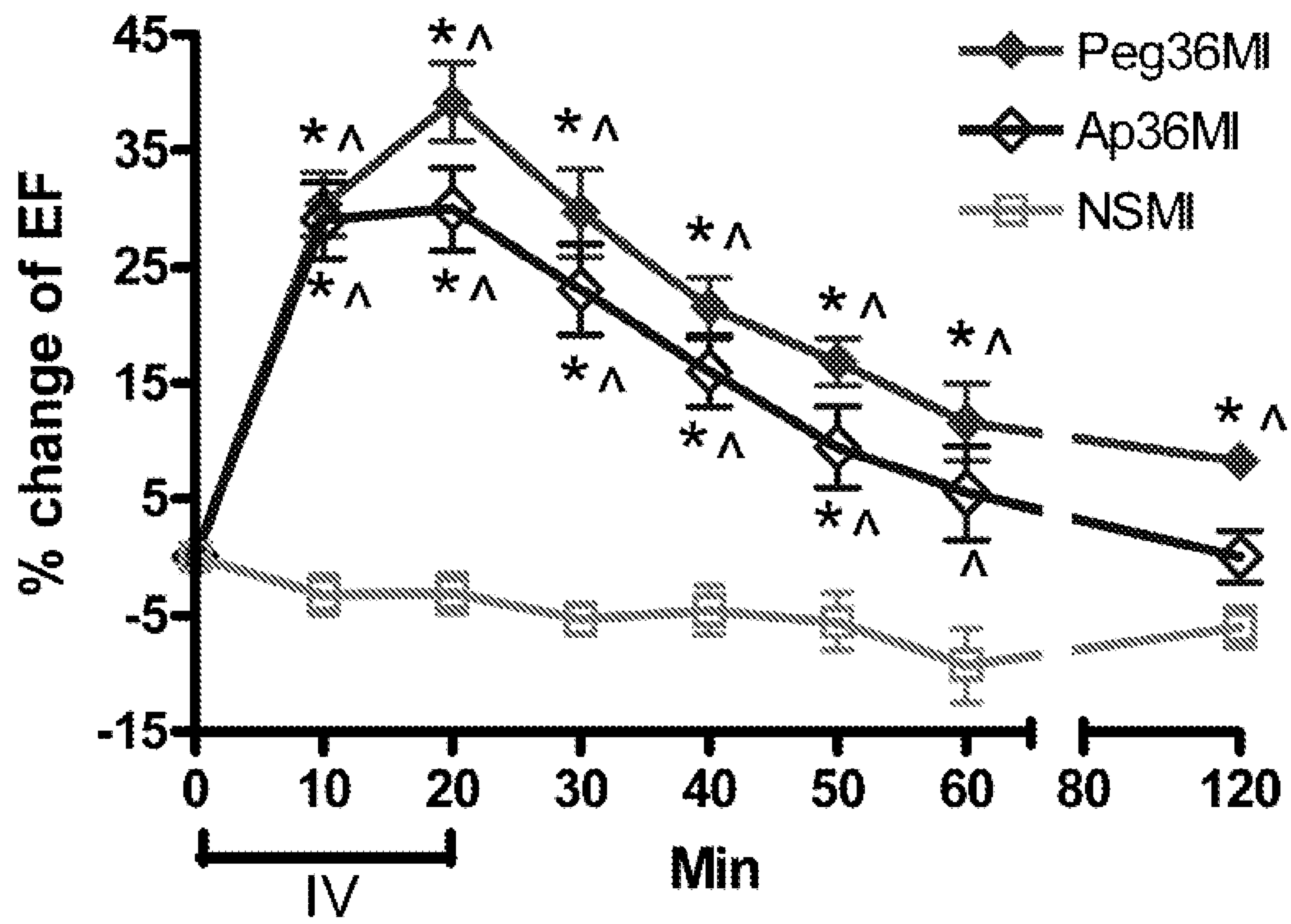


FIGURE 6

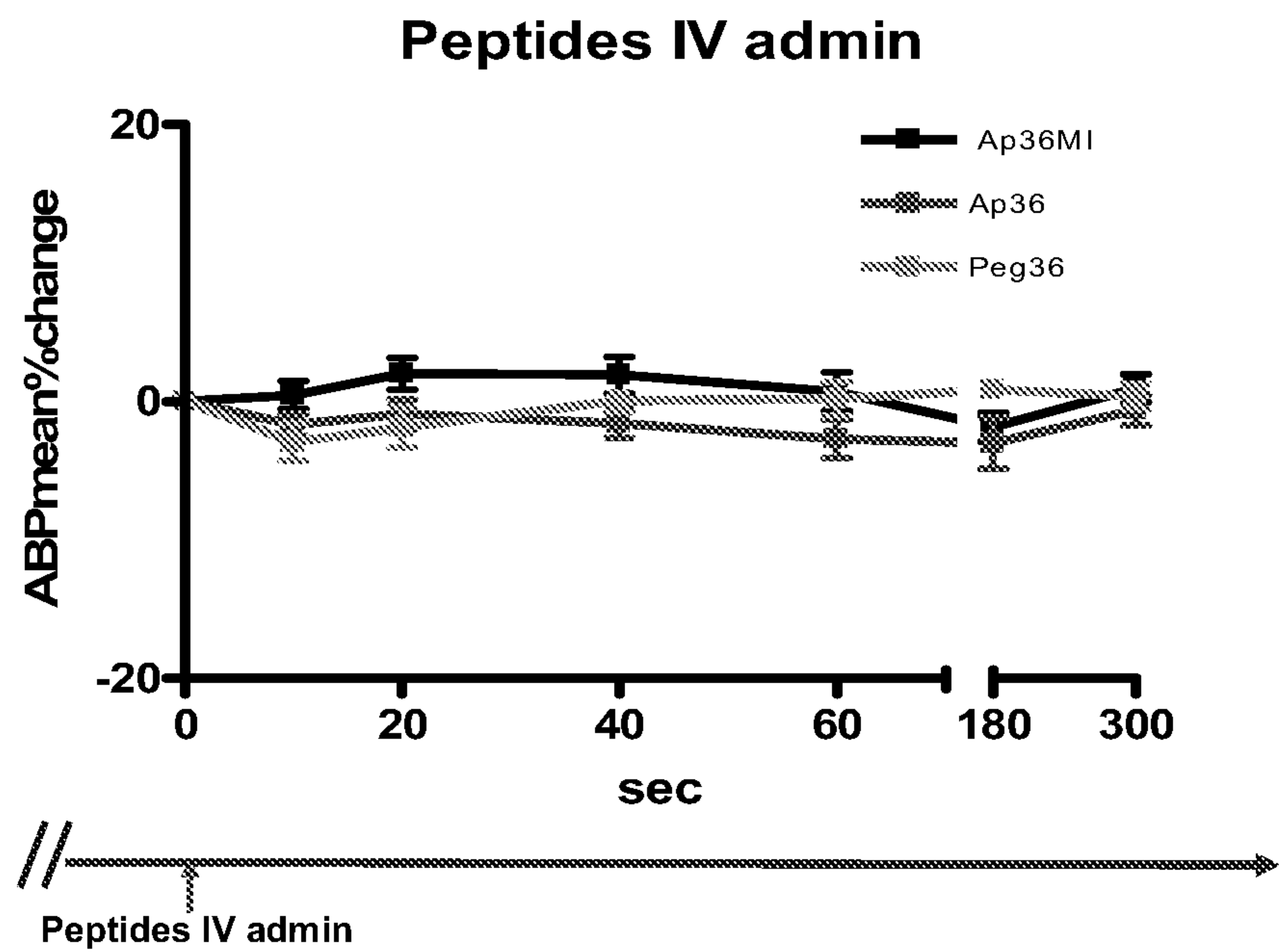


FIGURE 7

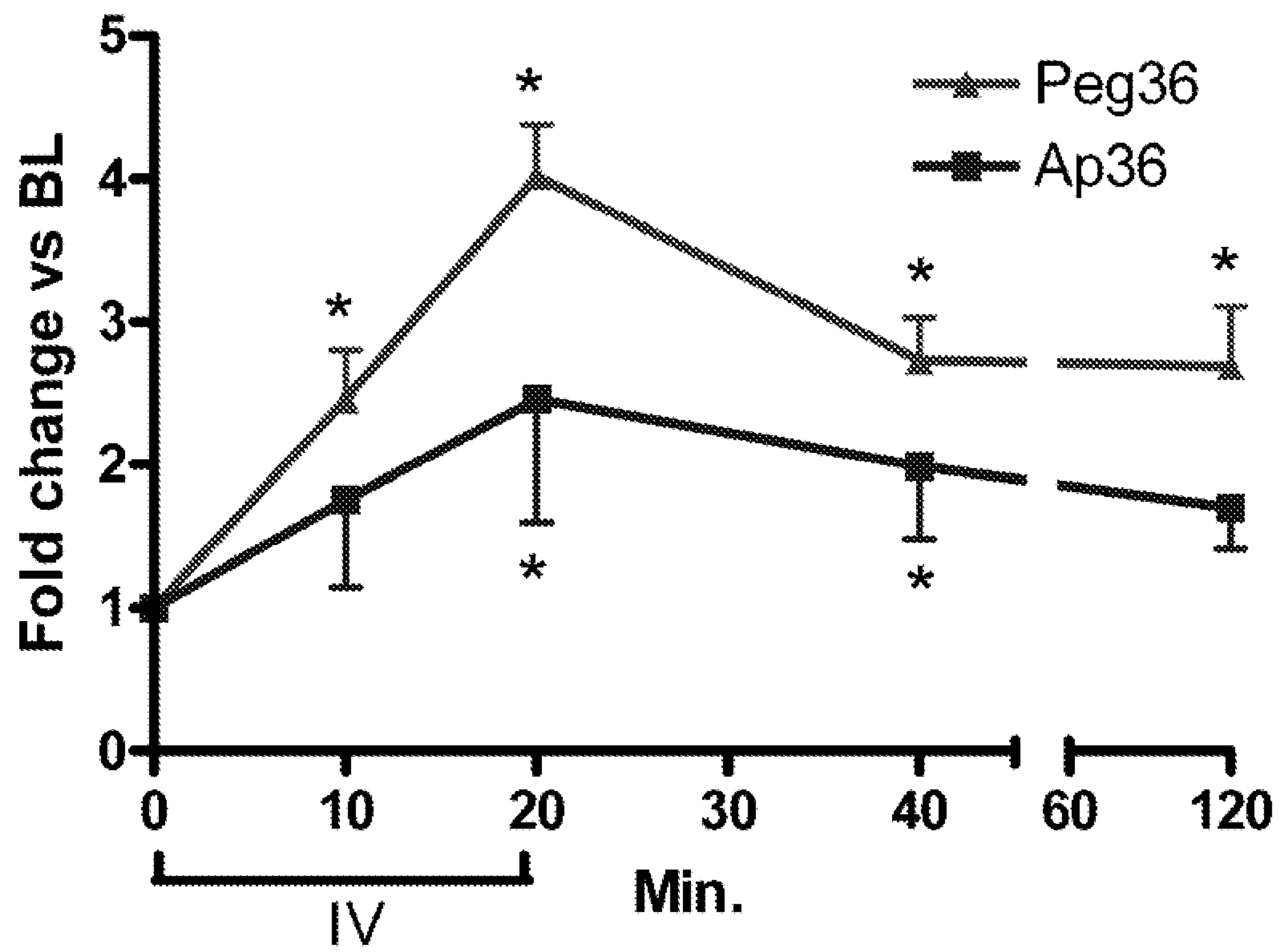


FIGURE 8