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(54) **VARIANT OF BPIFB4 PROTEIN**

VARIANTE DES BPIFB4-PROTEINS

VARIANTE DE PROTÉINE BPIFB4

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- **KINANE DENIS F ET AL: "Human variability in innate immunity.", PERIODONTOLOGY, vol. 45, 2007, pages 14-34, XP002698131, ISSN: 0906-6713**

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- **FARHOUD RAYATNIA ET AL: "Nitric oxide involvement in consolidation, but not retrieval phase of cognitive performance enhanced by atorvastatin in mice", EUROPEAN JOURNAL OF PHARMACOLOGY, vol. 666, no. 1-3, 1 September 2011 (2011-09-01), pages 122-130, XP055345826, NL ISSN: 0014-2999, DOI: 10.1016/j.ejphar.2011.05.017**
- **MEHRAK JAVADI-PAYDAR ET AL: "Atorvastatin improved scopolamine-induced impairment in memory acquisition in mice: Involvement of nitric oxide", BRAIN RESEARCH, ELSEVIER, AMSTERDAM, NL, vol. 1386, 17 February 2011 (2011-02-17), pages 89-99, XP028186558, ISSN: 0006-8993, DOI: 10.1016/J.BRAINRES.2011.02.057 [retrieved on 2011-02-23]**

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Description

Field of the Invention

[0001] The present invention relates *inter alia* to a polynucleotide coding for a variant of BPIFB4 protein (Bactericidal/Permeability Increasing protein family B, member 4) for use for the treatment of pathologies associated with endothelial dysfunction due to impaired endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) mediated vasodilatation.

Background of the Invention

[0002] Human BPIFB4 (also known as C20orf186; RY2G5; LPLUNC4) is a secreted protein member of the BPI/LBP/PLUNC-like family, which has been implicated in host defence processes against bacteria. The protein exists as two different isoforms of different length with amino acid sequences of 575 (Acc. P-59827-2) and 613 (Acc. EAW76337.1) amino acids (Bingle CD et al, Biochem Soc Trans. (2011) 39 (4): 977-83; Andrault J.-B et al, Genomics (2003) 82: 172-184; Bingle C.D et al., Hum. Mol. Genet. (2002) 11: 937-943; Bingle C.D et al, Protein Sci. (2004) 13: 422-430).

[0003] A number of single nucleotide polymorphisms have been described for this protein at the following sites, indicated with reference to the 575 amino acid sequence: rs2070325-Ile229Val, rs571391-Asn281Thr, rs7583529Phe488Leu and rs285097-Thr494Ile, that may lead to the generation of a number of different variants of the protein. The present inventors have identified and characterised a number of variants of BPIFB4. After a careful analysis of the haplotype phases (i.e. combination of the alleles) of the four polymorphisms described above, the present inventors have found that the most common haplotype (65% analyzed chromosomes) is the combination AACT that codes for amino acids Ile229/Asn281/Leu488/Ile494 (INLI); the second most frequent haplotype is the combination GCTC (30% chromosomes contain this haplotype) that codes for amino acids Val229/Thr281/Phe488/Thr494 (VTFT) and finally the combination of AATC is represented only in 2% of human Caucasian chromosomes that code for Ile229/Asn281/Phe488/Thr494 (INFT).

[0004] The vascular endothelium is formed by a layer of cells located between the vessel lumen and the vascular smooth muscle cells. These cells continuously produce NO, a soluble gas that is synthesized by the enzyme eNOS. This substance has a crucial role in the regulation of vascular homeostasis and endothelial function, including modulation of the vascular tone, regulation of local cell growth, and protection of the vessel from injurious consequences of platelets and cells circulating in blood.

[0005] A growing list of conditions have been associated with a decreased release of nitric oxide by the arterial wall either because of impaired synthesis by eNOS or excessive oxidative degradation (American Journal of Physiology, Endocrinology and Metabolism 2012 Mar 1; 302(5) and Current Vascular Pharmacology 2012 Jan; 10(1): pages 4-18). Most of these pathological conditions are associated with aging. For example, impairment of Nitric Oxide signalling has been reported in coronary spastic angina (Miyamoto Y et al. Hum Mol Genet. 2000 Nov 1; 9(18): pages 2629-37), thrombosis (Loscalzo J, Circulation Research. 2001; 88, pages 756-762), pulmonary hypertension (D'Uscio LD., Cardiovasc Res 2011, 92 (3), pages 359-360), pre-eclampsia (The Lancet, Volume 361, 9368, Pages 1511-1517), vasculitis (Kanwar JR et al., Curr Med Chem. 2009; 16(19): 2373-2394), cancer (Kanwar JR et al. Curr Med Chem. 2009; 16(19): pages 2373-2394), inflammatory disorders (Kanwar JR et al., Curr Med Chem. 2009; 16(19): pages 2373-2394), venous insufficiency (Förstermann U et al. Circulation. 2006; 113: pages 1708-1714), in genetic diseases with reduced eNOS activity and NO production, for example as for MTHFR gene variations (Lemarie CA et al., Am J Physiol Heart Circ Physiol 2011, vol. 300: H745-53), arterial hypertension (Sparacino-Watkins CE et al, Circulation., 2012; vol 125(23), pages 2824-6; Böger RH et al, Circulation. 2009, vol 119(12), pages 1592-600), atherosclerosis, diabetes mellitus, dyslipidemia, renal failure (Jiang B et al, Hum Gene Ther. 2012; 23(11), pages 1166-75 Ponnuswamy Pet al. PLoS One. 2012; 7(1):e30193; Vita JA. et al, Circulation. 2011, Vol 124(25), pages 906-12; Li ZL et al., PLoS One. 2012, Vol 7(6):e38787), metabolic syndrome (Quyyumi AA et al., Circulation. 1995, Vol 92: pages 320-326), stroke (Madden JA., Neurology. 2012 Sep 25;79(13 Suppl 1):S58-62), myocardial infarction (Nakata S et al, Circulation. 2008 Apr 29; Vol 117(17): pages 2211-23), erectile dysfunction (Bianca Rd et., PLoS One. 2012, Vol 7(2): e31019), neurodegenerative diseases and multiple sclerosis (Faraci FM., Circulation Research. 2006, Volume 99, pages 1029-1030; Wu M, et al, Glia. 2009, Vol 57(11), pages 1204-15), cognitive disorders (Rayatnia et al, Eur J Pharmacol. 2011, Vol 666(1-3), pages 122-30; Paydar et al, Brain Res. 2011; Vol 1386, pages 89-99), retinal degeneration, uveoretinitis, vascular retinopathy, cataracts and glaucoma (Chiou g et al. Journal of Ocular Pharmacology and Therapeutics. April 2001, 17(2): pages 189-198, Li Q et al, Invest Ophthalmol Vis Sci. 2010 Oct, 51(10): pages 5240-6, Kwak HJ et al, Mol Cells. 2001 Oct 31;12(2):pages 178-84).

[0006] WO2001/79269 (Grell) discloses a polypeptide of length 614 amino acids said to be a New Lipid Binding Protein 4 polypeptide.

[0007] The decreased production of NO and the consequent disequilibrium in endothelial function has been identified as one of the key factors responsible for the above pathological states. Thus, there have been efforts in the art to identify potential candidate therapies to reverse endothelial dysfunction by enhancing the release of nitric oxide from the endothelium.

[0008] Furthermore, an increase in eNOS activity/NO production has been demonstrated to be beneficial in

post-exercise fatigue in muscular dystrophy patients (Nature. 2008 Nov 27; 456, pages 511-515) and in the implantation of stents for vascular occlusions (Sharif F, et al. Mol Ther. 2008 Oct;16(10): pages 1674-80.).

[0009] The present inventors have now surprisingly identified that a specific variant of the BPIFB4 protein is associated with exceptional longevity. The inventors have further found that the variant identified is surprisingly able to increase the activation of eNOS and the production of NO in endothelial cells. These biological properties are dependent on the presence in the protein of four specific amino acids at positions 229, 281, 488 and 494 since replacement of any of these positions with different amino acids leads to loss of activity of the protein.

Summary of the Invention

[0010] Accordingly, the present invention provides a polynucleotide coding for

(i) a BPIFB4 protein consisting of SEQ ID NO: 1;
(ii) a BPIFB4 protein variant of the BPIFB4 protein of (i) having at least 95% homology to that of SEQ ID NO: 1; or
(iii) a fragment of the protein of (i) or the variant of (ii),

wherein said BPIFB4 protein variant or fragment comprises a Valine at the position corresponding to position 229 of SEQ ID NO: 1, a Threonine at the position corresponding to position 281 SEQ ID NO: 1, a Phenylalanine at the position corresponding to position 488 of SEQ ID NO: 1 and a Threonine at the position corresponding to position 494 of SEQ ID NO: 1 and said BPIFB4 protein, variant or fragment has activity in increasing the activity of eNOS and/or the production of NO for use in therapy.

[0011] Said homology in the amino acid sequence is preferably at least 99%.

[0012] According to a particularly preferred embodiment, the protein coded for by the said polynucleotide has the amino acid sequence of SEQ ID NO: 1.

[0013] According to an alternative preferred embodiment, the protein coded for by the said polynucleotide has an amino acid sequence corresponding to the sequence of SEQ ID NO 1, wherein one or more amino acids at positions different from positions 229, 281, 488 and 494 of SEQ ID NO 1 have been substituted by a conserved amino acid. By "conserved amino acid" it is meant an amino acid with functional and physicochemical properties equivalent to those of the original amino acid.

[0014] The invention further provides a polynucleotide for use in therapy wherein the polynucleotide has a nucleotide sequence coding for the above protein and a vector for use in therapy containing said polynucleotide operatively linked to expression control sequences. According to a preferred embodiment, said polynucleotide has the sequence of SEQ ID NO: 2.

[0015] The disclosure also provides a host cell that has been transformed with the above vector and it is able to express the protein coded for by the said polynucleotide.

[0016] In particular, an object of the invention is the polynucleotide or vector for use in the prevention, reduction of the risk of, amelioration and/or treatment of endothelial dysfunctions due to a decrease in the activity of eNOS and/or in the production of NO or of pathologies or conditions where it is beneficial to increase the activity of eNOS and/or the production of NO. According to a preferred embodiment, the above protein, polynucleotide or vector is for use in the prevention, reduction of the risk, amelioration or treatment of a pathology or condition selected from arterial hypertension, atherosclerosis, diabetes mellitus, dyslipidemia, renal failure, metabolic syndrome, stroke, myocardial infarction, erectile dysfunction, neurodegenerative diseases, multiple sclerosis, cognitive disorders, retinal degeneration, uveoretinitis, vascular retinopathy, cataracts, glaucoma, coronary spastic angina, thrombosis, pulmonary hypertension, pre-eclampsia, vasculitis, cancer, inflammatory disorders, venous insufficiency, genetic diseases with reduced eNOS activity and NO production, for example MTHFR gene variations, post-exercise fatigue in muscular dystrophy patients. According to a further preferred embodiment, the above polynucleotide or vector is for use as a co-adjuvant in the implantation of one or more stents, preferably medicated, for vascular occlusions.

[0017] Finally, the present invention provides a pharmaceutical composition comprising the polynucleotide of the disclosure in combination with pharmaceutically acceptable carriers and excipients.

[0018] Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Figures

[0019]

Figure 1 shows the sequence of the pRK5 vector encoding INFT hBPIFB4 (SEQ ID NO: 3) or VTFT hBPIFB4 (SEQ ID NO:1) used in Example 3, with the sequence of the BPIFB4 protein underlined and that of EGFP in italics.

Figure 2 shows detection of green fluorescent protein in mesenteric vessels perfused ex *vivo* with a plasmid encoding INFT BPIFB4 (left panel) or a control empty pRK5 plasmid (right panel) in Example 3.

Figure 3 represents BPIFB4 protein expression and eNOS activation in mesenteric vessels perfused with empty vector (EV), a plasmid encoding INFT hBPIFB4 or VTFT hBPIFB4. Panel a shows a Western blot of seven pooled experiments and detection of BPIFB4 (top) and P-eNOS S1177 (middle). Panel b shows quantification of BPIFB4 expression and panel c shows quantification of phosphorylation at serine 1177 of eNOS.

Figures 4, 5 and 6: panels 4a, 5a and 6a represent KCl-induced vasoconstriction observed in Example 3 in mesenteric vessels perfused ex *vivo* with an empty plasmid pRK5 plasmid (EV/Fig 4a), a pRK5 plasmid encoding INFT hBPIFB4 (INFT /Fig 5a), or a pRK5 plasmid encoding VTFT hBPIFB4 (VTFT /Fig 6a). Panels 4b, 5b and 6b represent phenylephrine-induced vasoconstriction observed in Example 3 in mesenteric vessels perfused ex *vivo* with an empty plasmid pRK5 plasmid (EV/ Fig 4b), a pRK5 plasmid encoding INFT hBPIFB4 (INFT / Fig 5b) or a pRK5 plasmid encoding VTFT hBPIFB4 (VTFT / Fig 6b). Panels 4c, 5c and 6c represent acetylcholine-induced vasodilatation observed in Example 3 in mesenteric vessels perfused ex *vivo* with an empty plasmid pRK5 plasmid (EV/ Fig 4c), a pRK5 plasmid encoding INFT hBPIFB4 (INFT/ Fig 5c) or a pRK5 plasmid encoding VTFT BPIFB4 (VTFT hBPIFB4/ Fig 6c). The results observed with the plasmid encoding VNFT hBPIFB4 (SEQ ID NO: 4), ITFT hBPIFB4 (SEQ ID NO: 5), VTLI hBPIFB4 (SEQ ID NO: 6) and INLI hBPIFB4 (SEQ ID NO: 7) observed on KCl-induced vasoconstriction, phenylephrine-induced vasoconstriction or acetylcholine-induced vasodilatation in mesenteric vessels perfused are superimposable to those obtained with the empty vector. (data not shown).

Figures 7 and 8: panels 7a and 8a represent the effect of the eNOS inhibitor L-NAME on acetylcholine-induced relaxation of vessels perfused ex *vivo* or with an empty pRK5 plasmid (EV/

Fig 7a) or a pRK5 plasmid encoding mutated VTFT hBPIFB4 (VTFT/ Fig 8a). Panel 8b represents recovery of vasorelaxation of vessels from methylenetetrahydrofolate reductase knockout mice ($Mthfr^{+/-}$) control ($Mthfr^{+/+}$) and knockout mice treated with either empty pRK5 plasmid ((Mthfr+/-EV) (Fig 7b) or a pRK5 plasmid encoding VTFT hBPIFB4 (($Mthfr^{+/-}$ - M) (Fig 8b).

Figure 9: panel a) shows a RT-PCR demonstrating induction of expression of BPIFB4 by H_2O_2 in HEK293T cells. Panel b), shows a Western blot of the phosphorylation on eNOS at Ser1177 in HEK293T cells expressing VTFT hBPIFB4 (VTFT) and in cells overexpressing INFT hBPIFB4 (INFT) or those exposed to an empty vector (EV). Panel c), top, shows β-actin-normalized ODs.

Detailed Description of the Invention

[0020] A first object of the present invention is a polynucleotide coding for

(i) a BPIFB4 protein consisting of SEQ ID NO: 1;
(ii) a BPIFB4 protein variant of the BPIFB4 protein of (i) having at least 95% homology to that of SEQ ID NO: 1; or
(iii) a fragment of the protein of (i) or the variant of (ii),

wherein said BPIFB4 protein variant or fragment comprises a Valine at the position corresponding to position 229 of SEQ ID NO: 1 (hereinafter referred to as Valine 229), a Threonine at the position corresponding to position 281 SEQ ID NO: 1 (hereinafter referred to as Threonine 281), a Phenylalanine at the position corresponding to position 488 of SEQ ID NO: 1 (hereinafter referred to as Phenylalanine 488) and a Threonine at the position corresponding to position 494 of SEQ ID NO: 1 (hereinafter referred to as Threonine 494) and said BPIFB4 protein, variant or fragment has activity in increasing the activity of eNOS and/or the production of NO for use in therapy..

[0021] Said homology is preferably at least 99%.

[0022] The amino acid sequence of the BPIFB4 protein variant may differ from that of SEQ ID NO: 1 due to the presence of additions, deletions or further substitutions of amino acids.

[0023] However, an essential feature of the variant is that it contains the above said four amino acids. In case of homologs that differ from SEQ ID NO: 1 due to deletions or additions of amino acids, the above four amino acids are present at the position that corresponds to its original position in SEQ ID NO:1. In case of homologs that differ from SEQ ID NO: 1 due to substitution of amino acids, the above four amino acids are present in the same position as in SEQ ID NO: 1. According to a preferred embodiment, the protein has an amino acid sequence corresponding to SEQ ID NO 1, wherein one or more amino acids at positions different from positions 229, 281, 488 and 494 of SEQ ID NO 1 have been substituted by a conserved amino acid. By "conserved amino acid" it is meant an amino acid with functional and physicochemical properties equivalent to those of the original amino acid.

[0024] Particularly preferred proteins have the amino acid sequence of known BPIFB4 proteins identified in *Homo Sapiens* (SEQ ID NO: 1) and *Pan Troglodytes* (Acc N XP525303) which has been modified so that it comprises a Valine at the position corresponding to position 229 of SEQ ID NO:1, a Threonine at the position corresponding to position 281 SEQ ID NO: 1, a Phenylalanine at the position corresponding to position 488 of SEQ ID NO: 1 and a Threonine at the position corresponding to position 494 of SEQ ID NO: 1.

[0025] According to a particularly preferred embodiment the BPIFB4 protein encoded by the polynucleotide for use in therapy of the invention has the sequence of SEQ ID NO:1. A protein having such sequence will be hereinafter called VTFT hBPIFB4.

[0026] A second object of the present invention is a polynucleotide for use in therapy of the invention coding for a protein having a sequence which consists of the amino acid sequence of a BPIFB4 protein, variant or fragment of the disclosure linked to an additional amino acid sequence able to impart to the protein particularly advantageous properties. Preferably, said additional amino acid sequence is useful for identifying the said BPIFB4 pro-

tein, variant or fragment or to target the said BPIFB4 protein, variant or fragment to a specific organ or tissue. Preferably said protein is a chimeric protein.

[0027] As will be described in detail in the experimental section, the present inventors have surprisingly found that the above VTFT hBPIFB4 is associated with exceptional longevity in three independent populations. The present inventors have further demonstrated that the beneficial effect of the mutant protein on life expectancy is a consequence of its ability to modulate vascular dysfunctions associated with aging. As demonstrated in the experimental section, this modulation is dependent on the presence of the specific four amino acids at positions corresponding to positions 229, 281, 488 and 494 of SEQ ID NO:1 in the VTFT hBPIFB4 of the disclosure.

[0028] As shown in Example 3, mouse mesenteric vessels were perfused ex *vivo* with an empty plasmid or plasmids encoding VTFT hBPIFB4 or proteins that differ from VTFT hBPIFB4 in that they show various substitutions at the 4 relevant amino acids: INFT hBPIFB4, having the amino acid sequence of SEQ ID NO: 3, which differs from that of VTFT hBPIFB4 in that it contains Isoleucine and an Asparagine at positions 229 and 281, respectively, VNFT hBPIFB4, having the amino acid sequence of SEQ ID NO: 4, which differs from that of VTFT hBPIFB4 in that it contains an Asparagine at position 281, ITFT hBPIFB4, having the amino acid sequence of SEQ ID NO: 5, which differs from that of VTFT hBPIFB4 in that it contains Isoleucine at position 229, VTLI hBPIFB4 , having the amino acid sequence of SEQ ID NO: 6, which differs from that of VTFT hBPIFB4 in that it contains a Leucine at position 488 and an Isoleucine at position 494, INLI hBPIFB4 , having the amino acid sequence of SEQ ID NO: 7, which differs from that of VTFT hBPIFB4 in that it contains Isoleucine at position 229, Asparagine at position 281, Leucine at position 488 and an Isoleucine at position 494. While VNFT hBPIFB4, ITFT hBPIFB4, VTLI hBPIFB4 and INLI hBPIFB4 did not show any effect on vascular function and INFT hBPIFB4 strongly inhibited any vascular function, blocking both vasoconstriction and vasodilatation, the VTFT BPIFB4 protein showed a weak effect on inhibition of vasoconstriction and a significant enhancement of vasodilatation. This effect has been demonstrated to be mediated by activation of eNOS through phosphorylation on serine 1177 and it is therefore associated with an increase in the release of NO by endothelial cells. The ability of VTFT hBPIFB4 to induce activation of eNOS has been corroborated in the cell line HEK293T (Example 5).

[0029] The above data have also been further confirmed in an animal model of vascular disease linked to impaired NO production, the heterozygotic Mthfr knockout mice, wherein the transfection of VTFT hBPIFB4 protein has been shown to restore NO release and endothelium-dependent vasodilatation response (Example 4).

[0030] A further object of the present disclosure is a fragment of the BPIFB4 protein or variant of the disclosure having a sequence comprising the above said Valine 229, Threonine 281, Phenylalanine 488 and Threonine 494.

[0031] Thanks to their biological activity, the above said BPIFB4 protein variant, protein or fragment of the disclosure may advantageously be used in the prevention, reduction of the risk of, amelioration and/or treatment of pathological conditions of the endothelium due to decreased production of NO or activity of eNOS or of pathologies or conditions where it is beneficial to increase the activity of eNOS and/or the production of NO.

[0032] Thus, a further object of the disclosure is the above said BPIFB4 protein variant, protein or fragment for use in therapy.

[0033] Preferably, the BPIFB4 protein variant, the protein or the fragment disclosed herein are for use in the prevention, reduction of the risk of, amelioration and/or treatment of an endothelial dysfunction due to release of NO from endothelial cells below the physiological levels or a decrease in the activity of eNOS or in clinical situations wherein it is beneficial to obtain an increase in the activation of eNOS and/or in the production of NO. According to a preferred embodiment of the disclosure, said BPIFB4 protein variant, said protein or said fragment of the disclosure are for use in the prevention, reduction of the risk, amelioration or treatment of a pathology selected from arterial hypertension, atherosclerosis, diabetes mellitus, dyslipidemia, renal failure, metabolic syndrome, stroke, myocardial infarction, erectile dysfunction, neurodegenerative diseases, multiple sclerosis, cognitive disorders, retinal degeneration, uveoretinitis, vascular retinopathy, cataracts, glaucoma, coronary spastic angina, thrombosis, pulmonary hypertension, pre-eclampsia, vasculitis, cancer, inflammatory disorders, venous insufficiency, genetic diseases with reduced eNOS activity and NO production, for example MTHFR gene variations.

[0034] According to an alternative preferred embodiment of the disclosure, said BPIFB4 protein, variant or fragment is for use in the improvement of post-exercise fatigue in muscular dystrophy patients and as a co-adjuvant in the implantation of one or more stents, preferably medicated, for vascular occlusions.

[0035] The BPIFB4 protein variant, the protein or the fragment according to the disclosure may be administered to a subject in need thereof, affected by one of the above pathologies or in the above clinical conditions, by oral, nasal, endovenous, topical, intra- or retro- ocular administration.

[0036] Accordingly, a further object of the disclosure is a pharmaceutical composition, preferably suitable for oral, nasal, endovenous topical, intra- or retro- ocular administration, comprising the BPIFB4 protein variant, the protein or the fragment of the disclosure in admixture with pharmaceutically acceptable carriers and/or excipients. Suitable formulations for the pharmaceutical composition of the disclosure are well known in the art and are, for example, described in "Remington's Pharmaceutical Sciences Handbook", Mack Publishing Company, Easton, Pennsylvania, last or Babizhayev MA. Drug Testing and

Analysis, Volume 4, Issue 6, pages 468-485, June 2012).

[0037] A particularly suitable pharmaceutical formulation for the administration of the BPIFB4 protein variant, the protein or the fragment according to the disclosure is based on synthetic copolymers, using polyaminoacidic and polysaccharidic structures, able to form reversible physical complexes with the BPIFB4 protein variant, the protein or the fragment thereof by electrostatic, hydrophobic or other physical interactions, and generate nanoaggregates from which the protein or fragment is released in intact form after administration. (Diaz-Fernandez YA et al, Biosens Bioelectron. 2010 Sep 15;26(1):29-35).

[0038] A further object of the present disclosure is a polynucleotide, preferably a DNA polynucleotide, coding for the amino acid sequence of the BPIFB4 protein variant, the protein or the polypeptide according to the present disclosure. According to a preferred embodiment, said polynucleotide has a sequence which comprises or consists of SEQ ID NO: 2 or the sequence of a fragment thereof comprising the nucleotides coding for the above said Valine 229, Threonine 281, Phenylalanine 488 and Threonine 494.

[0039] The above polynucleotide may be used in order to obtain expression of the mutated protein or polypeptide in host cells either *in vitro, ex vivo* or *in vivo* by means of a suitable expression vector comprising it.

[0040] Thus, a further object of the disclosure is a vector containing the above said polynucleotide of the disclosure operatively linked to expression control sequences.

[0041] According to a preferred embodiment, the BPIFB4 protein variant, the protein or the fragment of the disclosure is recombinantly produced in host cells transfected with the above said vector. According to this embodiment the vector of the disclosure is preferably one that it is suitable for high yield production of the protein or polynucleotide. For example, the pcDNA™3.3-TOPO® vector can be used for high level expression of the protein of the disclosure in adherent mammalian tissue culture cells following transient transfection, or high level expression of secreted protein using the Freestyle™ MAX CHO and Freestyle™ MAX 293 systems (Invitrogen INC.)

[0042] Thus, a further object of the present disclosure is host cells transfected with the above said vector of the disclosure.

[0043] A further object of the disclosure is a method of recombinantly producing the BPIFB4 protein variant, the protein or the fragment according to the disclosure comprising culturing the above said host cells under conditions allowing expression of the BPIFB4 protein variant, the protein or the fragment and recovering said BPIFB4 protein variant, protein or fragment.

[0044] Alternatively to direct administration as such, the BPIFB4 protein variant, the protein or the fragment of the disclosure may be expressed in the target tissue following administration, preferably via the endovenous, subcutaneous, intraocular or retroocular route, into a subject in need thereof of a vector according to the present disclosure, which is suitable to induce expression in said target tissue of the mutated protein or polypeptide. The target tissue may differ depending on the pathology to be treated and may be, for example, the endothelial tissue, the tissue of the liver, heart, kidney, eye or muscle.

[0045] According to this embodiment, the vector of the disclosure is one that is preferably suitable for transfection of the cells of the target tissue of interest following endovenous administration.

[0046] According to a particularly preferred embodiment, said vector is a viral vector, preferably an Adenovirus vector, more preferably a vector selected from AAV serotypes 1-9 vectors, on the basis of specificity for the target tissue of interest (Varadi K, et al, Gene Ther. (2012); 19 (8):800-9; Zincarelli C et al, Mol Ther. (2008), 16(6): 1073-80, Diaz-Fernandez YA et al, Oligonucleotides. 2010; 20(4): 191-8.).

[0047] Thus, a third object of the invention is the above said polynucleotide or vector of the disclosure for use in therapy. Preferably, said polynucleotide or vector is for use in the prevention, reduction of the risk of, amelioration or treatment of an endothelial dysfunction due to release of NO from endothelial cells below the physiological levels or a decrease in the activity of eNOS or in conditions wherein it is beneficial to obtain an increase in the activation of eNOS and/or in the production of NO. According to a preferred embodiment of the invention, said polynucleotide or vector is for use in the prevention, reduction of the risk of, amelioration or treatment of a pathology or condition selected from arterial hypertension, atherosclerosis, hypertension, diabetes mellitus, dyslipidemia, renal failure, metabolic syndrome, stroke, myocardial infarction, erectile dysfunction, neurodegenerative diseases, multiple sclerosis, cognitive disorders, retinal degeneration, uveoretinitis, vascular retinopathy, cataracts, glaucoma, coronary spastic angina, thrombosis, pulmonary hypertension, pre-eclampsia, vasculitis, cancer, inflammatory disorders, venous insufficiency, genetic diseases with reduced eNOS activity and NO production, for example MTHFR gene variations.

[0048] According to an alternative preferred embodiment of the invention, said polynucleotide or vector is for use in the improvement of post-exercise fatigue in muscular dystrophy patients and as a co-adjuvant in the implantation of one or more stents, preferably medicated, for vascular occlusions. A fourth object of the invention is a pharmaceutical composition, preferably suitable for endovenous, subcutaneous, intraocular or retroocular administration, comprising a vector according to the invention in admixture with pharmaceutically acceptable carriers and/or excipients. Suitable formulations for the pharmaceutical composition of the invention are well known in the art. As an example, polymeric-based nanosystems or polycomplex nanosystems may be used to deliver the vector of the disclosure (Murano E et al, Nat Prod Commun. (2011), 6(4): 555-72, Moustafine RI et al, Int J Pharm. 2012 Oct 3)..

[0049] The mean daily dosage of the BPIFB4 protein variant, the protein or the fragment or vector of the disclosure will depend upon various factors, such as the seriousness of the disease and the conditions of the patient (age, sex and weight). The skilled man may use technical means well known in the art in order to find the correct dosage amount and regime to ensure optimal treatment in each particular pathological condition.

[0050] The present invention will be better illustrated by the Examples that follow.

Examples

Example 1: Identification of the VTFT hBPIFB4 protein in three independent populations

[0051] A recently published Genome Wide Association Study (GWAS) conducted on a Southern Italian Centenarian (SIC) population has identified a number of genetic variants associated with long lived individuals (Malovini et al, Rejuvenation Research 2011; Vol. 14(3), pages 283-291).

[0052] In order to validate the top four variations reported in that study ($p<1\times10^{-4}$) a replication attempt was carried out in a first replication cohort recruited for the German Centenary Study (Keidorp et al; Aging Cell 2011; Vol 10, pages 622-8), comprising 1447 long-living individuals (LLIs) (age range of 95-110 years, mean age 98.8 years) and 1029 younger controls (age range 60-75 years and mean age 66,8 years). Thus, two non-synonymous single-nucleotide polymorphisms (SNPs), rs2070325 and rs571391, and two intronic markers, rs7583529 and rs285097, which tag the functional variants rs7917 and rs1695501, have been tested by Taqman Analysis.

[0053] In detail, DNA was extracted from peripheral blood (QIAamp DNA blood midi kit, Qiagen) of the individuals and genotyped with TaqMan probe on ABI 7900HT Real Time PCR (Applied Biosystems). For the screening, the following probes were used:

hCV25757827 for rs2070325;
hCV958887 for rs571391;
hCV28993331 for rs7583529; and
hCV3073023 for rs285097.

[0054] Data analysis was performed with Sequence Detection Systems (Applied Biosystems).

[0055] The statistical methods and procedures applied to the analysis of data deriving from the genome wide scan are described in Malovini A et al., Rejuvenation Res 2011, Vol. 14, pages 283-91.

[0056] Of the four variants tested, only rs2070325, which results in the amino acid change Ile229Val in BPIFB4, replicated the association observed in the SICs cohort under the recessive genetic model (OR=2.42, 95% CI=1.56-3.77, $p=5.98\times10^{-5}$) in this set of LLIs (OR=1.42, 95% CI=1.12-1.80, $p=5.3\times10^{-3}$, Bonferroni adjusted p=0.021).

[0057] This variant was then tested by Taqman analysis, as described above, for association in a second set, represented by a US based collection of 1461 LLIs (age range of 91-119 years, mean age 100.8) and 526 controls (age range of 0-35 years, mean age 28,2). Logistic regression confirmed the association of the above SNP also in this second replication set (OR:1.62, 95% CI=1.15-2.27, $p=3.7\times10^{-3}$).

[0058] Meta-analysis of association results was performed by the "meta" package implemented in R (http://cran.r-project.org/web/packages/meta/index.html). Positional and functional annotation of the identified SNPs were performed by the SNPNexus on-line resource.

[0059] Results from meta-analysis, combining the association statistics deriving from the evaluation of this marker in the German and US replication sets, revealed no statistically significant heterogeneity between the ORs estimated in the two populations (Q-statistic, p>0.05; heterogeneity index, I2 = 0%). According to these observations, association statistics were combined assuming a fixed effects model (OR = 1.49; 95% CI = 1.22-1.81; $p < 1\times10^{-4}$).

Example 2: Haplotype analysis of the BPIFB4 locus

[0060] Haplotype analyses revealed patterns of strong linkage disequilibrium (LD) within the BPIFB4 genomic locus, delimiting a region that is highly enriched in non-synonymous SNPs (Fig. S1 in the Supplementary Appendix). The rs2070325 variation (Ile229Val) of BPIFB4 tags rs2889732 (Asn288Thr), rs11699009 (Leu488Phe), and rs11696307 (Ile494Thr).

[0061] The three-dimensional structure of human BPIFB4 was predicted by homology modelling with the program I-TASSER, (REF: Ambrish Roy, Alper Kucukural, Yang Zhang. I-TASSER: a unified platform for automated protein structure and function prediction. Nature Protocols, vol 5, 725-738 (2010).) using as template Protein BPI from PDB (code 1EWF). All models were considered in the visual structural analysis, performed with the program PyMOL Version 1.2r3pre, Schrödinger, LLC (Molecular Graphics System). The above analysis revealed that Ile268Val and Asn320Thr are both located in putative protein-protein interaction site. To evaluate the effects of the variations, we predicted the structure of wild-type (WT) and mutated (Ile229Val, Asn281Thr, Leu488Phe, Ile494Thr) BPIFB4 proteins by homology modelling. BPIFB4 is structurally very similar to BPI and CETP, for which experimental structures are available and because of their structural similarities, we thought it reasonable to expect that BPIFB4 binds lipopolysaccharides in regions that are similar to those of the other two proteins. Our structural analysis revealed that Leu488Phe is located in a lipid-binding pocket whose size is predicted to decrease as a consequence of the mutation. The Ile494Thr mutation is located in a second

lipid-binding pocket, whose hydrophobicity is decreased by the substitution. In both cases, the mutation may result in a decreased ability to bind lipids.

[0062] In contrast, Ile229Val and Asn281Thr are located far from the lipid-binding sites of the structurally homologous proteins, so they probably affect functions such as interaction with other proteins, rather than lipid binding.

Example 3: Ex Vivo Vessel reactivity to INFT hBPIFB4 and VTFT hBPIFB4

[0063] To determine the role of the specific BPIFB4 variant identified on vessel function, we studied the effects of ex *vivo* transfection of mouse mesenteric vessels with a pRK5 vector encoding VTFT hBPIFB4 or proteins that differ from VTFT hBPIFB4 in that they show various substitutions at the 4 relevant amino acids: INFT hBPIFB4, having the amino acid sequence of SEQ ID NO: 3, which differs from that of VTFT hBPIFB4 in that it contains Isoleucine and an Asparagine at positions 229 and 281, respectively, VNFT hBPIFB4, having the amino acid sequence of SEQ ID NO: 4, which differs from that of VTFT hBPIFB4 in that it contains an Asparagine at position 281, ITFT hBPIFB4, having the amino acid sequence of SEQ ID NO: 5, which differs from that of VTFT hBPIFB4 in that it contains Isoleucine at position 229, VTLI hBPIFB4 , having the amino acid sequence of SEQ ID NO: 6, which differs from that of VTFT hBPIFB4 in that it contains a Leucine at position 488 and an Isoleucine at position 494 and INLI hBPIFB4 , having the amino acid sequence of SEQ ID NO: 7, which differs from that of VTFT hBPIFB4 in that it contains Isoleucine at position 229, Asparagine at position 281, Leucine at position 488 and an Isoleucine at position 494. The sequence of the pRK5 vectors used are reported in Figure 1 (a, sequence of the vector encoding wtBPIFB4 and GFP and b, sequence of the vector encoding VTFT hBPIFB4 and GFP) Second-order branches of the mesenteric arterial tree of C57BL6 mice were transfected as described previously (Vecchione C et al., J Exp Med 2005; Vol. 201, pages 1217-28).

[0064] Briefly, vessels (n=7) were placed in a Mulvany pressure system filled with Krebs solution to which was added 20μg of a pRK5 vector encoding either INFT or VTFT hBPIFB4. An empty plasmid was used as a negative control. Vessels were perfused at 100 mmHg for 1 hour then at 60 mmHg for 5 hours.

[0065] The efficiency of transfection was evaluated by the presence of green fluorescent protein (GFP) co-expression (Fig.2) and by Western blotting.

[0066] In details, Western blot analysis was performed on protein extracts from transfected perfused vessels (n=7 for each vector). Protein extracts were separated on 10% SDS-PAGE at 100V for 1h or on 4-12% SDS-PAGE at 100V for 2h and then transferred to a nitrocellulose or PVDF membrane. The membranes were incubated overnight with the following primary antibodies: anti-phospho-Ser1177 eNOS (Cell Signaling, rabbit mAb, 1:1000), anti-BPIFB4 (Abcam, rabbit polyclonal Ab, 1:200), and anti-β-actin (Cell Signaling, mouse mAb, 1:3000). The membranes were washed three times and then incubated for 1 or 2 h with the secondary antibody (horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG, Amersham Life Science) at 1:3000 dilution. The membrane was then washed four times and specific protein bands were detected with ECL Prime chemiluminescent agents (Amersham Life Science). Western blot data were analyzed using imaged software (developed by Wayne Rasband, National Institutes of Health, USA) to determine optical density (OD) of the bands. The OD reading was normalized to β-actin to account for variations in loading.

[0067] As shown in Figure 3, BPIFB4 protein was abundantly detected in vessels after perfusion with either INFT hBPIFB4- or VTFT hBPIFB4-encoding plasmids both wild type and VTFT hBPIFB4 being expressed in comparable amounts. On the contrary, vessels exposed to empty plasmids expressed a low level of native BPIFB4 protein.

[0068] In addition, vessels expressing VTFT hBPIFB4 but not INFT hBPIFB4 showed a strong induction of phosphorylation of eNOS on serine 1177, an activation site of the enzyme.

[0069] Vasoconstriction was assessed with KCl (80 mM) and increasing doses of phenylephrine (from 10^{-9}M to 10^{-6}M), as the percentage of lumen diameter change after drug administration. Vascular responses were tested before and after transfection. Endothelium-dependent and independent relaxations were assessed by measuring the dilatatory responses of mesenteric arteries to cumulative concentrations of acetylcholine (from 10^{-9}M to 10^{-5}M) and nitroglycerine (from 10^{-9}M to 10^{-5}M), respectively, in vessels precontracted with phenylephrine at a dose necessary to obtain a similar level of precontraction in each ring (80% of initial KCl-induced contraction). The maximal contraction evoked by phenylephrine was considered as the baseline for subsequent evoked vasorelaxations. Caution was taken to avoid endothelium damage: functional integrity was reflected by the response to acetylcholine (10^{-6}M).

[0070] Overexpression of INFT hBPIFB4 almost abolished the KCl- and phenylephrine-induced vasoconstrictions that could be elicited before exposure to the plasmids (fig. 5a). The absence of significant vasoconstriction impeded subsequent evaluation of vasorelaxation. In contrast, expression of VTFT hBPIFB4 partially rescued the inhibitory effects exerted by INFT hBPIFB4 on KCl and phenylephrine-induced vasoconstrictions: in fact, the vascular responses evoked by the agonists were reduced when compared with those observed before perfusion but they were not abolished (fig. 6a-6b). In addition, upon expression of VTFT hBPIFB4 there was a significant enhancement in acetylcholine-induced vessel vasodilatation compared with that observed before transfection (fig 6c), but no differences in nitroglycerin-evoked

smooth muscle relaxation (data not shown), indicating that this effect is due to an enhancement in endothelial function. No effect on vascular function was observed with VNFT hBPIFB4, ITFT hBPIFB4, VTLI hBPIFB4 and INLI hBPIFB4.

[0071] We examined the effect of L-NAME, an eNOS inhibitor, on vessels transfected with either an empty vector (Fig.7, panel a, EV) or VTFT hBPIFB4-encoding plasmids (Fig.8, panel a, VTFT). As expected, L-NAME blunted the vasodilatatory effect of acetylcholine in vessels perfused with empty plasmids, and this effect was more pronounced in vessels expressing VTFT hBPIFB4, indicating the presence of more NO in this latter condition.

Example 4: Effect of VTFT hBPIFB4 on in vivo model of vascular disease due to impairment of NO release

[0072] The above described experiments were also performed on mesenteric vessels from heterozygotic Mthfr mice and their control, as described in Lemarie CA et al., Am J Physiol Heart Circ Physiol 2011;Vol 300:H745-53. Mthfr+/- mice show dysfunction of eNOS which is associated with the downregulation of the longevity factor surtuin 1. Thus, we explored the effect of VTFT hBPIFB4 on the mesenteric vessels of these mice. As expected, acetylcholine-induced vasorelaxation was significantly reduced in $Mthfr^{+/-}$ mice compared with $Mthfr^{+/+}$ littermates after exposure to EV (Fig. 7, panel b), but no differences were observed in nitroglycerine-evoked vascular responses (data not shown). After exposure to VTFT hBPIFB4 -encoding plasmids $Mthfr^{+/-}$ - VTFT, endothelial relaxation of $Mthfr^{+/-}$ vessels was significantly improved, becoming comparable to that observed in $Mthfr^{+/+}$ vessels (fig.8b). This indicates that VTFT hBPIFB4 may have strong therapeutic effects in fighting vascular dysfunction (Fig. 8, panel b).

Example 5: Evaluation of eNOS modulation by BPIFB4 in Hek293T Cells

[0073] Human embryonic kidney cells (HEK293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% non-essential amino acids at 37% in a 5% CO2 atmosphere. Cells were plated at 0.25×10^6 per well in six-well plates, and 24h after plating were transfected using 10μl of Lipofectamine 2000 (LifeTechnologies) and 4μg of plasmids. After 24h, cells were serum-starved for 24h. During serum starvation, transfected cells were treated with 400μM H_2O_2 for 24h. Transcription of BPIFB4 was detected by extraction from the cells of total RNA with TRIzol (Ambion), retrotranscription (iScript Bio-Rad). cDNA was amplified with specific primers for *BPIFB4* (Fw: CTCTCCCCAAAATCCTCAACA, Rev: AGCCTCTCTGGGACTGGTTC) and *GAPDH* (Fw: GTGAAGGTCGGAGTCAACG, Rev: GGTGGAATCATATTGGAACATG).

[0074] Transcription of *BPIFB4* could be induced in HEK293T cells upon exposure to H_2O_2: this demonstrates a role of BPIFB4 in the stress response (Fig 9, panel a). Thus, we explored how BPIFB4 affected stress-mediated phosphorylation of eNOS on serine 1177.

[0075] Protein extracts were separated on 10% SDS-PAGE at 100V for 1h or on 4-12% SDS-PAGE at 100V for 2h and then transferred to a nitrocellulose or PVDF membrane. The membranes were incubated overnight with the following primary antibodies: anti-phospho-eNOS Ser1177 (Cell Signaling, rabbit mAb, 1:1000), and anti-β-actin (Cell Signaling, mouse mAb, 1:3000). The membranes were washed three times and then incubated for 1 or 2 h with the secondary antibody (Amersham Life Science horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG, 1:3000). The membranes were then washed four times and specific protein bands were detected with ECL Prime chemiluminescent agents (Amersham Life Science). Western blot data were analyzed using Imaged software (developed by Wayne Rasband, National Institutes of Health, USA) to determine optical density (OD) of the bands. The OD readings were normalized to β-actin to account for variations in loading.

[0076] As shown in Figure 9, panel b and c, eNOS became more activated upon exposure to H_2O_2 in HEK293T cells expressing VTFT hBPIFB4 compared with cells overexpressing INFT hBPIFB4 . This result corroborated that obtained on eNOS activation with the perfusion of vessels ex *vivo.*

Claims

1. A polynucleotide coding for

(i) a BPIFB4 protein consisting of SEQ ID NO: 1;
(ii) a BPIFB4 protein variant of the BPIFB4 protein of (i) having at least 95% homology to that of SEQ ID NO: 1; or
(iii) a fragment of the protein of (i) or the variant of (ii),

wherein said BPIFB4 protein variant or fragment comprises a Valine at the position corresponding to position 229 of SEQ ID NO: 1, a Threonine at the position corresponding to position 281 SEQ ID NO: 1, a Phenylalanine at a position corresponding to position 488 of SEQ ID NO: 1 and a Threonine at a position corresponding to position 494 of SEQ ID NO: 1 and said BPIFB4 protein, variant or fragment has activity in increasing the activity of eNOS and/or the production of NO for use in therapy.

2. A polynucleotide according to claim 1 wherein the polynucleotide is for use in (i) the prevention, reduction of the risk, amelioration or treatment of a pathology selected from arterial hypertension, atherosclerosis, diabetes mellitus, dyslipidemia, renal failure, metabolic syndrome, stroke, myocardial infarction,

erectile dysfunction, neurodegenerative diseases, multiple sclerosis, cognitive disorders, retinal degeneration, uveoretinitis, vascular retinopathy, cataract, glaucoma, coronary spastic angina, thrombosis, pulmonary hypertension, pre-eclampsia, vasculitis, cancer, inflammatory disorders, venous insufficiency; or (ii) for the improvement of post-exercise fatigue in muscular dystrophy or as a co-adjuvant in the implantation of one or more stents for vascular occlusions.

3. A polynucleotide for use according to claim 2 wherein the polynucleotide is for use in (i) the prevention, reduction of the risk, amelioration or treatment of a pathology selected from arterial hypertension, diabetes mellitus, dyslipidemia, renal failure, metabolic syndrome, stroke, myocardial infarction, erectile dysfunction, neurodegenerative diseases, multiple sclerosis, cognitive disorders, retinal degeneration, uveoretinitis, vascular retinopathy, glaucoma, coronary spastic angina, thrombosis, pulmonary hypertension, pre-eclampsia, vasculitis, inflammatory disorders and venous insufficiency; or (ii) for the improvement of post-exercise fatigue in muscular dystrophy or as a co-adjuvant in the implantation of one or more stents for vascular occlusions.

4. A polynucleotide for use according to any preceding claim, wherein the BPIFB4 protein, variant or fragment thereof is linked to an additional amino acid sequence to target the BPIFB4 protein, variant or fragment thereof to a specific organ or tissue.

5. A polynucleotide for use according to any preceding claim, wherein the polynucleotide codes for a BPIFB4 protein which consists of SEQ ID NO: 1.

6. A polynucleotide for use according to any preceding claim, wherein the polynucleotide comprises the nucleotide sequence of SEQ ID NO: 2 or a fragment thereof which comprises the nucleotides coding for Valine 229, Threonine 281, Phenylalanine 488 and Threonine 494 of SEQ ID NO: 1.

7. A polynucleotide for use according to claim 6, wherein the polynucleotide sequence consists of the amino acid sequence of SEQ ID NO: 2.

8. A vector containing a polynucleotide coding for

(i) a BPIFB4 protein consisting of SEQ ID NO: 1;
(ii) a BPIFB4 protein variant of the BPIFB4 protein of (i) having at least 95% homology to that of SEQ ID NO: 1; or
(iii) a fragment of the BPIFB4 protein of (i) or the variant of (ii),

which BPIFB4 protein variant or fragment is operatively linked to expression control sequences, wherein said BPIFB4 protein variant or fragment comprises a Valine at the position corresponding to position 229 of SEQ ID NO: 1, a Threonine at the position corresponding to position 281 SEQ ID NO: 1, a Phenylalanine at a position corresponding to position 488 of SEQ ID NO: 1 and a Threonine at a position corresponding to position 494 of SEQ ID NO: 1 and said BPIFB4 protein, variant or fragment has activity in increasing the activity of eNOS and/or the production of NO for use in therapy.

9. A vector according to claim 8, wherein the vector is for use (i) in the prevention, reduction of the risk, amelioration or treatment of a pathology selected from arterial hypertension, atherosclerosis, diabetes mellitus, dyslipidemia, renal failure, metabolic syndrome, stroke, myocardial infarction, erectile dysfunction, neurodegenerative diseases, multiple sclerosis, cognitive disorders, retinal degeneration, uveoretinitis, vascular retinopathy, cataract, glaucoma, coronary spastic angina, thrombosis, pulmonary hypertension, pre-eclampsia, vasculitis, cancer, inflammatory disorders, venous insufficiency; or (ii) for the improvement of post-exercise fatigue in muscular dystrophy or as a co-adjuvant in the implantation of one or more stents for vascular occlusions.

10. A vector for use according to any one of claims 8 to 9, wherein the BPIFB4 protein, variant or fragment thereof is linked to an additional amino acid sequence to target the BPIFB4 protein variant or fragment thereof to a specific organ or tissue.

11. A vector for use according to any one of claims 8 to 10, wherein the polynucleotide codes for a BPIFB4 protein which consists of SEQ ID NO: 1.

12. A vector for use according to any one of claims 8 to 11, wherein the polynucleotide comprises the nucleotide sequence of SEQ ID NO: 2 or a fragment thereof which comprises the nucleotides coding for Valine 229, Threonine 281, Phenylalanine 488 and Threonine 494 of SEQ ID NO: 1, optionally wherein the polynucleotide sequence consists of the amino acid sequence of SEQ ID NO: 2.

13. A vector for use according to any one of claims 8 to 12, wherein the vector is a viral vector, for example the vector is an adenovirus vector or is selected from AAV serotypes 1-9 vectors.

14. A pharmaceutical composition comprising a polynucleotide coding for

(i) a BPIFB4 protein consisting of SEQ ID NO: 1;
(ii) a BPIFB4 protein variant of the BPIFB4 protein of (i) having at least 95% homology to that

of SEQ ID NO: 1; or
(iii) a fragment of the protein of (i) or the variant of (ii),

wherein said BPIFB4 protein variant or fragment comprises a Valine at the position corresponding to position 229 of SEQ ID NO: 1, a Threonine at the position corresponding to position 281 SEQ ID NO: 1, a Phenylalanine at a position corresponding to position 488 of SEQ ID NO: 1 and a Threonine at a position corresponding to position 494 of SEQ ID NO: 1 and said BPIFB4 protein, variant or fragment has activity in increasing the activity of eNOS and/or the production of NO or a vector comprising said polynucleotide, admixed with a pharmaceutically acceptable excipient.

Patentansprüche

1. Polynukleotid, codierend für

(i) ein BPIFB4-Protein, das aus SEQ ID NO: 1 besteht;
(ii) eine BPIFB4-Proteinvariante des BPIFB4-Proteins von (i), die mindestens 95 % Homologie zu der aus SEQ ID NO: 1 aufweist; oder
(iii) ein Fragment des Proteins von (i) oder der Variante von (ii),

wobei die BPIFB4-Proteinvariante oder das Fragment ein Valin an der Position, die der Position 229 aus SEQ ID NO: 1 entspricht, ein Threonin an der Position, die der Position 281 aus SEQ ID NO: 1 entspricht, ein Phenylalanin an einer Position, die der Position 488 aus SEQ ID NO: 1 entspricht, und ein Threonin an einer Position, die der Position 494 aus SEQ ID NO: 1 entspricht, umfasst und das BPIFB4-Protein, die Variante oder das Fragment eine Wirkung zur Erhöhung der Wirkung von eNOS und/oder der Produktion von NO zur Verwendung in der Therapie aufweist.

2. Polynukleotid nach Anspruch 1, wobei das Polynukleotid zur Verwendung (i) bei der Vorbeugung, Verringerung des Risikos, Verbesserung oder Behandlung einer Pathologie, ausgewählt aus arterieller Hypertonie, Atherosklerose, Diabetes mellitus, Dyslipidämie, Nierenversagen, metabolischem Syndrom, Schlaganfall, Myokardinfarkt, erektiler Dysfunktion, neurodegenerativen Erkrankungen, Multipler Sklerose, kognitiven Störungen, Netzhautdegeneration, Uveoretinitis, vaskulärer Retinopathie, Katarakt, Glaukom, koronar-spastischer Angina, Thrombose, pulmonaler Hypertonie, Präeklampsie, Vaskulitis, Krebs, entzündlichen Erkrankungen, venöser Insuffizienz; oder (ii) zur Verbesserung von Ermüdung nach Anstrengung bei Muskeldystrophie oder als Co-Adjuvans bei der Implantation eines oder mehrerer Stents für Gefäßverschlüsse dient.

3. Polynukleotid zur Verwendung nach Anspruch 2, wobei das Polynukleotid zur Verwendung (i) bei der Vorbeugung, Verringerung des Risikos, Verbesserung oder Behandlung einer Pathologie, ausgewählt aus arterieller Hypertonie, Diabetes mellitus, Dyslipidämie, Nierenversagen, metabolischem Syndrom, Schlaganfall, Myokardinfarkt, erektiler Dysfunktion, neurodegenerativen Erkrankungen, Multipler Sklerose, kognitiven Störungen, Netzhautdegeneration, Uveoretinitis, vaskulärer Retinopathie, Glaukom, koronar-spastischer Angina, Thrombose, pulmonaler Hypertonie, Präeklampsie, Vaskulitis, entzündlichen Erkrankungen und venöser Insuffizienz; oder (ii) zur Verbesserung von Ermüdung nach Anstrengung bei Muskeldystrophie oder als Co-Adjuvans bei der Implantation eines oder mehrerer Stents für Gefäßverschlüsse dient.

4. Polynukleotid zur Verwendung nach einem der vorhergehenden Ansprüche, wobei das BPIFB4-Protein, die Variante oder das Fragment davon mit einer zusätzlichen Aminosäuresequenz verknüpft ist, um das BPIFB4-Protein, die Variante oder das Fragment davon auf ein spezifisches Organ oder Gewebe zu richten.

5. Polynukleotid zur Verwendung nach einem der vorhergehenden Ansprüche, wobei das Polynukleotid für ein BPIFB4-Protein codiert, das aus SEQ ID NO: 1 besteht.

6. Polynukleotid zur Verwendung nach einem der vorhergehenden Ansprüche, wobei das Polynukleotid die Nukleotidsequenz aus SEQ ID NO: 2 oder ein Fragment davon umfasst, das die Nukleotide umfasst, die für Valin 229, Threonin 281, Phenylalanin 488 und Threonin 494 aus SEQ ID NO: 1 codieren.

7. Polynukleotid zur Verwendung nach Anspruch 6, wobei die Polynukleotidsequenz aus der Aminosäuresequenz aus SEQ ID NO: 2 besteht.

8. Vektor, enthaltend ein Polynukleotid, codierend für

(i) ein BPIFB4-Protein, das aus SEQ ID NO: 1 besteht;
(ii) eine BPIFB4-Proteinvariante des BPIFB4-Proteins von (i), die mindestens 95 % Homologie zu der aus SEQ ID NO: 1 aufweist; oder
(iii) ein Fragment des BPIFB4-Proteins von (i) oder der Variante von (ii),

wobei die BPIFB4-Proteinvariante oder das Fragment operativ mit Expressionssteuersequenzen verknüpft ist, wobei die BPIFB4-Proteinvariante oder

das Fragment ein Valin an der Position, die der Position 229 aus SEQ ID NO: 1 entspricht, ein Threonin an der Position, die der Position 281 aus SEQ ID NO: 1 entspricht, ein Phenylalanin an einer Position, die der Position 488 aus SEQ ID NO: 1 entspricht, und ein Threonin an einer Position, die der Position 494 aus SEQ ID NO: 1 entspricht, umfasst und das BPIFB4-Protein, die Variante oder das Fragment eine Wirkung zur Erhöhung der Wirkung von eNOS und/oder der Produktion von NO zur Verwendung in der Therapie aufweist.

9. Vektor nach Anspruch 8, wobei der Vektor zur Verwendung (i) bei der Vorbeugung, Verringerung des Risikos, Verbesserung oder Behandlung einer Pathologie, ausgewählt aus arterieller Hypertonie, Atherosklerose, Diabetes mellitus, Dyslipidämie, Nierenversagen, metabolischem Syndrom, Schlaganfall, Myokardinfarkt, erektiler Dysfunktion, neurodegenerativen Erkrankungen, Multipler Sklerose, kognitiven Störungen, Netzhautdegeneration, Uveoretinitis, vaskulärer Retinopathie, Katarakt, Glaukom, koronar-spastischer Angina, Thrombose, pulmonaler Hypertonie, Präeklampsie, Vaskulitis, Krebs, entzündlichen Erkrankungen, venöser Insuffizienz; oder (ii) zur Verbesserung von Ermüdung nach Anstrengung bei Muskeldystrophie oder als Co-Adjuvans bei der Implantation eines oder mehrerer Stents für Gefäßverschlüsse dient.

10. Vektor zur Verwendung nach einem der Ansprüche 8 bis 9, wobei das BPIFB4-Protein, die Variante oder das Fragment davon mit einer zusätzlichen Aminosäuresequenz verknüpft ist, um die BPIFB4-Proteinvariante oder das Fragment davon auf ein spezifisches Organ oder Gewebe zu richten.

11. Vektor zur Verwendung nach einem der Ansprüche 8 bis 10, wobei das Polynukleotid für ein BPIFB4-Protein codiert, das aus SEQ ID NO: 1 besteht.

12. Vektor zur Verwendung nach einem der Ansprüche 8 bis 11, wobei das Polynukleotid die Nukleotidsequenz aus SEQ ID NO: 2 oder ein Fragment davon umfasst, das die Nukleotide umfasst, die für Valin 229, Threonin 281, Phenylalanin 488 und Threonin 494 aus SEQ ID NO: 1 codieren, wobei optional die Polynukleotidsequenz aus der Aminosäuresequenz aus SEQ ID NO: 2 besteht.

13. Vektor zur Verwendung nach einem der Ansprüche 8 bis 12, wobei der Vektor ein viraler Vektor ist, zum Beispiel der Vektor ein Adenovirus-Vektor ist oder aus Vektoren der AAV-Serotypen 1-9 ausgewählt ist.

14. Pharmazeutische Zusammensetzung, umfassend ein Polynukleotid, codierend für

(i) ein BPIFB4-Protein, das aus SEQ ID NO: 1 besteht;
(ii) eine BPIFB4-Proteinvariante des BPIFB4-Proteins von (i), die mindestens 95 % Homologie zu der aus SEQ ID NO: 1 aufweist; oder
(iii) ein Fragment des Proteins von (i) oder der Variante von (ii),

wobei die BPIFB4-Proteinvariante oder das Fragment ein Valin an der Position, die der Position 229 aus SEQ ID NO: 1 entspricht, ein Threonin an der Position, die der Position 281 aus SEQ ID NO: 1 entspricht, ein Phenylalanin an einer Position, die der Position 488 aus SEQ ID NO: 1 entspricht, und ein Threonin an einer Position, die der Position 494 aus SEQ ID NO: 1 entspricht, umfasst und das BPIFB4-Protein, die Variante oder das Fragment eine Wirkung zur Erhöhung der Wirkung von eNOS und/oder der Produktion von NO oder eines Vektors aufweist, der das Nukleotid, zugemischt zu einem pharmazeutisch annehmbaren Trägerstoff, umfasst.

Revendications

1. Polynucléotide codant pour

(i) une protéine BPIFB4 consistant en SEQ ID NO: 1 ;
(ii) une variante de protéine BPIFB4 de la protéine BPIFB4 de (i) ayant au moins 95 % d'homologie avec celle de SEQ ID NO: 1 ; ou
(iii) un fragment de la protéine de (i) ou de la variante de (ii),

dans lequel ladite variante ou ledit fragment de protéine BPIFB4 comprend une valine à la position correspondant à la position 229 de SEQ ID NO: 1, une thréonine à la position correspondant à la position 281 SEQ ID NO: 1, une phénylalanine à une position correspondant à la position 488 de SEQ ID NO: 1 et une thréonine à une position correspondant à la position 494 de SEQ ID NO: 1 et ladite protéine BPIFB4, une variante ou un fragment de celle-ci a une activité pour augmenter l'activité de eNOS et/ou la production de NO pour une utilisation en thérapie.

2. Polynucléotide selon la revendication 1, dans lequel le polynucléotide est destiné à être utilisé dans (i) la prévention, la réduction du risque, l'amélioration ou le traitement d'une pathologie choisie parmi l'hypertension artérielle, l'athérosclérose, le diabète sucré, la dyslipidémie, l'insuffisance rénale, le syndrome métabolique, l'accident vasculaire cérébral, l'infarctus du myocarde, la dysfonction érectile, les maladies neurodégénératives, la sclérose en plaques, les troubles cognitifs, la dégénérescence rétinienne, l'uvéorétinite, la rétinopathie vasculaire, la cataracte,

le glaucome, l'angor spastique coronaire, la thrombose, l'hypertension pulmonaire, la pré-éclampsie, la vascularite, le cancer, les troubles inflammatoires, l'insuffisance veineuse ; ou (ii) pour l'amélioration de la fatigue post-exercice dans la dystrophie musculaire ou comme co-adjuvant dans l'implantation d'un ou de plusieurs stents pour les occlusions vasculaires.

3. Polynucléotide destiné à être utilisé selon la revendication 2, dans lequel le polynucléotide est destiné à être utilisé dans (i) la prévention, la réduction du risque, l'amélioration ou le traitement d'une pathologie choisie parmi l'hypertension artérielle, le diabète sucré, la dyslipidémie, l'insuffisance rénale, le syndrome métabolique, l'accident vasculaire cérébral, l'infarctus du myocarde, la dysfonction érectile, les maladies neurodégénératives, la sclérose en plaques, les troubles cognitifs, la dégénérescence rétinienne, l'uvéorétinite, la rétinopathie vasculaire, le glaucome, l'angor spastique coronaire, la thrombose, l'hypertension pulmonaire, la pré-éclampsie, la vascularite, les troubles inflammatoires et l'insuffisance veineuse ; ou (ii) pour l'amélioration de la fatigue post-exercice dans la dystrophie musculaire ou comme co-adjuvant dans l'implantation d'un ou de plusieurs stents pour les occlusions vasculaires.

4. Polynucléotide destiné à être utilisé selon une quelconque revendication précédente, dans lequel la protéine BPIFB4, une variante ou un fragment de celle-ci est lié à une séquence d'acides aminés supplémentaire pour cibler la protéine BPIFB4, une variante ou un fragment de celle-ci vers un organe ou tissu spécifique.

5. Polynucléotide destiné à être utilisé selon une quelconque revendication précédente, dans lequel le polynucléotide code pour une protéine BPIFB4 qui consiste en SEQ ID NO: 1.

6. Polynucléotide destiné à être utilisé selon une quelconque revendication précédente, dans lequel le polynucléotide comprend la séquence nucléotidique de SEQ ID NO: 2 ou un fragment de celle-ci qui comprend les nucléotides codant pour la valine 229, la thréonine 281, la phénylalanine 488 et la thréonine 494 de SEQ ID NO: 1.

7. Polynucléotide destiné à être utilisé selon la revendication 6, dans lequel la séquence polynucléotidique consiste en la séquence d'acides aminés de SEQ ID NO: 2.

8. Vecteur contenant un polynucléotide codant pour

(i) une protéine BPIFB4 consistant en SEQ ID NO: 1 ;

(ii) une variante de protéine BPIFB4 de la protéine BPIFB4 de (i) ayant au moins 95 % d'homologie avec celle de SEQ ID NO: 1 ; ou

(iii) un fragment de la protéine BPIFB4 (i) ou de la variante de (ii),

dans lequel ladite variante ou ledit fragment de protéine BPIFB4 est lié de manière opérationnelle à des séquences de commande d'expression, dans lequel ladite variante ou ledit fragment de protéine BPIFB4 comprend une valine à la position correspondant à la position 229 de SEQ ID NO: 1, une thréonine à la position correspondant à la position 281 SEQ ID NO: 1, une phénylalanine à une position correspondant à la position 488 de SEQ ID NO: 1 et une thréonine à une position correspondant à la position 494 de SEQ ID NO: 1 et ladite variante ou ledit fragment de protéine BPIFB4 a une activité pour augmenter l'activité de eNOS et/ou la production de NO pour une utilisation en thérapie.

9. Vecteur selon la revendication 8, dans lequel le vecteur est destiné à être utilisé (i) dans la prévention, la réduction du risque, l'amélioration ou le traitement d'une pathologie choisie parmi l'hypertension artérielle, l'athérosclérose, le diabète sucré, la dyslipidémie, l'insuffisance rénale, le syndrome métabolique, l'accident vasculaire cérébral, l'infarctus du myocarde, la dysfonction érectile, les maladies neurodégénératives, la sclérose en plaques, les troubles cognitifs, la dégénérescence rétinienne, l'uvéorétinite, la rétinopathie vasculaire, la cataracte, le glaucome, l'angor spastique coronaire, la thrombose, l'hypertension pulmonaire, la pré-éclampsie, la vascularite, le cancer, les troubles inflammatoires, l'insuffisance veineuse ; ou (ii) pour l'amélioration de la fatigue post-exercice dans la dystrophie musculaire ou comme co-adjuvant dans l'implantation d'un ou de plusieurs stents pour les occlusions vasculaires.

10. Vecteur destiné à être utilisé selon l'une quelconque des revendications 8 à 9, dans lequel la protéine BPIFB4, une variante ou un fragment de celle-ci est lié à une séquence d'acides aminés supplémentaire pour cibler la protéine BPIFB4, une variante ou un fragment de celle-ci vers un organe ou tissu spécifique.

11. Vecteur destiné à être utilisé selon l'une quelconque des revendications 8 à 10, dans lequel le polynucléotide code pour une protéine BPIFB4 qui consiste en SEQ ID NO: 1.

12. Vecteur destiné à être utilisé selon l'une quelconque des revendications 8 à 11, dans lequel le polynucléotide comprend la séquence nucléotidique de SEQ ID NO: 2 ou un fragment de celle-ci qui comprend les nucléotides codant pour la valine 229, la

thréonine 281, la phénylalanine 488 et la thréonine 494 de SEQ ID NO: 1, éventuellement dans lequel la séquence polynucléotidique consiste en la séquence d'acides aminés de SEQ ID NO: 2.

13. Vecteur destiné à être utilisé selon l'une quelconque des revendications 8 à 12, dans lequel le vecteur est un vecteur viral, par exemple le vecteur est un vecteur adénovirus ou est choisi parmi les vecteurs AAV des sérotypes 1 à 9.

14. Composition pharmaceutique comprenant un polynucléotide codant pour

(i) une protéine BPIFB4 consistant en SEQ ID NO: 1 ;
(ii) une variante de protéine BPIFB4 de la protéine BPIFB4 de (i) ayant au moins 95 % d'homologie avec celle de SEQ ID NO: 1 ; ou
(iii) un fragment de la protéine de (i) ou de la variante de (ii),

dans lequel ladite variante ou ledit fragment de protéine BPIFB4 comprend une valine à la position correspondant à la position 229 de SEQ ID NO: 1, une thréonine à la position correspondant à la position 281 SEQ ID NO: 1, une phénylalanine à une position correspondant à la position 488 de SEQ ID NO: 1 et une thréonine à une position correspondant à la position 494 de SEQ ID NO: 1 et ladite variante ou ledit fragment de protéine BPIFB4 a une activité pour augmenter l'activité de eNOS et/ou la production de NO ou un vecteur comprenant ledit polynucléotide, mélangé avec un excipient pharmaceutiquement acceptable.

Figure 1

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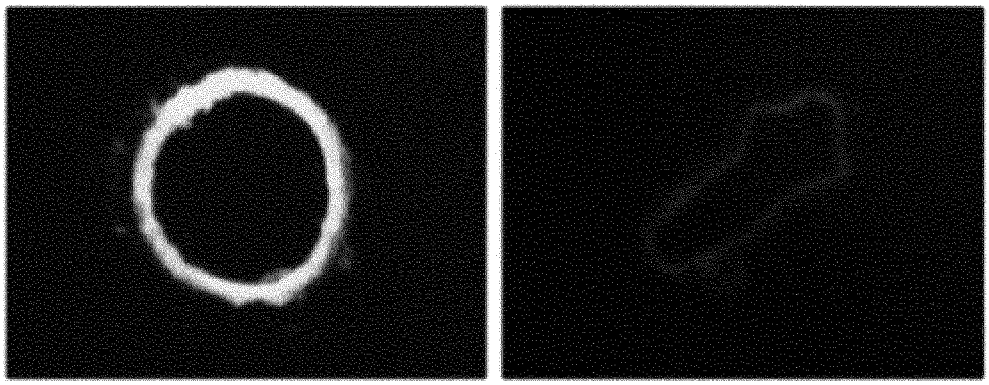

Figure 2

Figure 3

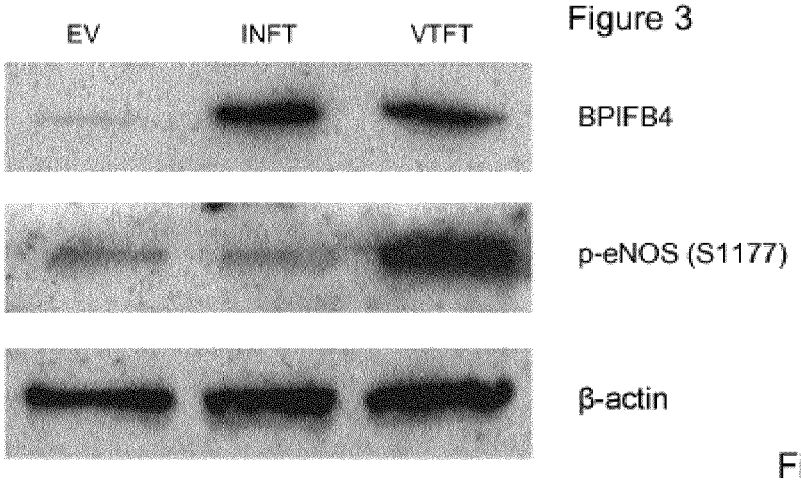

Figure 3a

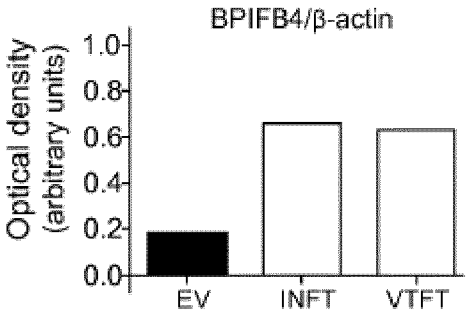

Figure 3b

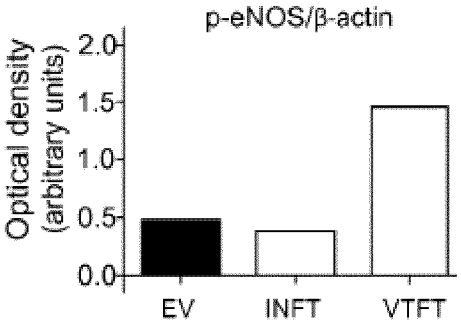

Figure 3c

Figure 4

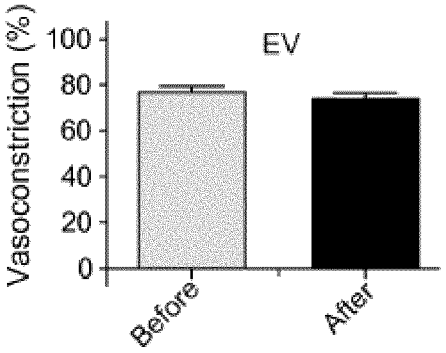

Figure 4a

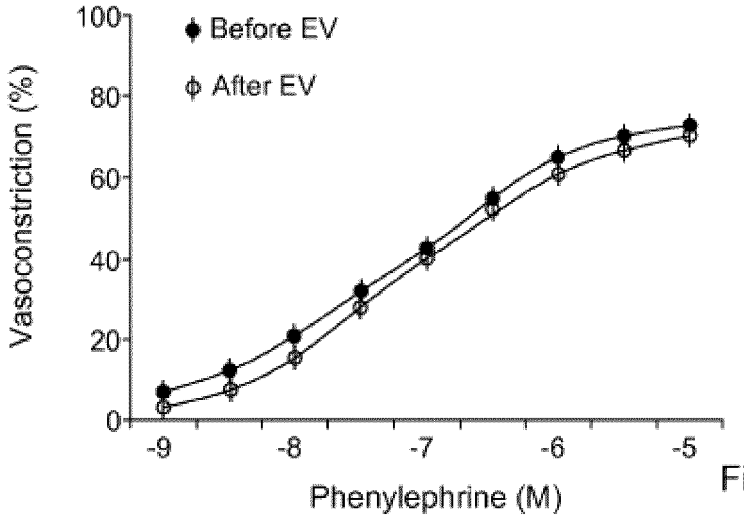

Figure 4b

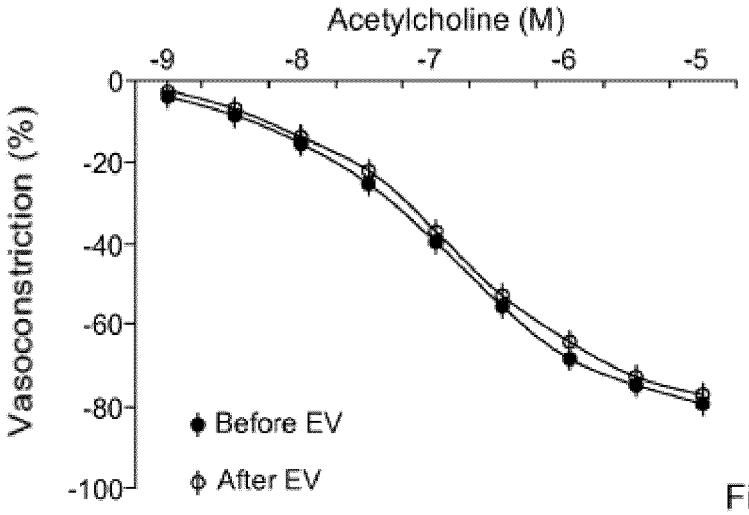

Figure 4c

Figure 5

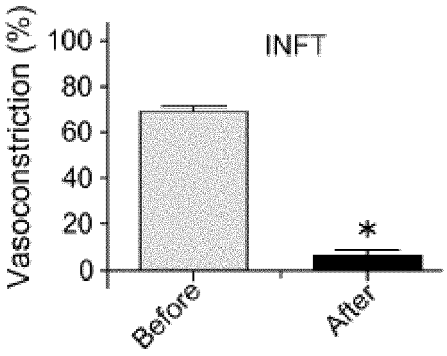

Figure 5a

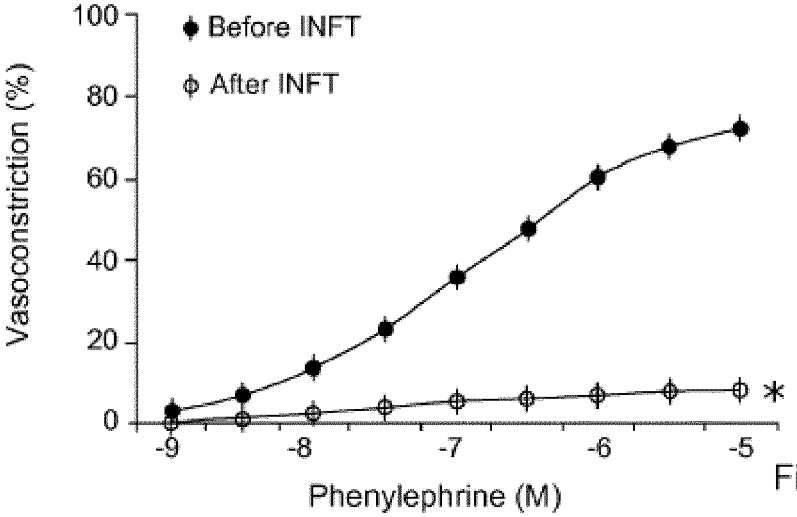

Figure 5b

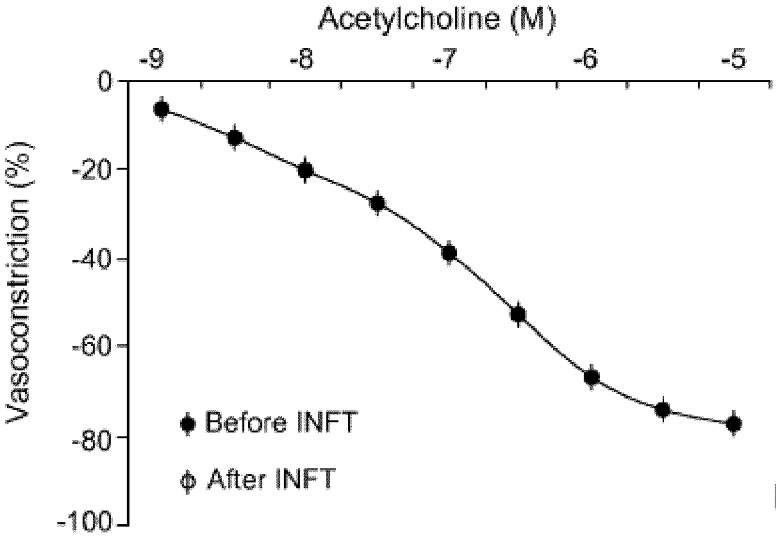

Figure 5c

Figure 6

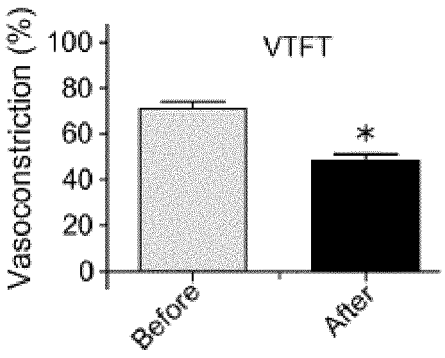

Figure 6a

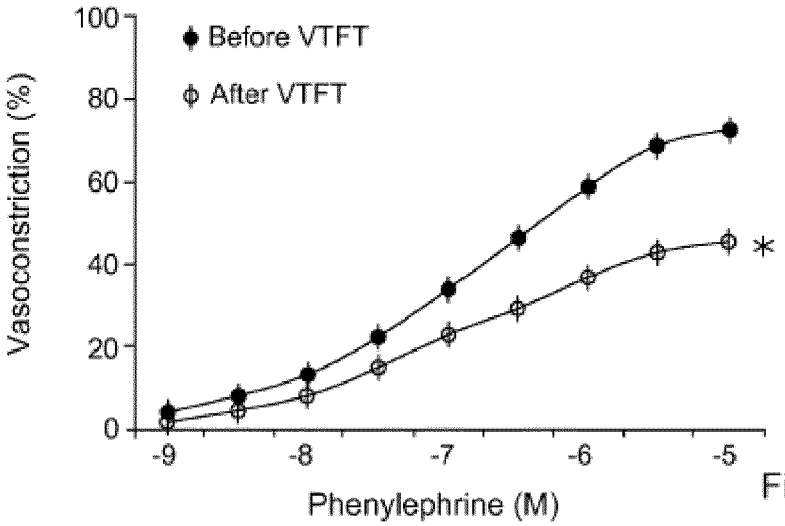

Figure 6b

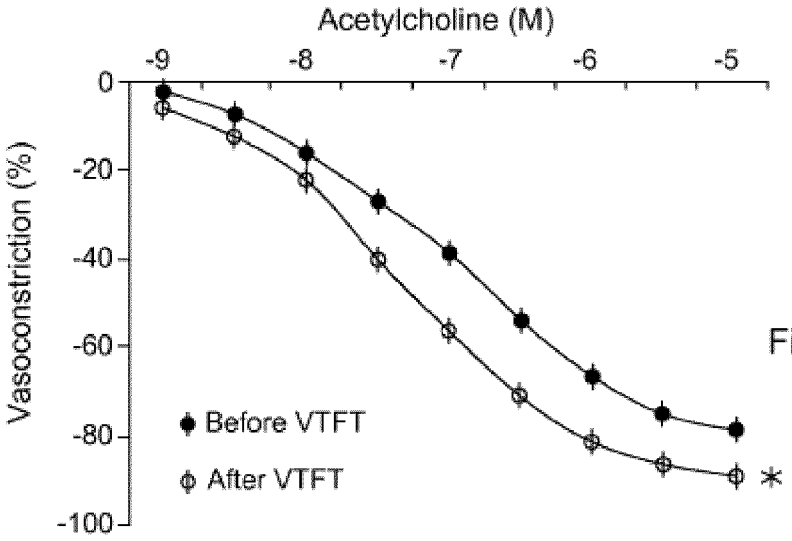

Figure 6c

Figure 7

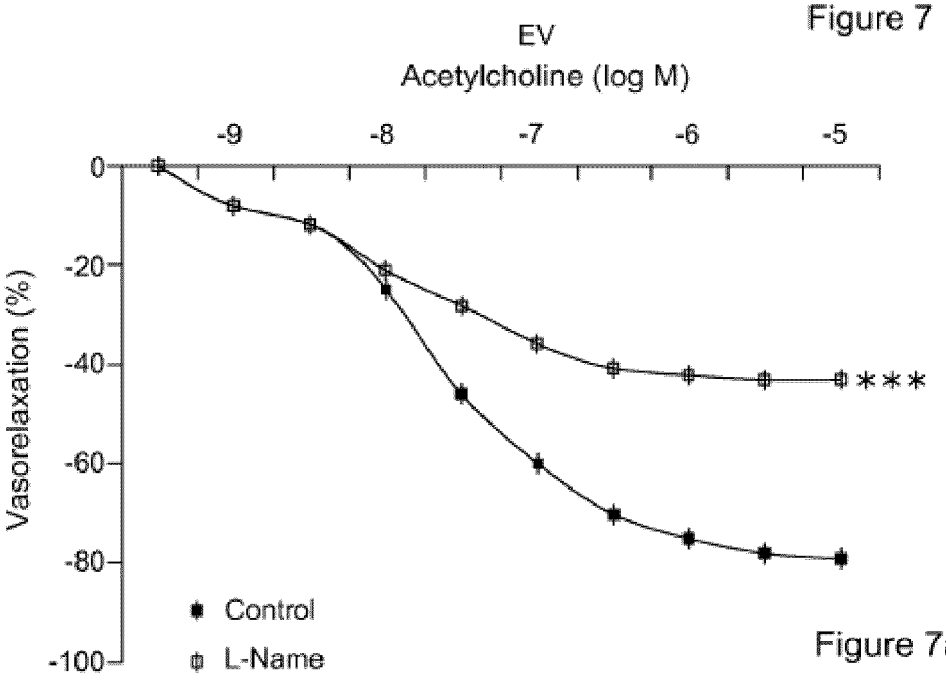

Figure 7a

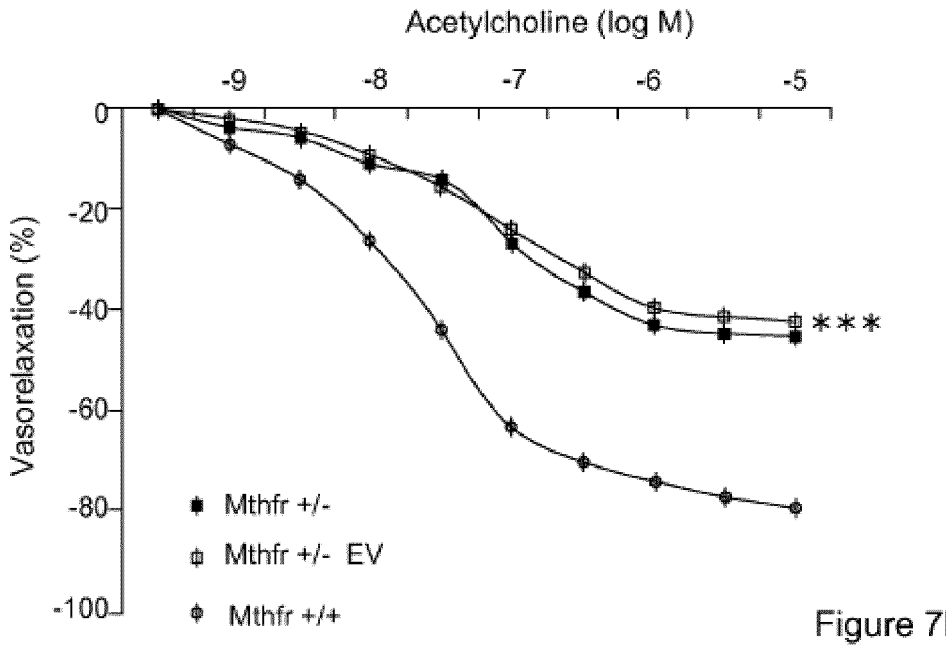

Figure 7b

Figure 8

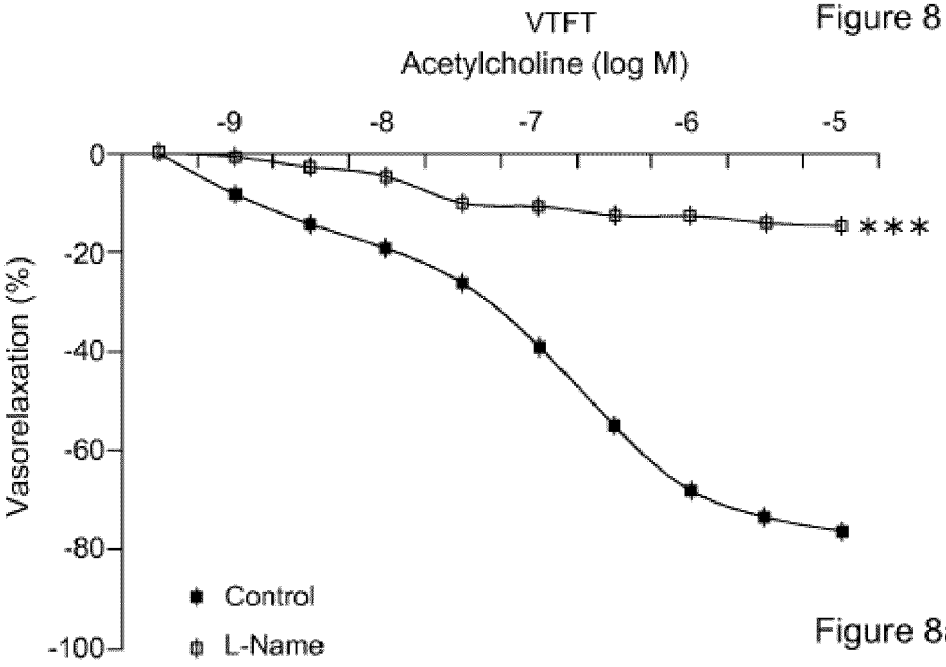

Figure 8a

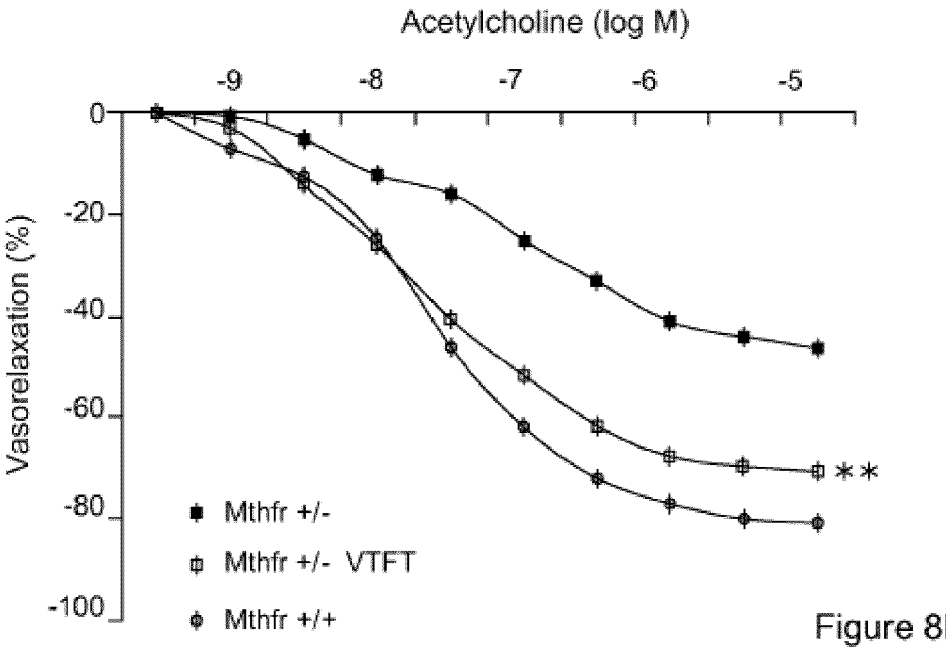

Figure 8b

Figure 9

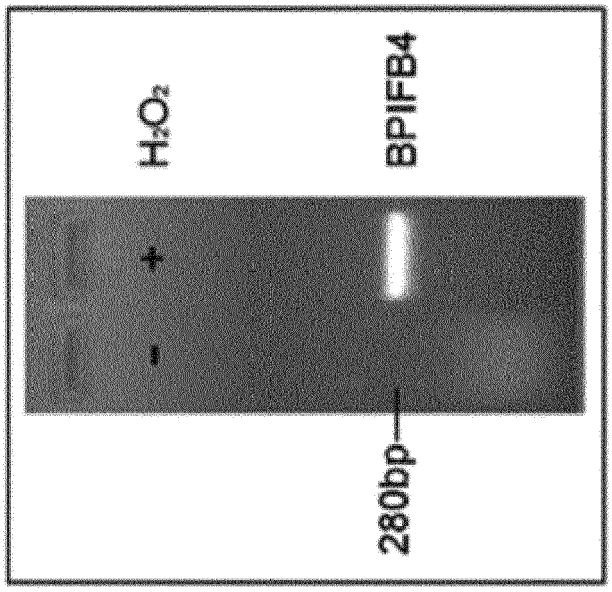

Figure 9a

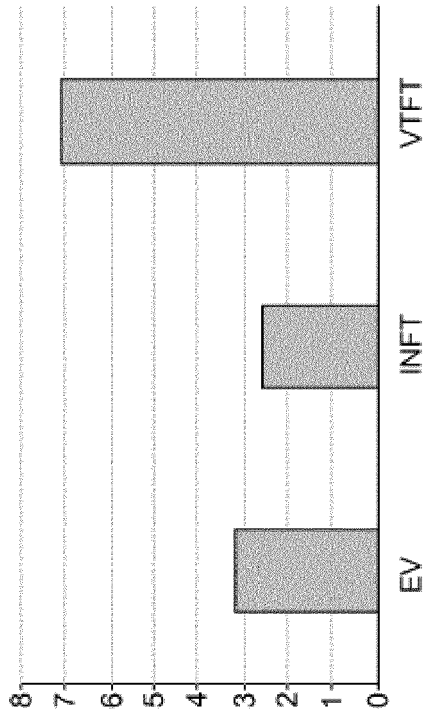

Figure 9b

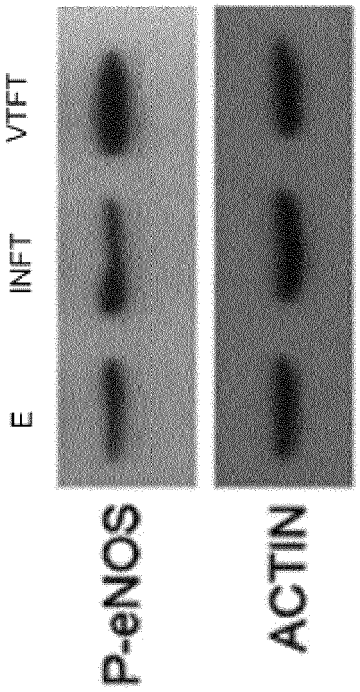

Figure 9c

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