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(54) Title: METHODS FOR TREATING AIRWAY SMOOTH MUSCLE DISORDERS IN SUBJECTS DESENSITIZED TO β -ADRENERGIC RECEPTOR AGONIST THERAPY

(57) Abstract: The present invention provides methods for treating airway smooth muscle disorders and/or bronchospasm using HSP20 polypeptides.



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**Methods for treating airway smooth muscle disorders in subjects desensitized to
 β -adrenergic receptor agonist therapy**

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Cross Reference

This application claims priority to U.S. Provisional Patent Application Serial No. 60/897,104 filed January 24, 2007, incorporated by reference herein in its entirety.

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Statement of Government Interest

This work was supported by NIH RO1HL58027 and NIH RO1HL58506, and thus the U.S. government has certain rights in the invention.

Field of the Invention

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This invention relates generally to the fields of cell biology, molecular biology, pharmaceuticals, and smooth muscle biology.

Background of the Invention

β -adrenergic receptor agonists (β -agonists) are the most commonly used therapy
20 for relief of acute bronchospasm in asthmatics. β -agonists stimulate the heterotrimeric G protein Gs, which in turn stimulates adenylyl cyclase, catalysing the hydrolysis of ATP into cyclic AMP. However, β -agonists therapy can cause alterations in β_2 -adrenergic receptor responsiveness in a time-dependent and cell-specific manner. Agonist-specific desensitization may also be responsible for the loss of prophylactic bronchoprotection and
25 deterioration of asthma control clinically observed with regular use of β -agonists. Airway inflammation or cytokine treatment contributes to β_2 -adrenergic receptor dysfunction and a loss of the relaxant effect of β agonists in ASM cells, tissues, and *in vivo* models. Adverse effects have been seen with inhaled β_2 -adrenergic agonist in patients with a genetic polymorphism that results in homozygosity for arginine, rather than glycine at
30 amino acid residue 16 of the β_2 -adrenergic receptor.

There is thus a need in the art for methods to effectively treat asthma in subjects that have become desensitized to beta agonist therapy.

A recently identified substrate protein of PKA is the small heat shock-related protein (HSP20) (Beall et al., *J Biol Chem* 274: 11344-11351, 1999); Rembold et al., *J Physiol* 524 Pt 3: 865-878, 2000). PKA leads to increases in the phosphorylation of HSP20 on serine 16 (Beall et al., 1999). Agonists that stimulate HSP20 phosphorylation, as well as analogues of phosphorylated HSP20 which contain a transduction domain (allowing for cellular penetration without altering membrane permeability), induce relaxation of smooth muscle from a variety of different species and different types of smooth muscles (Beall et al. 1999; Rembold et al., 2000; Woodrum et al. *J Vasc Surg* 37: 874-881, 2003; Tessier et al.. *J Vasc Surg* 40: 106-114, 2004; Flynn et al., *Journal of Applied Physiology* 98: 1836-1845, 2005; Flynn et al., *Faseb J* 17: 1358-1360, 2003.

While HSP20 phosphopeptides have been shown to induce smooth muscle cell relaxation, it has not been determined whether they can be used to effectively treat asthma and other airway smooth muscle disorders in subjects that have become desensitized to β -agonist treatment.

Summary of the Invention

In one aspect, the present invention provides methods for treating an airway smooth muscle disorder, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat an airway smooth muscle disorder of a polypeptide comprising an amino acid sequence according to general formula I

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 2)

wherein X2 is absent or comprises a transduction domain;

X3 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (SEQ ID NO:1);

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine,

hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

Z2 is selected from the group consisting of L and K; and

Z3 is selected from the group consisting of S, T, and K; and

X6 is absent or comprises a transduction domain.

In another aspect, the present invention comprises methods for treating bronchospasm, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat bronchospasm of a polypeptide comprising an amino

acid sequence according to general formula I

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 2)

wherein X2 is absent or comprises a transduction domain;

X3 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO:1**);

5 X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

Z2 is selected from the group consisting of L and K; and

10 Z3 is selected from the group consisting of S, T, and K; and

X6 is absent or comprises a transduction domain.

In another aspect, the present invention provides methods for treating an airway smooth muscle disorder, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat an airway smooth muscle disorder of a polypeptide comprising an amino acid sequence according to general formula II:

15

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 33)

wherein X2 is absent or comprises a cell transduction domain;

X3 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of **SEQ ID NO: 34**;

20 X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of **SEQ ID NO: 34**; and

X6 is absent or comprises a cell transduction domain.

25 In another aspect, the present invention provides methods for treating bronchospasm, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat bronchospasm of a polypeptide comprising an amino acid sequence according to general formula II:

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 33)

30 wherein X2 is absent or comprises a cell transduction domain;

X3 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of **SEQ ID NO: 34**;

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of SEQ ID NO: 34; and

X6 is absent or comprises a cell transduction domain.

5 **Brief Description of the Figures**

Figure 1A-D Western blot (A) and data graphs (B-D) showing that inhibition of PKA inhibits isoproterenol or forskolin induced phosphorylation of VASP and HSP-20, and dephosphorylation of cofilin in human airway smooth muscle cells.

10 **Figure 2A-F** Representative epifluorescence micrographs showing that inhibition of PKA inhibits ISO or forskolin-mediated stellation and stress fiber disruption in HASM cells.

Figure 3A-I Data graphs (A and I), representative epifluorescence micrographs (B-G), and Western blot (H) showing that phospho-HSP20 peptide causes stress fiber disruption and focal adhesion changes in GFP as well as PKI-GFP expressing HASM cells.

15 **Figure 4A-C** Data graphs showing that HSP20 phosphopeptide treatment leads to relaxation of carbachol contracted bovine airway smooth muscle.

Figure 5A-B Western blot (A) and data graph (B) showing that isoproterenol and forskolin reduce the levels of phospho-cofilin in bovine airway smooth muscle.

20 **Detailed Description of the Invention**

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), “Guide to Protein Purification” in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.)

30 In one aspect, the present invention provides methods for treating airway smooth muscle disorders, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat airway smooth muscle disorders of a

polypeptide comprising or consisting of an amino acid sequence according to general formula I

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 2)

wherein X2 is absent or comprises a transduction domain;

5 X3 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO:1**);

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

10 Z2 is selected from the group consisting of L and K; and

Z3 is selected from the group consisting of S, T, and K; and

X6 is absent or comprises a transduction domain.

In another aspect, the present invention provides methods for treating
15 bronchospasm, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat bronchospasm of a polypeptide comprising or consisting of an amino acid sequence according to general formula I

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 2)

wherein X2 is absent or comprises a transduction domain;

20 X3 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO: 1**);

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

25 Z2 is selected from the group consisting of L and K; and

Z3 is selected from the group consisting of S, T, and K; and

X6 is absent or comprises a transduction domain.

The methods of the invention provide airway smooth muscle disorder and
30 bronchospasm therapy capable of overcoming problems associated with β_2 -adrenergic receptor desensitization. Patients in prolonged beta-agonist therapy have a tendency to develop a tolerance to the bronchodilating effect of beta-agonists, which often causes them to increase the dosage and/or frequency of their medication. These patients thus can

become desensitized to beta-agonists, where a larger dose of the agonist is required to get an equivalent physiological response. Desensitization is one of the most clinically significant problems involving the beta-adrenergic receptor since an increase in dosage often leads to an increase in the kind, number, or severity of adverse effects (see e.g.

5 Lancet (1990) 336:1411-1412; Spitzer et al., N Engl J Med. (1992) 326(8):501-6).

As used herein, "desensitized" means that the β -agonist treatment does not (a) limit bronchospasm or one or more other symptoms in the subject, (b) worsens bronchospasm or one or more other symptoms in the subject, and/or (c) requires unacceptably high doses of β -agonist that lead to undesirable side effects, regardless of
10 the cause of such desensitization. In different embodiments, desensitization is caused or exacerbated by one or more of β -agonist treatment, inflammation, cytokine treatment, structural remodeling of airways, and genetic predisposition to desensitization (including but not limited to mutations in the β_2 -adrenergic receptor).

In a further embodiment, the subject has a genetic polymorphism that results in
15 homozygosity for arginine, rather than glycine at amino acid residue 16 of the β_2 -adrenergic receptor.

As used herein, "airway smooth muscle disorders" are those disorders involving abnormal spasm ("bronchospasm") of airway smooth muscle. Bronchospasm is caused by a sudden constriction of the smooth muscle in the walls of the bronchioles. Airway
20 smooth muscle disorders include, but are not limited to, asthma, chronic obstructive pulmonary disease (COPD) (including but not limited to emphysema and bronchitis) and anaphylaxis.

Asthma is a chronic disease of the respiratory system in which the airway periodically constricts, becomes inflamed, and is lined with excessive amounts of mucus.
25 Airway constriction is caused by bronchospasm, a constriction of bronchiole smooth muscle. Thus, symptoms of asthma include, but are not limited to bronchospasm, wheezing, shortness of breath, chest tightness, rapid breathing, prolonged expiration, rapid heart rate, cyanosis, excessive mucus buildup, and coughing.

COPD is a term for a group of respiratory tract disorders (including chronic
30 bronchitis and emphysema) characterized by chronic inflammation of the bronchi, bronchospasm, and airway obstruction. Approximately 15% of all chronic smokers develop COPD. Other risk factors include alpha 1-antitrypsin deficiency, byssinosis (exposure to cotton dust in inadequately ventilated working environments; commonly

occurs in workers who are employed in yarn and fabric manufacture industries), prolonged exposure to dusty environments (including but not limited to coal dust), air pollutant exposure (for example, those living in cities with excessive air pollution) and idiopathic disease. Symptoms of COPD include, but are not limited to bronchospasm, shortness of breath on exertion (or, at later stages, with minimal or no exertion), recurrent respiratory infections, coughing, wheezing, excessive sputum, dyspnea, and respiratory failure.

Emphysema is characterized by loss of elasticity of the lung tissue, destruction of structures supporting the alveoli, bronchospasm, and destruction of capillaries feeding the alveoli. Symptoms include, but are not limited to, bronchospasm, shortness of breath on exertion (typically when climbing stairs or inclines; at rest in late stages of the disease at rest), hyperventilation, an expanded chest, clubbing of the fingers (caused by hypoxia). Those at most risk for emphysema are the same as listed above for COPD.

Bronchitis is an obstructive pulmonary disorder characterized by inflammation of the bronchi of the lungs and bronchospasm. Chronic bronchitis is predominantly caused by smoking, and has also been linked to pneumoconiosis, excessive alcohol consumption and exposure to cold and draught, as well as other causes listed above for COPD. Symptoms of bronchitis include, but are not limited to, bronchospasm, an expectorating cough, shortness of breath, fatigue, mild fever, and mild chest pains.

Anaphylaxis is a severe allergic reaction that occurs when a person is exposed to an allergen to which they have already become sensitized. Minute amounts of allergens may cause a life-threatening anaphylactic reaction. Anaphylaxis may occur after ingestion, inhalation, skin contact or injection of an allergen. The most severe type of anaphylaxis—anaphylactic shock—will usually lead to death in minutes if left untreated. Symptoms of anaphylaxis include, but are not limited to, bronchospasm, hypotension, fainting, unconsciousness, hives, flushed appearance, angioedema (swelling of the face, neck and throat), vomiting, itching, and anxiety.

As used herein, “beta agonist therapy” means treatment with one or more beta₂ adrenergic receptor agonists including, but not limited to albuterol, levalbuterol, terbutaline, pirbuterol, procaterol, fenoterol, bitolterol mesylate, formoterol, isoproterenol, metaproterenol, bambuterol, and salmeterol.

The subject can be any mammal and is preferably human.

As used herein, “treating” the airway smooth muscle disorder and/or bronchospasm means accomplishing one or more of the following: (a) reducing severity

of the symptoms of the disorder; for example, in a subject suffering from asthma, reducing severity of symptoms including, but not limited to, bronchospasm, wheezing, shortness of breath, chest tightness, excessive mucus buildup, and coughing; (b) reducing development of symptoms; (c) reducing worsening of symptoms; and (d) reducing
5 recurrence of symptoms. Such “reducing” can be any amount of reduction that provides a therapeutic benefit compared to symptom severity, development, and recurrence in the absence of the treatment methods of the invention.

According to various embodiments of the polypeptides of general formula I, X4 is S, T, Y, D E, a phosphoserine mimic, or a phosphotyrosine mimic. It is more preferred
10 that X4 is S, T, or Y; more preferred that X4 is S or T, and most preferred that X4 is S. In these embodiments where X4 is S, T, or Y, it is most preferred that X4 is phosphorylated. When X4 is D or E, these residues have a negative charge that mimics the phosphorylated state. The polypeptides of the invention are optimally effective in the methods of the invention when X4 is phosphorylated, is a phosphoserine or phosphotyrosine mimic, or is
15 another mimic of a phosphorylated amino acid residue, such as a D or E residue. Examples of phosphoserine mimics include, but are not limited to, sulfoserine, amino acid mimics containing a methylene substitution for the phosphate oxygen, 4-phosphono(difluoromethyl)phenylalanine, and L-2-amino-4-(phosphono)-4,4-difluorobutanoic acid. Other phosphoserine mimics can be made by those of skill in the
20 art; for example, see Otaka et al., Tetrahedron Letters 36:927-930 (1995). Examples of phosphotyrosine mimics include, but are not limited to, phosphonomethylphenylalanine, difluorophosphonomethylphenylalanine, fluoro-O-malonyltyrosine and O-malonyltyrosine.

According to various embodiments of the polypeptides of general formula I, X3 is
25 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO:1**). If X3 consists of only one amino acid of the sequence, an “R” is present, since it is the carboxy-terminal amino acid of the sequence and it would be present at the amino terminus of the rest of the A(X4)APLP (**SEQ ID NO: 3**) sequence. If X3 consists of two amino acids of WLRR (**SEQ ID NO:1**), then the two amino acids added will be “RR”. Other variations will be
30 apparent to one of skill in the art based on the teachings herein.

Similarly, variations in the residues that can make up X5 will be apparent to one of skill in the art based on the teachings herein.

In a preferred embodiment, at least one of X2 and X6 comprises a transduction domain. As used herein, the term “transduction domain” means one or more amino acid

sequence or any other molecule that can carry the active domain across cell membranes. These domains can be linked to other polypeptides to direct movement of the linked polypeptide across cell membranes. In some cases the transducing molecules do not need to be covalently linked to the active polypeptide. In a preferred embodiment, the

5 transduction domain is linked to the rest of the polypeptide via peptide bonding. In this embodiment, any of the polypeptides as described above or below would include at least one transduction domain. In a further embodiment, both X2 and X6 comprise transduction domains. In a further preferred embodiment, the transduction domain(s) is/are selected from the group consisting of (R)₄₋₉ (SEQ ID NO: 4); GRKKRRQRRRPPQ

10 (SEQ ID NO: 5); AYARAAARQARA (SEQ ID NO: 6); DAATATRGRSAASRPTERPRAPARSASRPRRPVE (SEQ ID NO: 7); GWTLNSAGYLLGLINLKALAALAKKIL (SEQ ID NO: 8); PLSSIFSRIGDP (SEQ ID NO: 9); AAVALLPAVLLALLAP (SEQ ID NO: 10); AAVLLPVLLAAP (SEQ ID NO: 11); VTVLALGALAGVGVG (SEQ ID NO: 12); GALFLGWLGAAGSTMGAWSQP

15 (SEQ ID NO: 13); GWTLNSAGYLLGLINLKALAALAKKIL (SEQ ID NO: 14); KLALKLALKALKAAKLA (SEQ ID NO: 15); KETWWETWWTEWSQPKKKRKV (SEQ ID NO: 16); KAFAKLAARLYRKAGC (SEQ ID NO: 17); KAFAKLAARLYRAAGC (SEQ ID NO: 18); AAFAKLAARLYRKAGC (SEQ ID NO: 19); KAFAALAARLYRKAGC (SEQ ID NO: 20); KAFAKLAAQLYRKAGC

20 (SEQ ID NO: 21), AGGGGYGRKKRRQRRR (SEQ ID NO: 22), and YARAAARQARA (SEQ ID NO: 23), YGRKKRRQRRR (SEQ ID NO: 24), WLRRIKA (SEQ ID NO: 25), WLRIKAWLRRIKA (SEQ ID NO: 26); and WLRIKAWLRIKAWLRRIKA (SEQ ID NO: 27).

In a preferred embodiment of general formula I, the polypeptide comprises or

25 consists of X2-WLRRRA(pS)APLPGLK (SEQ ID NO: 28, SEQ ID NO: 29), wherein X2 is selected from the group consisting of YARAAARQARA (SEQ ID NO: 23) and YGRKKRRQRRR (SEQ ID NO: 24); and “pS” represents a phosphorylated serine. Thus, in one embodiment, the polypeptide comprises or consists of YARAAARQARAWLRRRA(pS)APLPGLK (SEQ ID NO: 28); in another embodiment,

30 the polypeptide comprises or consists of YGRKKRRQRRRWLRRRA(pS)APLPGLK (SEQ ID NO: 29).

In further embodiments, the polypeptide for use in the present invention comprises or consists of WLRIKAWLRRRA(pS)APLPGLK (SEQ ID NO: 30),

WLRRIKAWLRRIKAWLRRRA(pS)APLPGLK (SEQ ID NO: 31) or

WLRRIKAWLRRIKAWLRRIKAWLRRRA(pS)APLPGLK (SEQ ID NO: 32).

In another embodiment, the polypeptides for use in the methods of the invention comprise or consist of a sequence according to general formula II:

5 X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 33)

wherein X2 is absent or comprises a cell transduction domain;

X3 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of SEQ ID NO: 34;

10 X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of SEQ ID NO: 34; and

X6 is absent or comprises a cell transduction domain. Preferred embodiments of such transduction domains are as described above.

15 In a preferred embodiment, at least one of X2 and X6 comprise a transduction domain.

Thus, in various preferred embodiments of the polypeptide of general formula II, X4 is S, T, Y, D, E, a phosphoserine analog, or a phosphotyrosine analog. In a preferred embodiment, X4 is S, T, or Y. In a more preferred embodiment, X4 is S or T. In a most preferred embodiment, X4 is S.

20 In these embodiments where X4 is S, T, or Y, it is most preferred that X4 is phosphorylated. When X4 is D or E, these residues have a negative charge that mimics the phosphorylated state. The polypeptides of the invention are optimally effective in the methods of the invention when X4 is phosphorylated, is a phosphoserine or phosphotyrosine mimic, or is another mimic of a phosphorylated amino acid residue, such as a D or E residue.

One embodiment of the polypeptides for use in the methods of the invention comprises or consists of full length HSP20 (X2-SEQ ID NO: 34-X6) (SEQ ID NO: 35).

30 Met Glu Ile Pro Val Pro Val Gln Pro Ser Trp Leu Arg Arg Ala Ser Ala Pro Leu Pro Gly Leu Ser Ala Pro Gly Arg Leu Phe Asp Gln Arg Phe Gly Glu Gly Leu Leu Glu Ala Glu Leu Ala Ala Leu Cys Pro Thr Thr Leu Ala Pro Tyr Tyr Leu Arg Ala Pro Ser Val Ala Leu Pro Val Ala Gln Val Pro Thr Asp Pro Gly His Phe Ser Val Leu Leu Asp Val Lys His Phe Ser Pro Glu Glu Ile Ala Val Lys Val Val Gly Glu His Val Glu Val His Ala Arg His Glu Glu Arg Pro Asp Glu His Gly Phe Val Ala Arg Glu Phe His Arg Arg Tyr Arg

Leu Pro Pro Gly Val Asp Pro Ala Ala Val Thr Ser Ala Leu Ser Pro Glu Gly Val Leu Ser
Ile Gln Ala Ala Pro Ala Ser Ala Gln Ala Pro Pro Pro Ala Ala Ala Lys. (**SEQ ID NO: 34**)

Another embodiment comprises or consists of full length HSP20 with the serine at
position 16 substitute with aspartic acid (X2-**SEQ ID NO:36-X6**) (**SEQ ID NO: 37**):

5 Met Glu Ile Pro Val Pro Val Gln Pro Ser Trp Leu Arg Arg Ala **Asp** Ala Pro Leu
Pro Gly Leu Ser Ala Pro Gly Arg Leu Phe Asp Gln Arg Phe Gly Glu Gly Leu Leu Glu
Ala Glu Leu Ala Ala Leu Cys Pro Thr Thr Leu Ala Pro Tyr Tyr Leu Arg Ala Pro Ser Val
Ala Leu Pro Val Ala Gln Val Pro Thr Asp Pro Gly His Phe Ser Val Leu Leu Asp Val Lys
His Phe Ser Pro Glu Glu Ile Ala Val Lys Val Val Gly Glu His Val Glu Val His Ala Arg
10 His Glu Glu Arg Pro Asp Glu His Gly Phe Val Ala Arg Glu Phe His Arg Arg Tyr Arg
Leu Pro Pro Gly Val Asp Pro Ala Ala Val Thr Ser Ala Leu Ser Pro Glu Gly Val Leu Ser
Ile Gln Ala Ala Pro Ala Ser Ala Gln Ala Pro Pro Pro Ala Ala Ala Lys. (**SEQ ID NO: 36**)

Another embodiment of the polypeptide comprises or consists of full length
HSP20 with the serine at position 16 substitute with glutamic acid (X2-**SEQ ID NO:38-**
15 **X6**)(**SEQ ID NO: 39**):

Met Glu Ile Pro Val Pro Val Gln Pro Ser Trp Leu Arg Arg Ala **Glu** Ala Pro Leu
Pro Gly Leu Ser Ala Pro Gly Arg Leu Phe Asp Gln Arg Phe Gly Glu Gly Leu Leu Glu
Ala Glu Leu Ala Ala Leu Cys Pro Thr Thr Leu Ala Pro Tyr Tyr Leu Arg Ala Pro Ser Val
Ala Leu Pro Val Ala Gln Val Pro Thr Asp Pro Gly His Phe Ser Val Leu Leu Asp Val Lys
20 His Phe Ser Pro Glu Glu Ile Ala Val Lys Val Val Gly Glu His Val Glu Val His Ala Arg
His Glu Glu Arg Pro Asp Glu His Gly Phe Val Ala Arg Glu Phe His Arg Arg Tyr Arg
Leu Pro Pro Gly Val Asp Pro Ala Ala Val Thr Ser Ala Leu Ser Pro Glu Gly Val Leu Ser
Ile Gln Ala Ala Pro Ala Ser Ala Gln Ala Pro Pro Pro Ala Ala Ala Lys. (**SEQ ID NO: 38**)

It is further preferred that the polypeptides are phosphorylated, most preferably at
25 residue 16, or contain phosphorylation mimics at the position of amino acid residue 16.

Other preferred embodiments of the polypeptides are the following:

X2-**SEQ ID NO: 40-X6** (**SEQ ID NO: 41**), wherein (**SEQ ID NO: 40**) is Trp
Leu Arg Arg Ala Ser Ala Pro Leu Pro Gly Leu Lys (preferably with the Ser residue
phosphorylated)

30 X2-**SEQ ID NO: 42-X6** (**SEQ ID NO: 43**), wherein (**SEQ ID NO: 42**) is Trp
Leu Arg Arg Ala Asp Ala Pro Leu Pro Gly Leu Lys; and

X2-**SEQ ID NO: 44-X6** (**SEQ ID NO: 45**), wherein (**SEQ ID NO: 44**) is Trp Leu
Arg Arg Ala Glu Ala Pro Leu Pro Gly Leu Lys.

In each of these further embodiments of the polypeptides, X2 and X6 are optional transduction domains; it is preferred that at least one of X2 and X6 comprises a transduction domain.

5 The term "polypeptide" is used in its broadest sense to refer to a sequence of subunit amino acids, amino acid analogs, or peptidomimetics linked by peptide bonds, except where noted. The polypeptides described herein may be chemically synthesized or recombinantly expressed.

10 Preferably, the polypeptides of the present invention are chemically synthesized. Synthetic polypeptides, prepared using the well known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc ($N\alpha$ -amino protected $N\alpha$ -t-butyloxycarbonyl) amino acid resin with the standard deprotecting, neutralization, coupling and wash protocols of the original solid phase procedure of Merrifield (1963, J. Am. Chem. Soc. 85:2149-2154), or the base-labile $N\alpha$ -amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids first described by Carpino and Han (1972, J. Org. Chem. 37:3403-3409). Both Fmoc and Boc $N\alpha$ -amino protected amino acids can be obtained from Sigma, Cambridge Research Biochemical, or other chemical companies familiar to those skilled in the art. In addition, the polypeptides can be synthesized with other $N\alpha$ -protecting groups that are
20 familiar to those skilled in this art.

Solid phase peptide synthesis may be accomplished by techniques familiar to those in the art, or using automated synthesizers. The polypeptides of the invention may comprise D-amino acids (which are resistant to L-amino acid-specific proteases in vivo), a combination of D- and L-amino acids, and various "designer" amino acids (e.g., β -methyl amino acids, $C\alpha$ -methyl amino acids, and $N\alpha$ -methyl amino acids, etc.) to convey
25 special properties. Synthetic amino acids include ornithine for lysine, and norleucine for leucine or isoleucine.

In addition, the polypeptides can have peptidomimetic bonds, such as ester bonds, to prepare peptides with novel properties. For example, a peptide may be generated that
30 incorporates a reduced peptide bond, i.e., $R_1-CH_2-NH-R_2$, where R_1 and R_2 are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a polypeptide would be resistant to protease activity, and would possess an extended half-life in vivo.

The polypeptides may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers, adjuvants, etc., prior to being disposed on the heparin coating. The polypeptides may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers, or may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanolic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidone, dextran sulfate, heparin-containing gels, and/or polyvinyl alcohol prior to being disposed on the heparin coating.

An “amount effective” is an amount sufficient to promote the desired therapeutic outcome, and can be determined by an attending physician in light of other relevant medical issues for the subject. In general, an amount effective of the polypeptides that can be employed ranges between about 0.01 $\mu\text{g/kg}$ body weight and about 10 mg/kg body weight, preferably ranging between about 0.05 $\mu\text{g/kg}$ and about 5 mg/kg body weight.

The polypeptides of the invention may be used as the sole active agent, or may be used in combination with other active agents. In one embodiment, the methods further comprise administering one or more further compounds useful for promoting smooth muscle relaxation and/or reduction in other symptoms of the disorder being treated, including but not limited to anti-inflammatories (including but not limited to dexamethasone, prednisone, fludrocortisone, hydrocortisone, flunisolide, triamcinolone, beclomethasone, methylprednisolone, prednisolone, leukotriene inhibitors such as zafirlukast and zileuton; cromolyn sodium, and nedrocromil) and inhibitors of heat shock protein 27 (HSP27). Increases in the phosphorylation of HSP27 are associated with smooth muscle contraction, and transfection of cells with dominant active phosphorylated mutants of HSP27 leads to stress fiber formation. Furthermore, increases in the phosphorylation of HSP27 are associated with smooth muscle cell migration. HSP20, in contrast, promotes vasorelaxation, and the data presented herein demonstrates that phosphorylated analogues of HSP20 lead to a loss of stress fiber formation, and inhibit smooth muscle cell proliferation and migration. Thus, the data indicate that HSP20 and HSP27 have opposing functions. Therefore, the combined use of one or more polypeptides of the invention and an inhibitor of HSP27 will have enhanced efficacy in carrying out the methods of the invention. As used herein, an “inhibitor” of HSP27

includes HSP27 antibodies, anti-sense HSP27 nucleic acids, or small molecule inhibitors of the phosphorylation of HSP27, such as SB203580 (available from SmithKline Beecham).

For use herein, the active agents may be administered by any suitable route, including orally, parentally, transdermally, or via aerosol delivery. The term parenteral as used herein includes, subcutaneous, intravenous, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally. Solid dosage forms for oral administration may include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compound may be admixed with at least one inert diluent such as sucrose, lactose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, e.g., lubricating agents such as magnesium stearate. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings. Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs containing inert diluents commonly used in the art, such as water. Such compositions may also comprise adjuvants, such as wetting agents, emulsifying and suspending agents and sweetening, flavoring and perfuming agents.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

25

Examples

Summary

Activation of the cyclic AMP/cyclic AMP-dependent protein kinase (PKA) pathway leads to relaxation of airway smooth muscle (ASM). The small heat shock-related protein (HSP20) is a substrate of PKA. The purpose of this study was to examine the role of HSP20 in mediating ASM relaxation. Human ASM cells were engineered to constitutively express a green fluorescent protein (GFP)-PKA inhibitory fusion peptide (PKI-GFP) or GFP alone (control). Activation of the cAMP-dependent signalling pathways by isoproterenol (ISO) or forskolin (FSK) led to increases in the

phosphorylation of HSP20 in GFP but not PKI-GFP cells. Forskolin treatment in GFP but not PKI-GFP cells led to a loss of central actin stress fibres and decreases in the number of focal adhesion complexes. This loss of stress fibres was associated with dephosphorylation of the actin depolymerizing protein cofilin in GFP but not PKI-GFP cells. To confirm that phosphorylated HSP20 plays a role in PKA-induced ASM relaxation, intact strips of bovine ASM (BASM) were pre-contracted with serotonin and treated with ISO. Activation of the PKA pathway led to relaxation of BASM, which was associated with increases in the phosphorylation of HSP20. Finally, treatment with phospho-peptide mimetics of HSP20 possessing a protein transduction domain relaxed pre-contracted BASM strips. Both phosphorylated HSP20 and phosphorylated cofilin have phosphopeptide motifs that bind to the 14-3-3 scaffolding protein. Hence, we speculate that phosphorylated HSP20 competes with phosphorylated cofilin for binding to 14-3-3 leading to activation of cofilin and actin de-polymerization. These data suggest that one possible mechanism by which HSP20 mediates ASM relaxation is via regulation of actin filament dynamics.

Introduction

To investigate the potential role of HSP20 in PKA-induced airway smooth muscle relaxation, stable lines of human ASM (HASM) cells expressing GFP or a GFP chimera of the PKA inhibitory peptide PKI (PKI-GFP; (Guo *et al.*, 2005) were generated. These cells were used to determine morphologic and biochemical changes in response to PKA stimulation. In addition, intact bovine ASM (BASM) was used to further elucidate the role of PKA in β -agonist-induced BASM relaxation. Given the established effect of activation of PKA on cytoskeletal dynamics (loss of actin stress fibres), we hypothesized that one of the possible mechanisms of Ca^{2+} -independent vasorelaxation is via a thin filament (actin) regulatory mechanism involving PKA-dependent phosphorylation of HSP20.

MATERIALS AND METHODS

Materials

Cell culture reagents including F-12 HAM's media, L-glutamine, BSA, G418, phosphate buffered saline (PBS) and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Dithiothreitol (DTT), HEPES, Trizma base, Triton X-100, formaldehyde, Tween-20, CaCl_2 , sodium nitroprusside, forskolin (FSK), and

isoproterenol (ISO) were purchased from Sigma Chemical Co. (St. Louis, MO). Pre-cast acryl amide gels, sodium dodecyl sulfate, Tris-glycine-SDS buffer (TGS), Tris-glycine (TG) and prestained Precision Blue Protein Standards were purchased from Bio-Rad (Hercules, CA). Phospho-HSP20 peptides, (YARAAARQARAWLRRA(pS)APLPGLK-COOH) (**SEQ ID NO: 28**) and HSP20 control peptides where the phospho serine is replaced with alanine (YARAAARQARAWLRRAAAPLPGLK-COOH) (**SEQ ID NO: 46**) were synthesized by UCB-Bioproducts-Lonza (Cambridge, MA) and American Peptide Co, (Sunnyvale, CA). Mouse anti-HSP20 antibodies were from Advanced Immunochemical, Inc., (Long Beach, CA); rabbit anti-phospho Ser 16-HSP20 antibodies were generated against the phospho-peptide WLRRA(pS)APLPGLK (**SEQ ID NO:47**)(17); mouse anti-VASP antibodies were from BD Biosciences (San Jose, CA); rabbit anti-PKG antibody was purchased from Stressgen (Victoria, BC); rabbit anti-actin were from Sigma Aldrich Co, (St. Louis, MO); anti GAPDH antibodies were obtained from Abcam Inc (Cambridge, MA); rabbit anti-phospho-cofilin 2 (ser 3) antibodies were from UpState Biotechnology (Charlottesville, VA), and rabbit anti-cofilin 2 antibodies were from Santa Cruz Biotechnology, (Santa Cruz, CA).

Cell Culture and Agonist Treatment

HASM lines stably expressing GFP or PKI-GFP were generated by retroviral infection at Wake Forest University and maintained as described previously. HASM cultures were grown in F-12 HAM's media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 25 mM HEPES, 12 mM NaOH, 1.7 mM CaCl₂, 100 units/mL penicillin, 100 µg/mL streptomycin and 300 µg/mL G418 (complete media). Cultures were used for 4-5 passages after transfection and phenotype changes were monitored by the expression of cGMP dependent protein kinase (PKG). Prior to treatment with agonists cells were serum starved for 24 h in complete media lacking FBS and containing 0.1% BSA. Serum starved cells plated in 100 mm dishes were stimulated with vehicle (control), 10 µM FSK, or 1 µM ISO for 10 min. Cells were rinsed once with PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4), harvested by scraping, snap-frozen in liquid nitrogen and stored at -80° C. For stress fiber experiments cells were plated onto 18 mm cover glasses in 60-mm dishes 1 day prior to experiment so that they were 65–75% confluent before agonist treatment. Serum starved cells were treated with agonists, 50 µM phospho-HSP20 peptides or 50 µM HSP20 control peptides as indicated, washed three times in PBS and fixed for 30 min in 4%

(w/v) formaldehyde prepared in PBS. Following fixation, cells were washed three times in PBS then permeabilized in 0.1% (v/v) Triton X-100 in PBS for 15 min at room temperature (RT) with gentle rocking then washed three times in PBS.

5 Immunofluorescence Microscopy and Immunoblotting

Formaldehyde-fixed and detergent-permeabilized cells were processed for indirect immunofluorescence microscopy by adding Alexa568-conjugated phalloidin (1:1000 in PBS, Invitrogen) and DAPI (1:1000) and letting cells gently rock in the dark at RT for 1 h. Following three washes with PBS, labelled cells were viewed using a Zeiss
10 Axiovert 200M epifluorescence microscope (Carl Zeiss, Thornwood, N.Y., USA) equipped with a Xcite light source, and GFP autofluorescence and Alexa 568-conjugated phalloidin fluorescence images were obtained using filter sets for exciting GFP (ex 492/18 nm) and Alexa 568 (ex 572/23 nm) and an Axiocam HR digital camera. Images were acquired processed using Axiovision 4.5 and Photoshop 7.0 software packages,
15 respectively.

For immunoblot analysis, control and agonist-treated cells were scraped from a 100 mm culture dish into 100 μ L UDC buffer (8M urea, 4% CHAPS, 10 mM DTT) and the samples rotated for 2 hr at RT. Samples were clarified by centrifugation at $\sim 8,000 \times g$ for 10 min. Protein concentrations were determined using the Bradford assay (Pierce
20 Chemical, Rockford, IL). Unless indicated otherwise, 20 μ g protein was separated on 4–20% polyacrylamide mini-gels in 1x TGS buffer (25 mM Tris·Cl pH 8.3, 192 mM glycine, 0.1% wt/vol SDS) at 120 V for 1.5 h. Electrophoretic transfer of proteins from the gels onto polyvinylidene difluoride membranes was carried out in 1x TG buffer (25 mM Tris·Cl pH 8.3, 192 mM glycine) at 50 volts for 12 h at 4°C. The blot was
25 subsequently incubated with one of the following primary antibodies: mouse anti-HSP20 (1:4,000 dilution); rabbit anti-phospho Ser 16-HSP20 (1:500); mouse anti-VASP (1:2000); rabbit anti-PKG (1:1000); rabbit anti-actin (1:500); anti GAPDH (1:300); rabbit anti-phospho-cofilin (1:2000), rabbit anti-cofilin (1:1000) and either of one corresponding secondary antibodies: AlexaFluor 680-conjugated affinity-purified goat anti-mouse
30 secondary antibody (Invitrogen, Carlsbad, CA) or IRDye800-conjugated affinity-purified goat anti-rabbit secondary antibody (Rockland Scientific, Gilbertsville, PA). Membranes were scanned and the intensities of selected bands were directly quantified by the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE).

Focal adhesion measurement

HASM cells were seeded and cultured overnight, with transfer to no serum (culture medium supplemented with 1mg/ml bovine serum albumin) medium overnight prior to experimentation. Cells were either untreated or treated with 100 nM Hep I (thrombospondin peptides, positive control), 10 μ M FSK, 25 μ M phospho-HSP20 peptides or 25 μ M HSP20 control peptides as described above. Cells were fixed and examined (at least 250 cells per condition) for the presence or absence of focal adhesions by Interference reflection microscopy (IRM). The percentage of cells positive for focal adhesions was determined in a minimum of 3 independent experiments. A cell was scored positive for focal adhesions if it contained at least five focal adhesions.

Procurement of bovine airway smooth muscle tissue

Fresh bovine lung was obtained from a local abattoir (South Western Processing, Queen Creek, AZ). The tissue was immediately placed in HEPES buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 4.7 mM KCl; 1.0 mM MgSO₄; 1.0 mM NaH₂PO₄; 1.5 mM CaCl₂; and 10 mM glucose) and stored on ice during transfer to the laboratory. Briefly, a secondary airway passage was dissected from bovine lung and cut open longitudinally. The airway was then pinned at each corner to a dissection tray. Epithelium was removed from the smooth muscle tissue by gentle rubbing with a cotton-tipped applicator. A 1 cm wide by 3 cm long strip of ASM (tangential to air flow) was then carefully dissected from the structure of the airway passage. 1.5-2 mm wide cross-sectional strips were then cut for use in the muscle bath.

Physiologic measurements

Bovine ASM strips were suspended in a muscle bath containing a bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO₄, 1.0 mM NaH₂PO₄, 10 mM glucose, 1.5 mM CaCl₂, and 25 mM Na₂HCO₃; pH 7.4), and equilibrated with 95% O₂/5% CO₂ at 37°C. Force measurements were obtained with a Kent Scientific (Litchfield, CT) force transducer (TRN001) interfaced with PowerLab from AD Instruments (Colorado Springs, CO). Data were recorded with Chart software, version 5.1.1 (AD Instruments). Each strip was washed every 15 min with 37°C bicarbonate buffer for the first h of equilibration, and the length progressively adjusted until maximal tension was obtained. The tissue was allowed to equilibrate for 2 additional h without disturbance before

experimentation was initiated. Each strip was initially tested for viability with high extracellular potassium (110mM KCl, with equimolar replacement of NaCl in bicarbonate buffer), the maximal tension obtained was taken as 100%, and tension obtained with agents (agonists, antagonists) was calculated. At the end of the experiments, all strips were washed and contracted with KCl to ensure viability of the tissues.

To determine the role of endogenous HSP20 in bovine airway smooth muscle relaxation, simultaneous physiologic water bath experiments were conducted using identical strips of airway smooth muscle dissected from the same tissue. These strips were placed in vials of bicarbonate buffer and equilibrated in a physiologic water bath with 95% O₂/5% CO₂ at 37° C for 3 hrs. The muscle strips were washed with fresh buffer each 15 min for the first h, and then left undisturbed for the subsequent 2 h. Strips were then contracted with 1 μM serotonin. At 5 min after the serotonin dose, one strip was snap frozen and pulverized in liquid nitrogen, and the other strip was relaxed with 1 μM ISO for 5 min or 10 μM FSK for 5 min. Strips were quick frozen in the same manner, along with an untreated control strip. All samples were stored at -80° C for later analysis using one or two-dimensional gel electrophoresis.

Determination of HSP20 phosphorylation in bovine ASM tissue

Proteins from frozen muscle strips were extracted in buffer containing 8M urea, 10 mM dithiothreitol, 4% CHAPS (UDC buffer). The mixtures were vortexed at room temperature overnight, and then centrifuged at 14,000 rpm for 15 min. For one dimensional separation equal amounts (20 μg) of proteins were placed in Laemmli sample buffer (Bio-Rad laboratories, Inc. Hercules, CA) containing 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, heated for 5 min at 100°C and separated by SDS-PAGE as described above. For two-dimensional analysis, 75 μg of extracted proteins were separated by first dimension isoelectric focusing (IEF) using the Protean IEF Cell (Bio-Rad) and second dimension using SDS-PAGE and probed with anti HSP20 antibodies.

Statistical analysis

Values are reported as mean $\pm$ standard error of the mean (SE). Statistical analysis was performed by unpaired Student's *t* test or one-way *ANOVA* followed by Tukey's post test (GraphPad Software, Inc. San Diego, CA). The criterion for

significance was $P < 0.05$. Stress values are calculated with the following formula:
Stress [Newtons (N)/m²] = force (g) x 0.0987 / area, where area is equal to the wet weight
[mg / length (mm at maximal length)] divided by 1.055.

5 RESULTS

Inhibition of PKA inhibits ISO-induced phosphorylation of HSP20 in human airway smooth muscle cells

To determine whether PKA mediates phosphorylation of HSP20 in ASM, HASM cells stably expressing either GFP (control) or the PKI-GFP chimera were generated as described previously (**Guo et al., Biochemistry** 44: 13771-13782, 2005). VASP phosphorylation by PKA at Ser 157 causes a significant mobility shift on one dimensional SDS-PAGE gels and was used as a marker of intracellular PKA activity in the HASM cells. GFP and PKI HASM cells were stimulated with the β -agonist ISO and the phosphorylation of VASP and HSP20 determined by immunoblotting. HASM cell lines were treated with 1 μ M isoproterenol (panel A, ISO, lanes 2 and 4) or 10 μ M forskolin (panel A, FSK, lanes 6 and 8) for 10 min and snap frozen. Whole cell lysates (20 μ g) of control and ISO or FSK-treated cells expressing GFP (lanes 1, 2, 5, 6) or PKI-GFP (lanes 3, 4, 7, 8) were separated by SDS-PAGE and probed with the indicated antibodies. The positions of VASP and phosphorylated VASP are indicated by arrows on the left). Bands were normalized to actin and ratio of PhosphoVASP/VASP (panel B), phosphoHSP20/HSP20 (panel C), and phosphocofilin/cofilin (panel D) was determined, n=4-6; * $p < 0.05$ with respect to control.

Treatment with 1 μ M ISO for 10 min increased VASP phosphorylation in the control GFP cells, but not in PKI-GFP cells (Figure 1A, B). Similarly, ISO led to increases in the phosphorylation of HSP20 in the control GFP cells but not in PKI-GFP cells (Figure 1A, C), suggesting the inhibition of PKA in PKI HASM cells. FSK (10 μ M-10 min), which activates PKA through direct activation of adenylyl cyclase, also led to increases in HSP20 and VASP phosphorylation in GFP cells and not in PKI-GFP cells (Figure 1A,B,C).

30

PKA inhibition and cofilin dephosphorylation in HASM cells

Cofilin is an actin depolymerizing protein which is activated upon dephosphorylation by phosphatases such as slingshot and chronophin. Phosphorylated

cofilin contains a binding motif for the scaffolding protein 14-3-3, and association of cofilin with 14-3-3 protects cofilin from dephosphorylation. HSP20 also contains a binding motif for 14-3-3 and binding of 14-3-3 protein to phosphopeptide analogs of HSP20 prevents the association of cofilin with 14-3-3. Increases in cAMP levels have been demonstrated to cause cofilin dephosphorylation. To assess the role of PKA in the regulation of cofilin phosphorylation in ASM, GFP and PKI-GFP HASM cells were treated with either 1 μ M ISO for 10 min or 10 μ M FSK for 10 min and phosphorylation of cofilin was analyzed by immunoblotting. FSK or ISO treatment significantly decreased phospho-cofilin levels in control GFP cells, but had no effect in PKI-GFP cells (Figure 1A, D).

Inhibition of PKA prevents changes in morphology and focal adhesion complexes in human airway smooth muscle cells

To investigate the role of PKA-mediated phosphorylation of HSP20 in the regulation of central actin stress fibers, serum starved HASM cells were treated with ISO or FSK and stress fiber formation was analyzed by fluorescence microscopy. Figure 2 shows representative epifluorescence micrographs of HASM control (panel A and D), 1 μ M ISO for 30 min (panel B and E) or 10 μ M FSK for 30 min (panel C and F) cells expressing GFP (A B, C) or PKI-GFP (D, E, F). Cells grown for 24 hr in serum-free medium were treated with the agents and then washed, processed for microscopy and labeled with Alexa 586-conjugated phalloidin to reveal the actin cytoskeleton and DAPI to reveal nucleus, bar =50 μ m.

Treatment with 1 μ M ISO for 30 min or 10 μ M FSK for 30 min led to disruption of stress fibers, with only cortical actin remaining, in HASM cells that express GFP alone (Figure 2 B, C). However, ISO or FSK treatment did not lead to stress fiber disruption in PKI-GFP expressing cells (Figure 2 E, F)).

Loss of stress fibers has been associated with a loss of focal adhesion complexes (2). To examine the effect of PKA on focal adhesion complexes, HASM cells were treated with FSK (10 μ M, 30 min) and loss of focal adhesions were measured using interference reflection microscopy. HepI peptide [from thrombospondin-1&2 with focal adhesion disassembly activity] was used as a positive control. Figure 3 shows the experiment. HASM cells were grown as described in the methods section. Panel A summarizes data obtained with cells that were serum starved overnight and either

untreated (control) or treated with 100 nM hep I (thrombospondin peptides, positive control), forskolin (FSK, 10 μ M), or 25 μ M phospho-HSP20 peptide (HSP20 peptide) containing a protein transduction domain and the active phosphorylation site of HSP20 or HSP20 control peptide (HSP20 control) for 30 min. Cells were fixed and examined (at least 250 cells per condition) for the presence or absence of focal adhesions by interference reflection microscopy (IRM). The percentage of cells positive for focal adhesions was determined in 5 independent experiments (n=5, * p< 0.05). Panels B-G are representative epifluorescence micrographs of HASM control (panel B and E), 50 μ M phospho-HSP20 peptide (panel C and F), 50 μ M HSP20 control peptide (panel D, G) for 24 h in cells expressing GFP (B, C, D) or PKI-GFP (E, F, G). Cells grown for 24 hr in serum-free medium were treated with the agents and then washed, processed for microscopy and labelled with Alexa 586-conjugated phalloidin to reveal the actin cytoskeleton and DAPI to reveal nucleus, bar =50 μ m . Panel H shows data obtained from HASM cell lines that were untreated (lanes 1 and 4) or treated with 50 μ M phospho-HSP20 peptide for 30 min or 24 hr (lanes 2, 3, 5, 6) and snap frozen. Whole cell lysates (20 μ g) of control and treated cells expressing GFP (panel H, lanes 1, 2, 3) or PKI-GFP (panel H, lanes 4, 5, 6) were separated by SDS-PAGE and probed with the indicated antibodies. Panel I shows a densitometric measurements of the bands which were normalized to GAPDH and ratio of phospho cofilin/cofilin was determined, and plotted along with results obtained from treatment of GFP and PKI-GFP cells with HSP20 control peptides. n=3; * p < 0.05 with respect to control.

Hep I treatment led to decreases in focal adhesions in both GFP and PKI-GFP cells (Figure 3 A). HepI activates cGMP pathways leading to activation of cGMP-dependent kinase (PKG). PKG is not inhibited by PKI. Forskolin stimulation led to decreases in focal adhesions in GFP cells, but not in PKI-GFP cells (Figure 3 A).

To examine the role of HSP20 phosphorylation in the regulation of cell morphology and focal adhesion disassembly, HASM cells were treated with a transducible synthetic phospho-peptide analog of HSP20 containing the active phosphorylation site of HSP20 [(serine 16 which is phosphorylated during active relaxation, WLRR(pS)APLPGLK) (**SEQ ID NO: 47**)] attached to a protein transduction domain (PTD, YARAAARQARA) (**SEQ ID NO: 23**) or a control peptide where phospho serine is replaced with alanine and stress fiber formations and focal adhesion disassemblies were measured. Treatment with the phospho-HSP20 peptides,

led to changes in morphology, loss of stress fibers and decreases in focal adhesion complexes (Figure 3 A- G) in both GFP- and PKI-GFP-expressing cells. There were no changes in morphology, stress fibers or focal adhesion complexes in cells treated with the peptide controls. To directly assess the role of phospho-HSP20 peptides in the regulation of cofilin phosphorylation, GFP and PKI-GFP HASM cells were treated with 50 μ M phospho-HSP20 peptides or control peptides for either 30 min or 24 h and phosphorylation of cofilin was analyzed by immunoblotting. Phospho-HSP20 peptide treatment significantly decreased phospho-cofilin levels in control GFP as well as in PKI-GFP cells (Figure 3, H, I). There were no decreases in phospho-cofilin levels in cells treated with the HSP20 control peptides (Figure 3, I). Taken together, these data suggest that the effects of the phospho-HSP20 peptides are downstream of PKA.

Stimulation of HSP20 phosphorylation with ISO in intact bovine ASM

To confirm that phosphorylated HSP20 plays a role in PKA-induced ASM relaxation, isolated intact strips of bovine ASM were equilibrated in a muscle bath. The strips were pre-contracted with serotonin (5-hydroxytryptamine, 5HT) then treated with ISO, with force generation continuously recorded. Parallel strips were similarly treated, snap-frozen, and phosphorylation of HSP20 was subsequently analyzed by immunoblotting.

Isolated bovine airway smooth muscle strips were suspended in a muscle bath and equilibrated for 2 hrs in bicarbonate buffer as described in the methods section. The muscle strips were treated with cumulative log doses (0.01 to 1 μ M) of serotonin (SER), followed by cumulative log doses (0.01 to 1 μ M) of isoproterenol (ISO) and force generated was recorded. Cumulative data obtained when the force was converted to stress and decrease in stress minus that of the untreated control was converted to a percentage of the maximal serotonin (1-5 μ M) contraction, and plotted against the concentration of ISO [(mean $\pm$ SEM), $n = 3$, * = $P < 0.05$.]. Bovine ASM strips that were equilibrated in a water bath and contracted with 1 μ M serotonin (SER) for 5 min, followed by 1 μ M ISO for 5 min. The strips were quick frozen, pulverized, and proteins analyzed by two dimensional (2D) gel electrophoresis and immunoblotting. Data was derived from experiments using bovine ASM strips that were equilibrated in a muscle bath as described above and precontracted with 5 μ M serotonin. The strips were then relaxed with increasing doses (0.1 mM-2 mM) of phospho-HSP20 peptide or HSP20

control peptide analogs. The percent contraction was calculated as above and plotted against HSP20 peptide or control peptide concentration [(mean $\pm$ SEM), $n = 3$, * $P = 0.034$ at 2 mM P20 peptide compared to 2 mM control peptide].

5 Serotonin treatment led to a dose-dependent increase in force and ISO induced a dose-dependent relaxation in bovine ASM. When related to 110 mM KCL induced contraction, 0.1, 1 and 10 μ M serotonin generated 6.3 ± 1 ; 25.1 ± 5.5 , and 62.7 ± 15.4 %, respectively. Two-dimensional gel electrophoresis and western blot analysis demonstrate increases in the phosphorylation of HSP20 in response to ISO stimulation.

10 To examine the direct role of HSP20 phosphorylation on airway smooth muscle relaxation, bovine ASM strips were pre-contracted with either serotonin (1 or 5 μ M) or increasing doses of carbachol (0.1, 0.5 and 5 μ M) and treated with phospho-HSP20 peptides or HSP20 control peptides. Transduction of serotonin pre-contracted strips of bovine ASM with phospho-HSP20 peptides led to a dose-dependent decrease in stress which was significant at 2 mM phospho-HSP20 peptide (45.7 ± 7.6 % compared to 90.0 ± 1.4 %) while there was no significant decrease in stress with HSP20 control peptides. 15 The decreases in stress generated by the phospho-HSP20 peptide with respect to maximum KCl contraction were 41.5 ± 3.6 ; 39.2 ± 1.7 ; 35.7 ± 1.7 ; 29.3 ± 3.2 , and 20.2 ± 2.4 % for 0, 0.1, 0.5, 1 and 2 mM phospho-HSP20 peptide, respectively.

20 Similarly, phospho-HSP20 peptides and not the control peptides led to a dose dependent decrease in stress in carbachol pre-contracted strips. Panels A&B show data derived from experiments in which bovine ASM strips were equilibrated in a muscle bath as described above and precontracted with increasing doses of (a=0.01, b=0.05 and c= 0.1 μ M) carbachol (CCh). The strips were then relaxed with increasing doses (d=0.5, e=1, f=2, g=3, and h=4 mM of phospho-HSP20 peptide (P20 peptide) analogs or HSP20 25 control (P20 control) peptide analogs. Panel C shows cumulative data obtained from the effect of P20 peptide when bovine ASM strips were contracted with 0.1, 0.5 or 5 μ M doses of carbachol. The percent contraction was calculated as described above and plotted against phospho-HSP20 peptide or HSP20 control peptide concentration [(mean $\pm$ SEM), $n = 3$ for each concentration of carbachol, * $P < 0.001$ when P20 control is compared to 0.1 μ M + 3 and 4 mM P20 peptide and $p = < 0.05$ when P20 control is compared to 0.5 30 μ M + 4 mM P20 peptide].

However, the peptide analogs did not lead to complete relaxation of bovine ASM. Phospho-HSP20 peptides were more effective on strips contracted with lower

concentration of carbachol (0.1 μ M) that generated 40-60% of KCl contraction when compared to the higher concentration (0.5 or 5 μ M) that generated 80-120% of KCl contraction. When bovine ASM was contracted with 0.5 μ M carbachol significant relaxation was achieved only at 4 mM phospho-HSP20 peptide compared to control peptide (75.11 $\pm$ 3.5 and 97.63 $\pm$ 3.4%, respectively) whereas at 0.1 μ M carbachol significant relaxation was achieved at 3 and 4 mM phospho-HSP20 peptide (55.57 $\pm$ 9.7 and 42.5 $\pm$ 12.3 for 3 and 4 mM HSP20 peptide compared to 96.9 $\pm$ 3.0 and 97.6 $\pm$ 3.4% for 3 and 4 mM control peptide) . There was no significant difference in the response of the control peptide to 0.1, 0.5 or 5 μ M carbachol (data not shown).

To determine whether the activation of PKA led to changes in cofilin phosphorylation, bovine ASM strips were pre-contracted with 5 μ M serotonin and treated with 1 μ M ISO or 10 μ M FSK for 5 min and the phosphorylation of cofilin was analyzed by SDS-PAGE and western blotting. Isolated bovine airway smooth muscle strips were suspended in a muscle bath and equilibrated for 3 h in bicarbonate buffer as described in the methods section. Bovine ASM strips were contracted with 5 μ M serotonin (SER) for 5 min, 5 μ M serotonin (SER) for 5 min followed by 1 μ M isoproterenol (ISO) or 5 μ M serotonin for 5 min followed by 10 μ M forskolin (FSK) for 5 min. The strips were quick frozen, pulverized, and the proteins separated by SDS-PAGE and probed with the indicated antibodies. Panel A of Figure 5 shows a representative blot. Bands were normalized to β actin and ratio of phospho cofilin/cofilin (panel B) was determined, n=3; * p < 0.05 with respect to serotonin alone. These data demonstrate that activation of PKA by ISO or FSK led to a significant decrease in the phosphorylation of cofilin (Figure 5 A, B).

DISCUSSION

In this investigation, we used cultured human airway smooth muscle cells that expressed a peptide inhibitor of PKA to assess events downstream of PKA signaling. Treatment of human airway smooth muscle cells with activators of PKA led to increases in the phosphorylation of the PKA substrates VASP and HSP20 in cells expressing GFP alone but not in cells expressing PKI-GFP. This was associated with distinct morphologic changes, decreases in actin stress fibers and focal adhesion complexes, in the GFP but not in PKI-GFP cells. There were decreases in the phosphorylation of the

actin depolymerizing protein, cofilin in the GFP but not in PKI-GFP cells. Hence, one possible mechanism to explain the loss of actin stress fibers and focal adhesions is activation of cofilin. PKA inhibition has been demonstrated earlier in HASM cells expressing PKI, where treatment with ISO or FSK caused only a small percentage of VASP to shift relative to that observed for the GFP-expressing lines, and agonist-stimulated CRE-Luc activity in these cells was abolished (**Guo et al.**, *Biochemistry* 44: 13771-13782, 2005).

In this study, we demonstrate that the phospho-peptide analogs of HSP20 lead to loss of stress fibers, decreases in focal adhesion complexes, and decreases in cofilin phosphorylation in both GFP and PKI-GFP expressing human airway smooth muscle cells. These data suggest that the events upstream of PKA can be bypassed by directly introducing phospho-peptide analogs of at least one of the substrates of PKA and that the mechanism of action may be competition for binding to the scaffolding protein 14-3-3. However, the effect of phospho-HSP20 peptides on cofilin dephosphorylation was more pronounced in PKI-GFP compared to GFP expressing cells suggesting that other pathways may also be involved in dephosphorylation of cofilin or that the expression of PKI led to alterations in the expression or activity of other signalling events. ISO induced actin depolymerization in airway smooth muscle cells has been reported to be by PKA-dependent as well as PKA-independent pathways involving Src kinase and G_s. Cofilin is an actin depolymerizing protein that when phosphorylated binds to the intracellular scaffolding protein, 14-3-3. When displaced from 14-3-3, cofilin becomes dephosphorylated and activated as an actin depolymerizing protein. Binding of 14-3-3 protein to phospho-peptide analogs of HSP20 prevents the association of cofilin with 14-3-3. It is likely that cyclic nucleotide dependent relaxation of smooth muscles includes multiple and redundant pathways and mechanisms.

β -agonist- or nitrovasodilator- induced phosphorylation of HSP20 has been demonstrated to mediate relaxation of vascular smooth muscle from various tissues. Phosphorylation of HSP20 also inhibits agonist mediated contraction, intimal hyperplasia and platelet aggregation (**McLemore et al.**, *Surgery* 136: 573-578, 2004; , **Tessier et al.**, *J Vasc Surg* 40: 106-114, 2004;, **Woodrum et al.**, *Am J Physiol* 277: H931-939., 1999). Here we have also demonstrated that ISO induced relaxation of intact bovine airway smooth muscle is associated with phosphorylation of HSP20. Phospho-peptide analogs of HSP20 also relaxed bovine ASM demonstrating a direct role for HSP20 in ASM

relaxation. However, the analogs did not completely relax airway smooth muscles. This may be due to the fact that the phospho-peptide analogs do not contain the complete structure of the HSP20 molecule and hence require higher concentrations than the intact, phosphorylated HSP20 molecule (Flynn et al., *Journal of Applied Physiology* 98: 1836-1845, 2005). Another possibility is that PKA phosphorylates other proteins to enable more complete relaxation. For example, β_2 -adrenergic relaxation of airway smooth muscle has been associated with PKA dependent and independent regulation of large-conductance, calcium activated potassium channels (K_{ca}) resulting in hyperpolarization as well as mechanisms involving calcium sensitivity of the contractile elements due to activation of myosin light chain phosphatase. The effect of ISO in nonhuman airways has also been demonstrated to involve enhanced Ca^{2+} pump activity to decrease $[Ca^{2+}]_i$ and myosin light chain phosphatase activation to decrease Ca^{2+} sensitivity of the contractile apparatus. Activation of PKA by ISO or FSK led to a significant decrease in the phosphorylation of cofilin in bovine ASM. These results suggest that ISO induced PKA mediated phosphorylation of HSP20 may induce actin depolymerization through cofilin dephosphorylation in ASM and this may be one of the mechanisms for Ca^{2+} independent relaxation of ASM along with the other mechanisms such as Ca^{2+} desensitization and myosin phosphatase activation.

β -agonists are the most commonly used therapy for relief of acute bronchospasm in asthmatics. It is widely assumed that β -agonists mediate their effect primarily by increasing cAMP concentration through activation of β_2 -adrenergic receptor-adenylyl cyclase pathway. Several mediators of inflammation and therapies such as β -agonists themselves can cause alterations in β_2 -adrenergic receptor responsiveness in a time-dependent and cell-specific manner. Agonist-specific desensitization may also be responsible for the loss of prophylactic bronchoprotection and deterioration of asthma control clinically observed with regular use of β -agonists. It has also been reported that airway inflammation or cytokine treatment contributes to β_2 -adrenergic receptor dysfunction and a loss of the relaxant effect of β agonists in ASM cells, tissues, and *in vivo* models. Adverse effects have been documented with inhaled β_2 -adrenergic agonist in patients with a genetic polymorphism that results in homozygosity for arginine, rather than glycine at amino acid residue 16 of the β_2 -adrenergic receptor. A recent meta-analysis that compares anticholinergics and β_2 -adrenergic agonists indicated that treatment with β_2 -adrenergic agonists led to a 2-fold increase in respiratory deaths.

Collectively, these studies suggest that regulation of proximal transmembrane signalling events in the β_2 -adrenergic receptor-Gs-adenylyl cyclase pathway may limit the efficacy of beta-agonist therapy. In the present study we demonstrate that β -agonist-induced relaxation of ASM is associated with increases in the phosphorylation of HSP20. In addition, phospho-peptide analogs of HSP20 linked to a transduction domain can produce the same morphologic and physiologic responses as do activation of the cAMP/PKA pathway. Thus, transducible peptide analogs of HSP20 that act in the same manner as the physiologic downstream PKA effector HSP20, represent a potential treatment approach to bronchospasm capable of overcoming problems associated with β_2 -adrenergic receptor desensitization.

We claim:

1. A method for treating an airway smooth muscle disorder, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat an airway smooth muscle disorder of a polypeptide comprising an amino acid sequence according to general formula I

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 2)

wherein X2 is absent or comprises a transduction domain;

X3 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO:1**);

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine,

- 10 hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

Z2 is selected from the group consisting of L and K; and

Z3 is selected from the group consisting of S, T, and K; and

- 15 X6 is absent or comprises a transduction domain.

2. The method of claim 1, wherein X4 is phosphorylated.

3. The method of claim 2, wherein X4 is S.

4. The method of claim 3 wherein X3 is WLRR (**SEQ ID NO: 1**).

5. The method of claim 4 wherein X5 is GLK.

- 20 6. The method of claim 5 wherein either X2 or X6 comprises a transduction domain.

7. The method of claim 6 wherein the transduction domain is selected from the group consisting of YARAAARQARA (**SEQ ID NO: 23**), YGRKKRRQRRR (**SEQ ID NO: 24**), WLRIKAWLRIKA (**SEQ ID NO: 26**), and WLRIKAWLRIKAWLRIKA (**SEQ ID NO: 27**).

- 25 8. The method of claim 6 wherein the polypeptide comprises YARAAARQARAWLRRRA(pS)APLPGLK (**SEQ ID NO: 28**).

9. The method of claim 6 wherein the polypeptide consists of YARAAARQARAWLRRRA(pS)APLPGLK (**SEQ ID NO: 28**).

10. The method of any one of claims 1-9 wherein the airway smooth muscle disorder is selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), emphysema, bronchitis, and anaphylaxis.

- 30 11. The method of any one of claims 1-10 wherein the airway smooth muscle disorder is asthma.

12. A method for treating bronchospasm, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat bronchospasm of a polypeptide comprising an amino acid sequence according to general formula I

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 2)

5 wherein X2 is absent or comprises a transduction domain;
 X3 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO:1**);
 X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine,
 hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;
 X5 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,
 10 wherein Z1 is selected from the group consisting of G and D;
 Z2 is selected from the group consisting of L and K; and
 Z3 is selected from the group consisting of S, T, and K; and
 X6 is absent or comprises a transduction domain.

13. The method of claim 11, wherein X4 is phosphorylated.

15 14. The method of claim 12, wherein X4 is S.

15. The method of claim 13 wherein X3 is WLRR (**SEQ ID NO: 1**).

16. The method of claim 14 wherein X5 is GLK.

17. The method of claim 15 wherein either X2 or X6 comprises a transduction domain.

20 18. The method of claim 16 wherein the transduction domain is selected from the group consisting of YARAAARQARA (**SEQ ID NO: 23**), YGRKKRRQRRR (**SEQ ID NO: 24**), WLRIKAWLRIKA (**SEQ ID NO: 26**), and WLRIKAWLRIKAWLRIKA (**SEQ ID NO: 27**).

19. The method of claim 16 wherein the polypeptide comprises
 25 YARAAARQARAWLRRRA(pS)APLPGLK (**SEQ ID NO: 28**).

20. The method of claim 16 wherein the polypeptide consists of
 YARAAARQARAWLRRRA(pS)APLPGLK (**SEQ ID NO: 28**).

21. The method of any one of claims 12-20 wherein the bronchospasm is associated with a disorder selected from the group consisting of asthma, chronic obstructive
 30 pulmonary disease (COPD), emphysema, bronchitis, and anaphylaxis.

22. The method of any one of claims 12-20 wherein the bronchospasm is associated with asthma.

23. A method for treating an airway smooth muscle disorder, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective

to treat an airway smooth muscle disorder of a polypeptide comprising an amino acid sequence according to general formula II:

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 33)

wherein X2 is absent or comprises a cell transduction domain;

5 X3 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of **SEQ ID NO: 34**;

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

10 X5 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of **SEQ ID NO: 34**; and

X6 is absent or comprises a cell transduction domain.

24. The method of claim 23, wherein X4 is phosphorylated.

25. The method of claim 24, wherein X4 is S.

26. The method of claim 25 wherein either X2 or X6 comprises a transduction
15 domain.

27. The method of claim 26 wherein the transduction domain is selected from the group consisting of YARAAARQARA (**SEQ ID NO: 23**), YGRKKRRQRRR (**SEQ ID NO: 24**), WLRIKAWLRIKA (**SEQ ID NO: 26**), and WLRIKAWLRIKAWLRIKA (**SEQ ID NO: 27**).

20 28. The method of any one of claims 23-27 wherein the airway smooth muscle disorder is selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), emphysema, bronchitis, and anaphylaxis.

29. The method of any one of claims 23-27 wherein the airway smooth muscle disorder is asthma.

25 30. A method for treating bronchospasm, comprising administering to a subject that is desensitized to beta agonist therapy an amount effective to treat bronchospasm of a polypeptide comprising an amino acid sequence according to general formula II:

X2-X3-A(X4)APLP-X5-X6 (SEQ ID NO: 33)

wherein X2 is absent or comprises a cell transduction domain;

30 X3 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of **SEQ ID NO: 34**;

X4 is selected from the group consisting of S, T, Y, D, E, hydroxylysine, hydroxyproline, phosphoserine analogs and phosphotyrosine analogs;

X5 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of **SEQ ID NO: 34**; and

X6 is absent or comprises a cell transduction domain.

31. The method of claim 30, wherein X4 is phosphorylated.

5 32. The method of claim 31, wherein X4 is S.

33. The method of claim 32 wherein either X2 or X6 comprises a transduction domain.

34. The method of claim 33 wherein the transduction domain is selected from the group consisting of YARAAARQARA (**SEQ ID NO: 23**), YGRKKRRQRRR (**SEQ ID NO: 24**), WLRRIKAWLRRRIKA (**SEQ ID NO: 26**), and
10 WLRRIKAWLRRRIKA (**SEQ ID NO: 27**).

35. The method of any one of claims 30-34 wherein the bronchospasm is associated with a disorder selected from the group consisting of asthma, chronic obstructive pulmonary disease (COPD), emphysema, bronchitis, and anaphylaxis.

15 36. The method of any one of claims 30-34 wherein bronchospasm is associated with asthma.

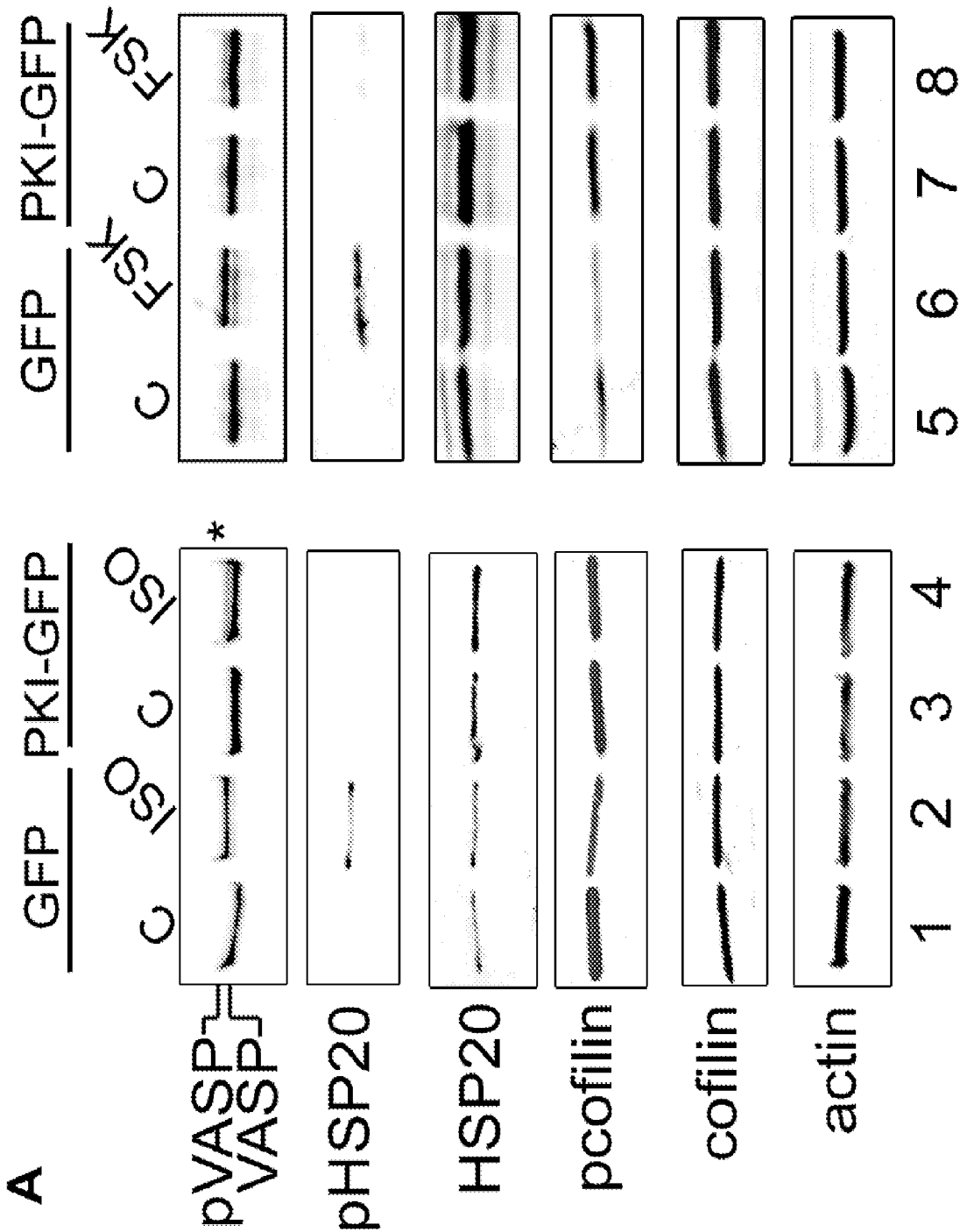


Fig.1

B

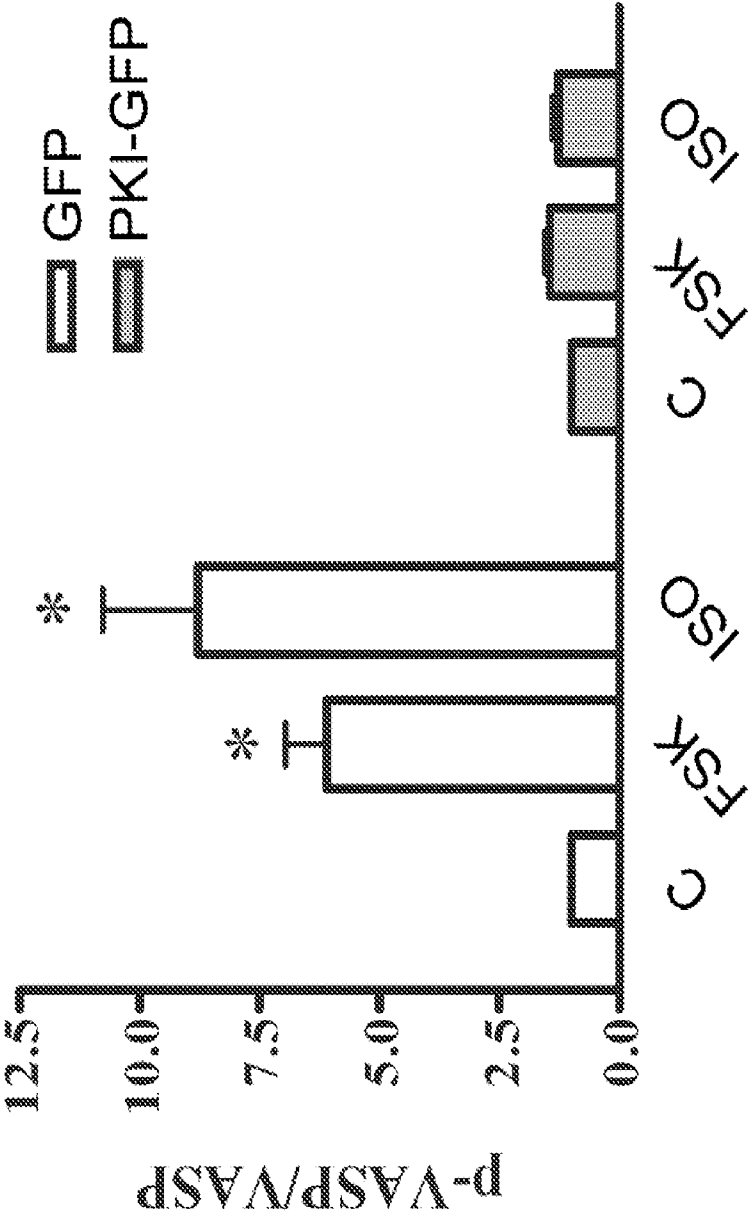


Fig. 1

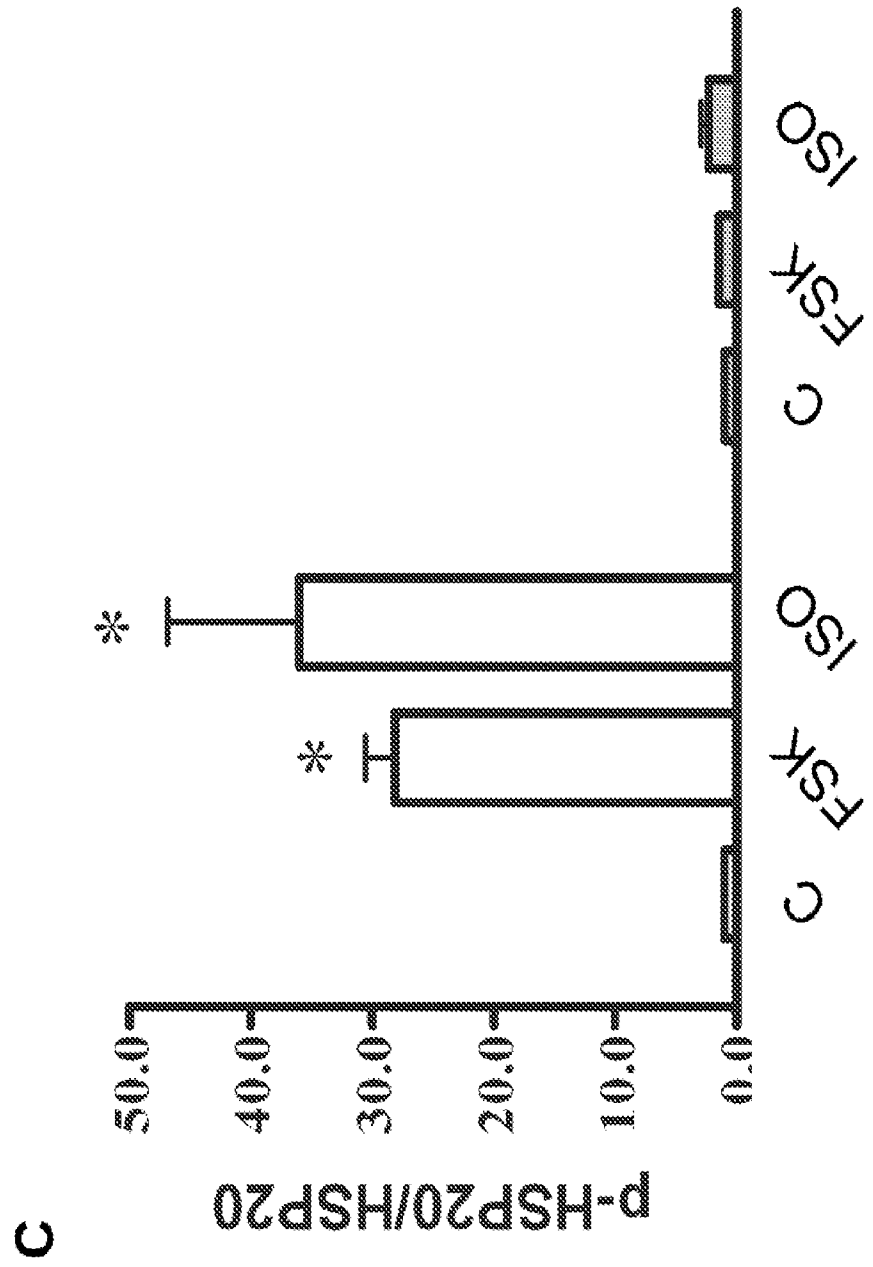


Fig.1

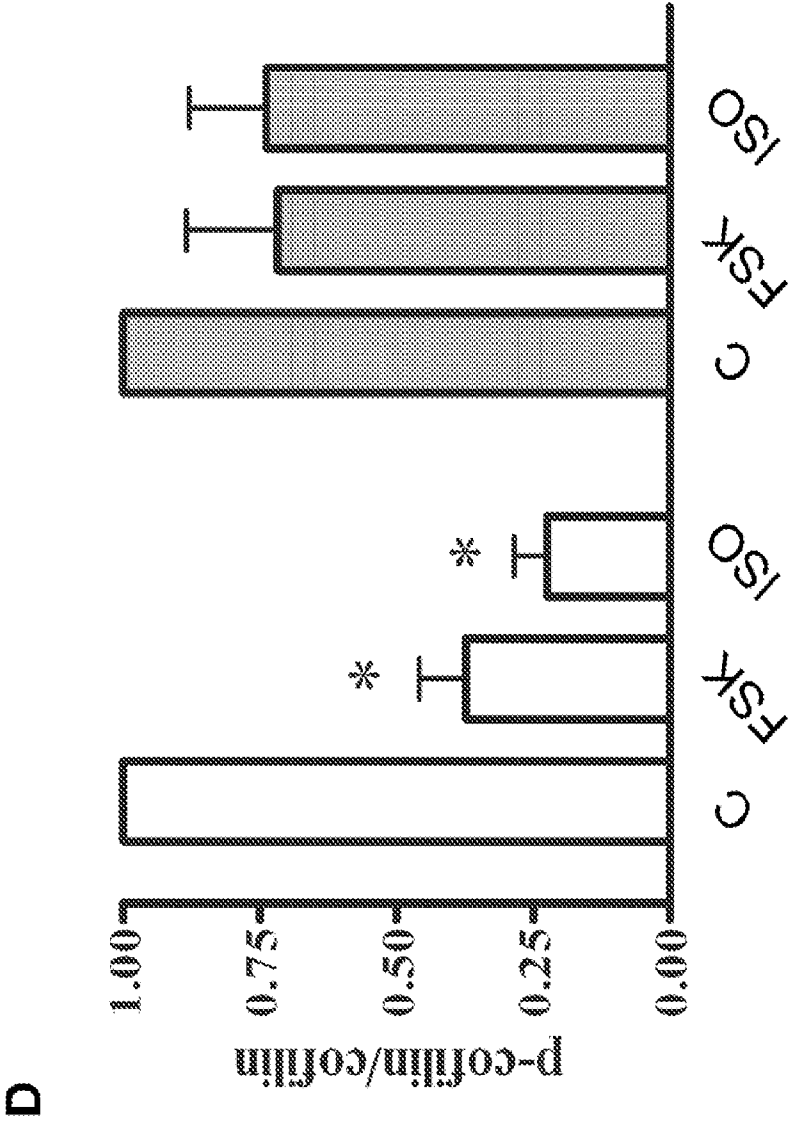
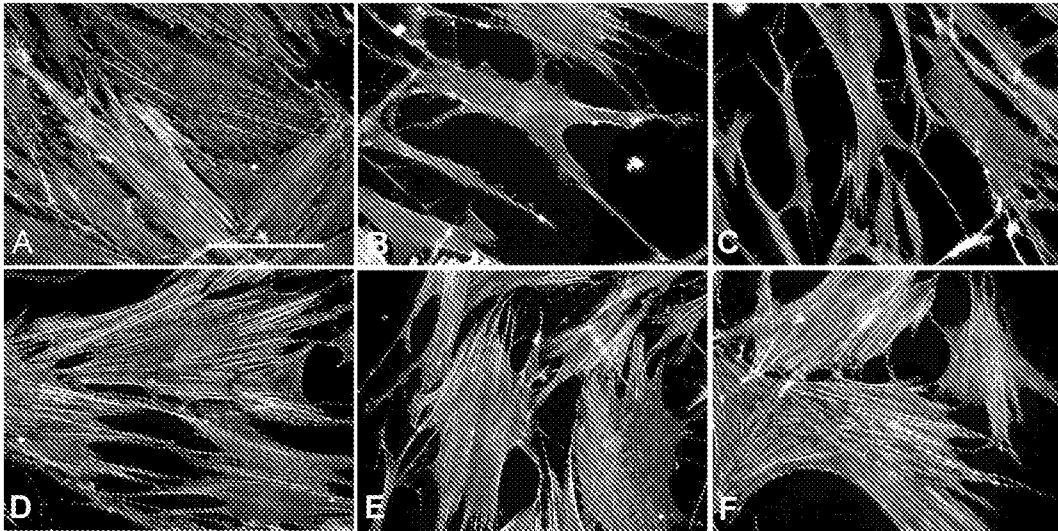


Fig.1

Figure 2:

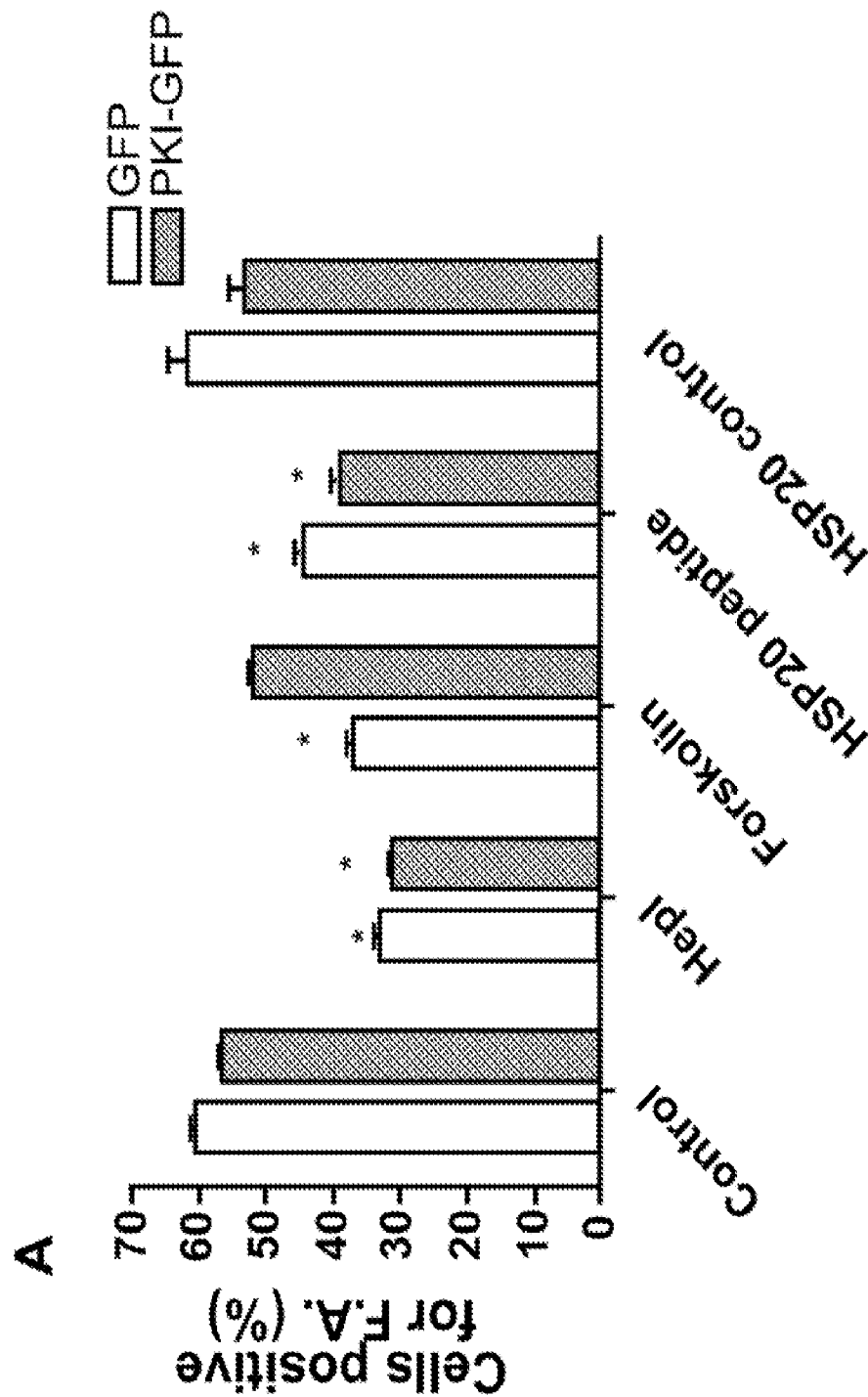


Fig. 3

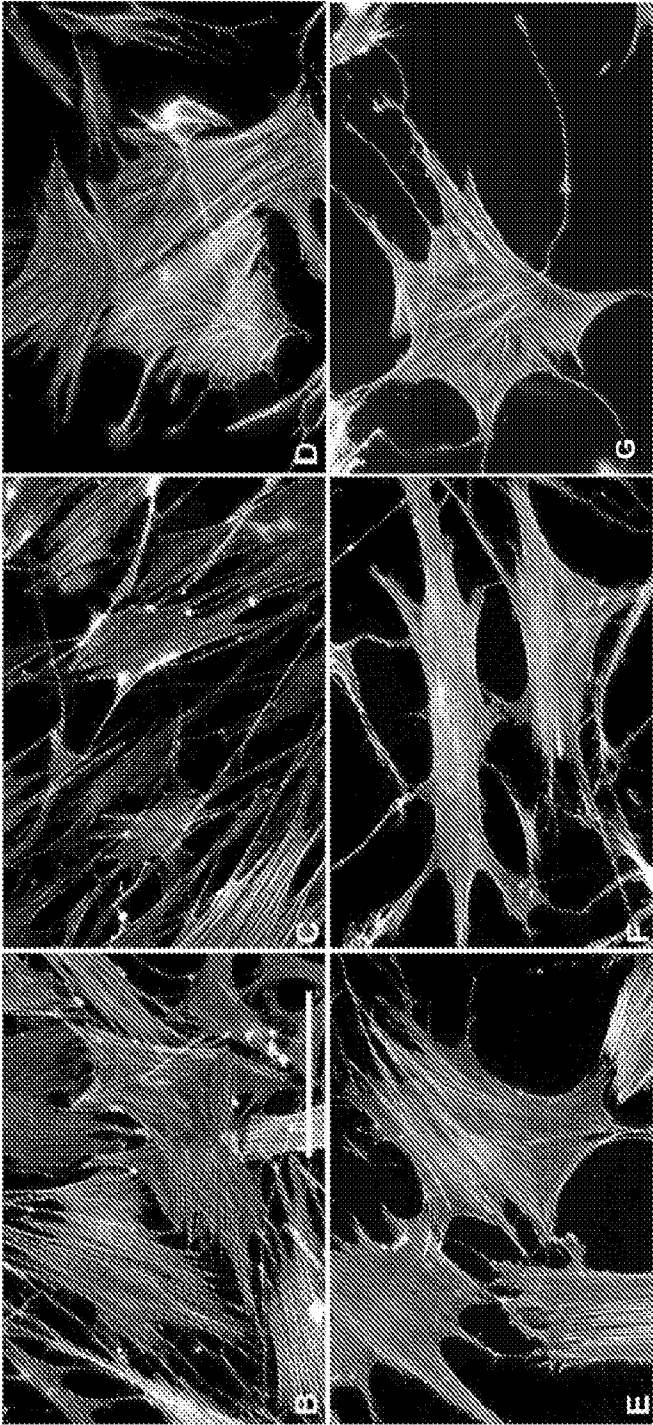
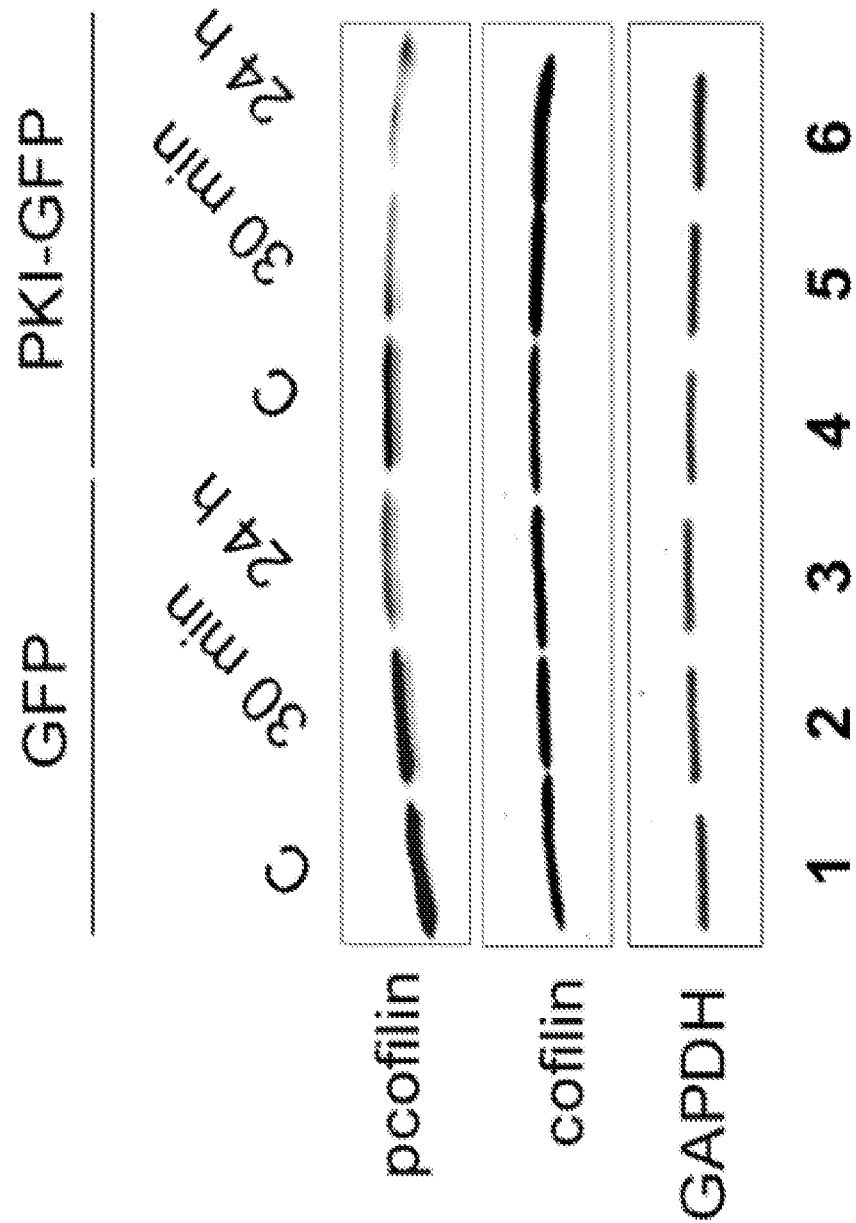


Fig. 3

Fig. 3
H



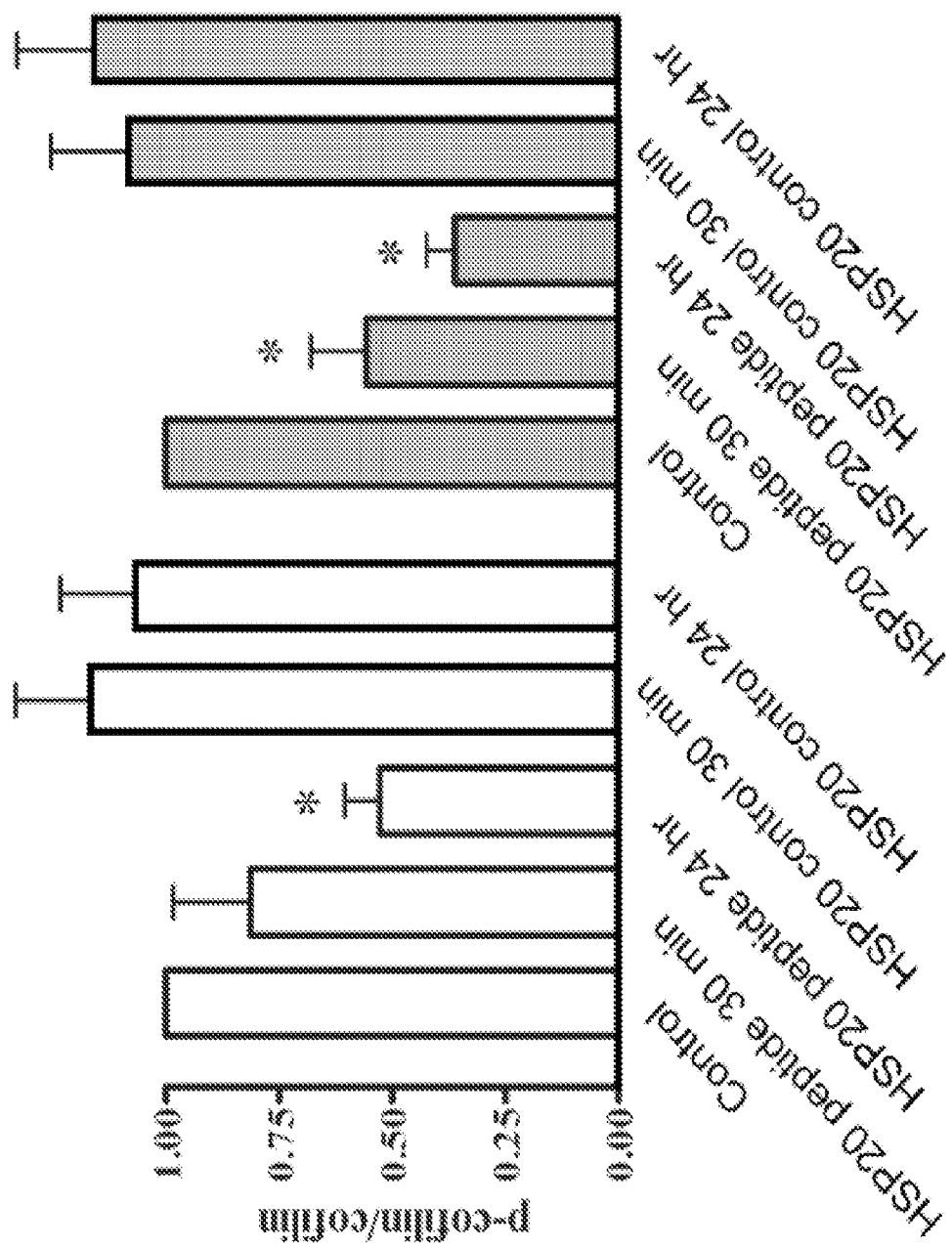


Fig. 3

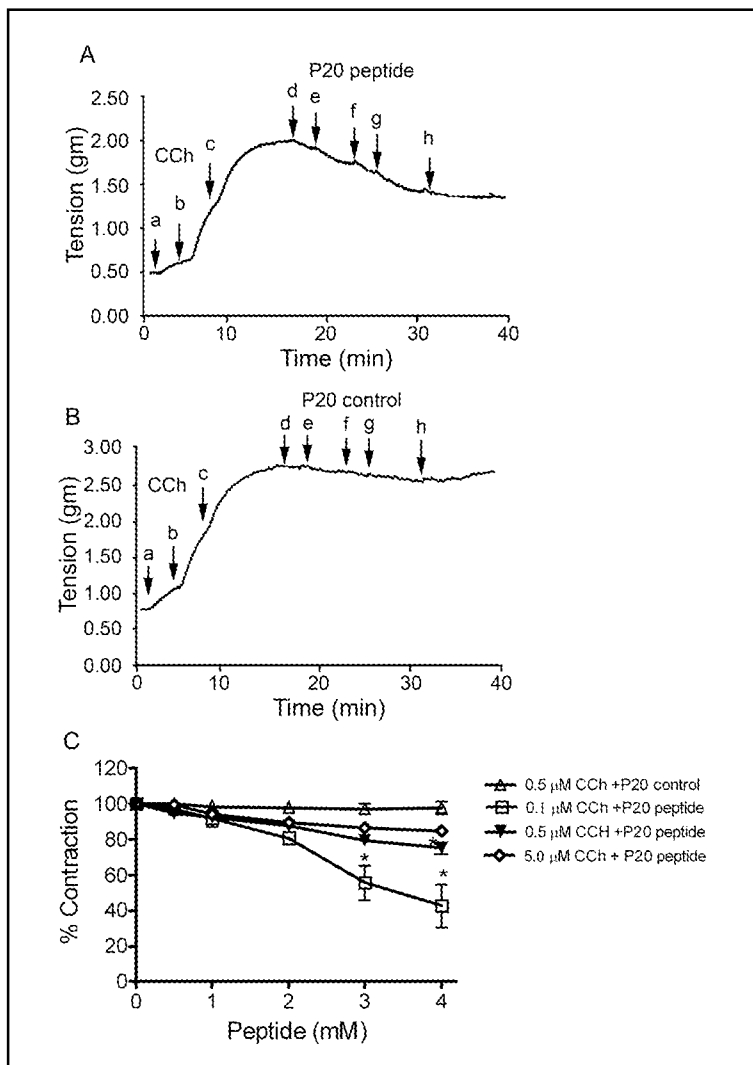
Figure 4:

Figure 5:

