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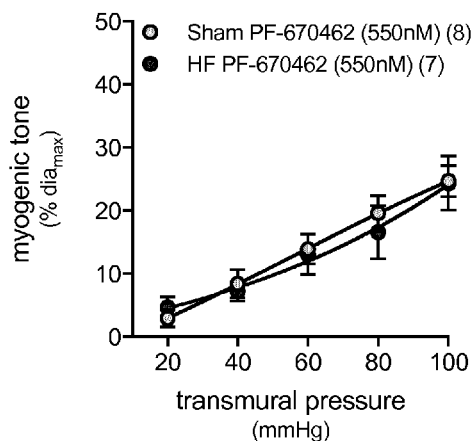
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(54) Title: USE OF CASEIN KINASE 1 INHIBITORS FOR TREATING VASCULAR DISEASES

Fig. 1B



(57) Abstract: The present invention relates to the use of casein kinase 1 inhibitors for treating vascular diseases, preferably peripheral vascular diseases, and to corresponding treatment methods.

## Use of casein kinase 1 inhibitors for treating vascular diseases

The present invention relates to the use of casein kinase 1 inhibitors for treating vascular diseases, preferably peripheral vascular and cardiovascular diseases. The present invention also relates to corresponding treatment methods.

5 Despite significant investment into basic and clinical cardiovascular research, cardiovascular disease remains the most devastating and challenging health issue worldwide: it is responsible for over 17,500,000 deaths each year (equaling 47% of all deaths caused by non-communicable diseases). The global costs for managing cardiovascular diseases will rise to \$1.04 trillion by 2030, thereby transforming this  
10 health issue into a serious threat to the global economy.

Hypertension is the #1 risk factor for all cardiovascular diseases: it increases the risk of heart attack, heart failure (HF), stroke and kidney failure. As many as 1 in 3 people have high blood pressure. However, since hypertension has few signs or symptoms,  
15 most cases remain undiagnosed. We do not understand why people develop hypertension: only 10% of hypertension cases can be explained. Understanding hypertension is key to preventing and treating cardiovascular disease.

Microvascular research studies the structure and function of the smallest blood  
20 vessels (pre- capillary arterioles and post-capillary venules) in health and disease. Resistance arteries are “hotspots” within the cardiovascular system that regulate both mean arterial pressure (MAP) and tissue perfusion, and are responsible for generating the largest portion of the total peripheral resistance (TPR). Consequently, changes to their structure and function (e.g. through diseases or aging) immediately  
25 affect tissue perfusion and MAP. Resistance arteries are an untapped opportunity for improving cardiovascular health. Understanding the structural and molecular basis of microvascular function in health and disease will unlock a range of new therapeutic strategies.

30 Current treatments that target the microvasculature are sub-standard as they fail to improve organ function and are difficult to titrate to effective doses without incurring significant side- effects. As an example, augmented myogenic tone in HF elevates

TPR and induces an “afterload mismatch” that induces deleterious long-term effects on ventricular topography and cardiac function. In fact, even a short-term augmentation in afterload induces significant infarct expansion. Consequently, several strategies that reduce cardiac afterload in HF have been evaluated, however, with limited success. While reducing cardiac afterload may confer cardiac benefits, vasodilators (e.g., nitric oxide) bear the risk of eliminating myogenic responsiveness and dangerously lowering TPR and blood pressure.

***Myogenic responsiveness and impacts on systemic hemodynamics:*** In 1902,

Sir William Bayliss discovered that transmural pressure elevation causes resistance arteries to “writhe like a worm” (i.e., constrict)<sup>1</sup>. This observation, later termed the myogenic response<sup>2-5</sup>, describes this dynamic adjustment of blood vessel diameter to changes in perfusion pressure. As the etymology of its name implies (myo = muscle, genic = generated), the myogenic response originates from the vascular wall smooth muscle layer<sup>6-8</sup>; anatomically, the myogenic response is a property of pre-capillary small arteries and arterioles (i.e., small diameter “resistance arteries” are functionally distinct from large diameter conduit arteries).

On the systemic level, Ohm’s law indicates that resistance artery myogenic reactivity will have significant impact on TPR and MAP<sup>9,10</sup>. This positions resistance arteries as a “functional hotspot” in several disease processes hallmarked by changes in tissue perfusion and/or systemic hemodynamics, including cardiomyopathy<sup>11</sup>, HF<sup>12,13</sup>, diabetes<sup>14,15</sup> and hypertension<sup>16</sup>. In vivo, skeletal muscle resistance arteries prominently modulate TPR, as this vascular bed forms the body’s largest circulatory network<sup>17</sup>.

***TNF reverse signaling as a regulator of myogenic responsiveness:*** In our

seminal study investigating skeletal muscle resistance arteries<sup>18</sup>, we demonstrated that tumour necrosis factor (TNF), and more specifically the membrane-bound version (mTNF), is a constitutive mechanosensor driving the myogenic response in skeletal muscle resistance arteries under a non-pathological setting. Accordingly, acutely deleting the TNF gene in smooth muscle cells or scavenging TNF with Etanercept attenuates skeletal muscle resistance artery myogenic responsiveness and hence, reduces systemic blood pressure. Notably, Etanercept’s inhibitory effect

on skeletal muscle artery myogenic responsiveness is conserved across five distinct species, including humans. Mechanistically, mTNF transduces the mechanical load imposed on vascular smooth muscle cells into an outside-in signal (i.e., a “reverse signal” through TNF) that connects to the established intracellular myogenic signaling elements (e.g., ERK 1/2 and sphingosine kinase 1). This non-canonical mTNF reverse signaling mechanism appears to be unique to skeletal muscle resistance arteries<sup>18</sup>.

**Casein Kinase 1 is a Modulator of TNF Reverse Signaling:** TNF’s cytoplasmic domain does not possess discernible enzymatic function and hence, signals through associated proteins including casein kinase 1 (CK1). Remarkably, evolutionary pressure has conserved TNF’s CK1 phosphorylation site across several species, suggesting that it serves a critical function<sup>19</sup>. In this regard, TNF phosphorylation acts as an “activity switch” and provides a flexible mechanism to modulate TNF reverse signaling functions.

CK1 is a family of 7 ubiquitously expressed monomeric serine/threonine protein kinases<sup>20</sup>. While all CK1 isoforms possess a highly conserved kinase domain, they differ significantly in their non-catalytic N- and C-terminal domains<sup>21,22</sup>: these domains play crucial roles in regulating kinase activity, kinase localization and substrate specificity *in vivo*<sup>21-23</sup>. Since CK1 family isoforms display similar substrate specificity *in vitro*<sup>24</sup>, their distinct biological functions *in vivo* (e.g., chromosome segregation, spindle formation, circadian rhythms, nuclear import, Wnt pathway signaling and cell survival/apoptosis) arise almost entirely from differences in localization, docking sequences and interaction partners<sup>22</sup>.

All CK1 isoforms are constitutively active and therefore, are classified as “second messenger-independent kinases”. Canonically, CK1 phosphorylates a “primed” (pre-phosphorylated) consensus sequence S(P)-X-X-S, where “S(P)” represents the “primed” phosphoserine, “X” represents any amino acid and “S” represents the target serine that CK1 phosphorylates<sup>20</sup>. Since efficient substrate recognition requires a phosphorylated serine residue, CK1 frequently phosphorylates substrates in conjunction with other kinases<sup>20</sup>, an aspect consistent with a hierarchical phosphorylation mechanism<sup>25</sup>.

The technical problem underlying the present invention is to provide a novel regimen for improving myogenic responsiveness in the peripheral vascular system.

5 According to the invention the term “improving myogenic responsiveness in the peripheral vascular system” is meant an improvement of the myogenic responsiveness especially in a disease state or condition, respectively, in which the myogenic responsiveness is deteriorated, in particular in the context of the specific diseases and conditions, respectively, as outlined in more detail below. Compared to  
10 a healthy subject, the myogenic responsiveness in the peripheral vascular system of subjects showing a deteriorated myogenic responsiveness in the peripheral vascular system, said myogenic responsiveness is normalized or at least changed in the direction of a normalized value.

15 The solution to the above technical problem is provided by the embodiments of the present invention as disclosed in the claims, the present description and the accompanying figures.

The inventors identified superior means to reduce TPR by selectively targeting  
20 mechanisms that modulate discrete portions of myogenic reactivity. The vascular bed-specific *modulation* of myogenic responsiveness improves organ blood flow and function at full preservation of normal physiological regulatory mechanisms.

In a first aspect the present invention provides a use of a casein kinase 1 (CK1) inhibitor  
25 having a higher inhibitory effect on CK1 $\delta$  than on CK1 $\epsilon$  for the manufacture of a medicament for the prevention and/or treatment of a vascular and/or cardiovascular disease, wherein the medicament reduces total peripheral resistance in a subject suffering from said vascular and/or cardiovascular disease.

30 In a second aspect the present invention provides a method for the prevention and/or treatment of a vascular and/or cardiovascular disease by reducing total peripheral resistance in a subject suffering from said vascular and/or cardiovascular disease, the method comprising the step of administering an effective amount of at least one CK1 inhibitor having a higher inhibitory effect on CK1 $\delta$  than on CK1 $\epsilon$  to the subject.

35

According to the present invention, compounds that alter vascular smooth muscle CK1 expression activity have impact on mTNF reverse signaling and myogenic responsiveness, and as a consequence, total peripheral resistance, tissue blood flow, and systemic blood pressure. Since altered myogenic reactivity is a hallmark of numerous diseases (e.g., heart failure, subarachnoid haemorrhage, diabetes, stroke, sepsis), targeting microvascular CK1 activity/expression has the potential to improve microvascular myogenic responsiveness and systemic hemodynamics in diverse diseases.

- 10 More specifically, the present invention provides the use of casein kinase 1 (CK1) inhibitors, i.e. one or more CK1 inhibitors may be used, for the prevention and/or treatment of vascular and/or cardiovascular diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, 15 cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis, subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred.
- 20 According to the invention, a CK1 inhibitor is a compound that reduces the expression and/or activity of CK1.

Preferred CK1 inhibitors for use in the invention are inhibitors selective for CK1 isoforms  $\delta$  (CK1 $\delta$ , in other instances also referred to as "CK1D") and/or  $\epsilon$  (CK1 $\epsilon$ , in 25 other instances also referred to as "CK1 E"). In certain embodiments of the invention, it is preferred when the CK1 inhibitor has a stronger inhibitory effect on CK1 $\delta$  than on CK1 $\epsilon$ . Particularly suitable CK1 inhibitors for use in the invention are CK1 inhibitors disclosed in WO 2014/023271, more preferably the CK1 inhibitors D4476, PF670462, IC261 and PF 4800567. Highly preferred CK1 inhibitors for use in the invention are 30 PF-670462 and PF-4800567, which may also be used in combination. Other useful CK1 inhibitors in the context of the invention are OK1 $\delta$  selective inhibitors disclosed in Salado et al. (2014 J. Med. Chem. 2014, 57, 2755-2772, especially those shown in Fig. 1, Table 1 and Fig. 2 thereof, with the compound M3-15 being most preferred. It is to be understood that the present invention is also directed to the use of

pharmaceutically acceptable salts, solvates, esters salts of such esters, as well as any other adduct or derivative which upon administration to a patient in need is capable of providing, directly or indirectly, a Ck1 inhibitor for use in the invention or a metabolite or residue thereof.

5

According to the invention, CK1 inhibitors inhibit CK1 expression/activity and reduce mTNF reverse signaling in the vascular smooth muscle cells of peripheral resistance arteries, in particular in patients suffering from diseases like vascular and cardiovascular diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis, subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred.

15

According to the invention, CK1 inhibitors reduce smooth muscle Erk1/2 phosphorylation and sphingosine-1 -phosphate signaling in diseases like heart failure, in particular in patients suffering from diseases like vascular and cardiovascular diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis, subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred.

20

25

According to the invention, CK1 inhibitors inhibit CK1 expression/activity and reduce peripheral myogenic reactivity, in particular in patients suffering from diseases like vascular and cardiovascular diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic

30

disease, venous thrombosis, subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred. According to the invention, CK1 inhibitors reduce total peripheral resistance, in particular in patients suffering from diseases like vascular and cardiovascular

5 diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis,

10 subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred.

According to the invention, CK1 inhibitors reduce systemic blood pressure, in particular in patients suffering from diseases like vascular and cardiovascular

15 diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis,

20 subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred.

According to the invention, CK1 inhibitors increase blood flow in skeletal muscle, mesenteric, renal, and coronary circulations, in particular in patients suffering from

25 diseases like vascular and cardiovascular diseases (CVDs) such as coronary artery diseases (CAD) (angina and myocardia infarction (commonly known as a heart attack)), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic

30 disease, venous thrombosis, subarachnoid haemorrhage and hypertension, whereby heart failure, subarachnoid haemorrhage and hypertension are particularly preferred.

According to the invention, the effect of CK1 inhibitors is limited to myogenic tone; catecholamine-induced vasoconstriction is not affected in diseases like heart failure, subarachnoid haemorrhage, and hypertension

5 According to the invention, the effect of CK1 inhibitors is limited to the peripheral and coronary circulation; cerebrovascular hemodynamics is not affected in diseases such as those as described above, in particular in heart failure, subarachnoid haemorrhage, and hypertension.

10 The present invention is also directed to a CK1 inhibitor, preferably a CK1 inhibitor selected from those as outlined in more detail above, for use in the prevention and/or treatment of vascular and/or cardiovascular diseases as described above.

The present invention is furthermore directed to a method for preventing and/or  
15 treating of a vascular disease and/or cardiovascular disease as described above comprising administering an effective amount of at least one CK1 inhibitor, preferably at least one CK1 inhibitor selected from those as outlined in more detail above, to a patient, preferably a mammalian patient, in particular a human patient in need thereof.

20 In the present invention, it is shown that the effect of CK16/e-selective inhibition plateaus and thus, it is concluded that maximal CK1 inhibition is capable of only partially attenuating myogenic vasoconstriction. In response to this comment, we conducted an additional experiment to confirm CK1 inhibition efficacy in the heart  
25 failure (HF) setting as an exemplified condition amenable to the inventive treatment using CK1 inhibitors.

According to the invention one or more CK1 inhibitor(s) can be used in its/their free form. In other embodiments the CK1 inhibitor(s) (i.e. at least one CK1 inhibitor) as  
30 used in the present invention is present in a pharmaceutical composition comprising said at least one CK1 inhibitor typically in combination with at least one pharmaceutically acceptable excipient, diluent, carrier and/or vehicle.

The effective amount of the CK1 inhibitor to be applied in the method and uses of the invention, i.e. the specific effective dose level for any particular patient or organism will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed, and like factors well known in the medical arts.

Preferred doses in the context of the invention, in particular with reference to the preferred compounds as referred to herein, more specifically PF-4800567 and PF-670462, are about 1 to about 300 mg per kg body weight (hereinafter referred to as "mg/kg"), preferably about 10 to about 100 mg/kg, more preferably about 20 to about 70 mg/kg, particularly preferred about 25 to about 45 mg/kg such as 30 mg/kg. The dose may be administered once or more often, such as twice or thrice daily. Preferably, doses are administered once or twice daily. Preferred administration of the one or more CK1 inhibitor(s) takes place once daily.

It is known that many cardiovascular incidents regularly take place during the resting state, i.e. at night when human patients are concerned. Therefore, the at least one CK1 inhibitor is preferably administered according to a regime that provides for a maximal pharmacological effect of the at least one CK1 during the resting state of the treated subject, more preferably at or around mid rest phase, whereby "around mid rest phase" is preferably from about - 2 to about + 2 hours of mid rest phase, more preferably from about - 1 to about + 1 hour of mid rest phase, even more preferred from about - 0.5 to about + 0.5 hours of mid rest phase. It is clear for the person skilled in the art that the regime providing maximal pharmacological effect of the at least one CK1 inhibitor depends on the chosen CK1 inhibitor. For example, a CK1 inhibitor displaying comparatively fast degradation of the active compound may be administered once daily at a suitable time before rest phase (i.e. before bed time for human patients) such as 3, 4, 5, or 6 hours before rest phase of the patient. In alternative embodiments where the chosen CK1 inhibitor is a compound displaying slow degradation the CK1 inhibitor can be administered through a controlled release

composition ensuring that the inhibitor has maximal effect, typically maximal bioavailable concentration in the treated patient during the rest phase, preferably at or around mid rest phase (preferably according to the definition given above).

Appropriate pharmaceutical compositions and/or dose and administration regimens employing, e.g. the specific CK1 inhibitors as outlined in more detail herein, for providing the above-described maximal effect during the resting phase are known to the skilled person.

The administration route for the CK1 inhibitor is not particularly critical and the chosen route depends on the individual CK1 inhibitor compound or compounds applied and the subject to be treated. Preferably the CK1 inhibitor(s) is/are administered systemically such as orally or by i.v. administration, which oral administration particularly preferred.

The terms "patient" and "subject" are used herein interchangeably and each mean an animal, preferably a mammal, and most preferably a human.

The at least one CK1 inhibitor, preferably in the present of a pharmaceutical composition as outlined above, for use according to the invention may be administered using any amount and any route of administration effective for treating the cerebrovascular condition. It is understood according to the invention that the term "treating" or "treatment" means that the severity of the condition does at least not progress as compared to the non-treated condition, preferably the severity of the condition does not progress, more preferably, the severity of the condition is lessened, even more preferred substantially lessened, and, ideally, the condition is cured to a substantial extent. Preferably, the severity of the condition according to the invention is reduced at least by 30 %, more preferably by at least 50 %, particularly by at least 70 %, even more preferred by at least 90 %, with complete cure of the condition being the most preferred outcome of the inventive treatment.

Any reference herein to known prior art does not, unless the contrary indication appears, constitute an admission that such prior art is commonly known by those skilled in the art to which the invention relates, at the priority date of this application.

Where ever it is used, the word “comprising” is to be understood in its “open” sense, that is, in the sense of “including”, and thus not limited to its “closed” sense, that is the sense of “consisting only of”. A corresponding meaning is to be attributed to the corresponding words “comprise”, “comprised” and “comprises” where they appear.

5

The Figures show:

Fig. 1: **(A)** Prior to treatment, myogenic tone in cremaster arteries isolated from mice with HF (n=6) is augmented relative to arteries isolated from sham control (n=8). **(B)** Following treatment with 550nM PF670462 *in vitro* (30 minutes), myogenic tone in both groups is attenuated to same level. Thus, the HF-mediated augmentation of myogenic tone is eliminated (i.e., the post-treatment curves overlap) and the new tone level is only modestly reduced compared to the sham.

15 Fig. 2: shows that cytosolic portion of mTNF regulates reverse signaling.

Fig. 3: shows that casein kinase 1 regulates mTNF-mediated myogenic responsiveness.

20 Fig. 4: shows that casein kinase 1 delta regulates myogenic responsiveness.

Fig. 5: shows that CK1 D regulates circadian oscillations in myogenic responsiveness.

25 Fig. 6: shows that CK1 D inhibition improves microvascular dysfunction and cardiac performance in HF.

The present invention is further illustrated by the following non-limiting examples.

30 EXAMPLES

**Example 1: CK1 inhibition reduces myogenic responsiveness in vitro and in vivo**

Referring to Fig. 1, microvascular smooth muscle cells were cultured from mouse mesenteric arteries and ERK1/2 phosphorylation was assessed by standard western blotting. Murine cremaster skeletal muscle resistance arteries were assessed by pressure myography. mTNF reverse signalling was induced by the intrinsically  
 5 activity TNF type I receptor, sTNFR1-Fc.

In microvascular smooth muscle cells, sTNFR1-Fc increases phosphorylation of ERK1/2. sTNFR1-Fc-induced ERK1/2 phosphorylation is abolished by both the pan CK1 inhibitor CKI-7 and the specific CK1 $\delta$  inhibitor PF-670462. These findings were  
 10 verified in cremaster skeletal muscle resistance arteries. In vitro application of CKI-7 abrogates sTNFR1-Fc-induced mTNF reverse signalling and reduces myogenic responsiveness. CK1 inhibition did not affect myogenic responsiveness in TNF<sup>-/-</sup> arteries. In vitro PF-670462 also reduces myogenic responsiveness and when applied in vivo, PF-67062 reduced myogenic responsiveness in isolated cremaster  
 15 arteries. Importantly, none of the above treatments affected agonist-induced vasoconstriction.

### **Example 2: Cytosolic portion of mTNF regulates reverse signaling**

Referring to Fig. 2, human embryonic kidney (HEK) cells were transiently transfected for 24hrs and stimulated with the TNF antibody Adalimumab (Humira™) for 5 min. mTNF reverse signaling is indicated by ERK1/2 phosphorylation, assessed by western blot. mTNF reverse signaling is not stimulated unless the HEK cells express a tumour necrosis factor (TNF) plasmid construct (NT = non-transfected); truncation  
 25 of TNF's intracellular domain (amino acids 2-25) abolishes reverse signaling (Trunc TNF). Importantly, the cytoplasmic tail of mTNF contains the casein kinase 1 (CK1) recognition site (S(P)-X-X-S) that is removed in Trunc TNF, indicating the necessity for CK1 binding in mTNF reverse signaling.

\* in Fig. 2 indicates P<0.05 by unpaired Student's t- test, n=6-12 biological replicates.

### **Example 3: Casein kinase 1 regulates mTNF-mediated myogenic responsiveness**

Referring to Fig. 3, mouse cremaster skeletal muscle resistance arteries were isolated and cannulated for pressure myography. Stepwise increases in transmural pressure (20-100mmHg in 20mmHg steps) elicit myogenic vasoconstriction. (Fig. 3a) The pan-casein kinase 1 (CK1 ) inhibitor CKI-7 (10mM in vitro) reduces myogenic responsiveness. (Fig. 3b) Vessel health is not compromised by CKI-7 as phenylephrine-induced vasoconstriction remains intact. (Fig. 3c) CKI-7 elicits dose-dependent reductions in myogenic responsiveness (n=4-6 vessels). (Fig. 3d) CKI-7 is not effective in the absence of TNF signaling (i.e., TNF knockout arteries, TNF KO). (Fig. 3e) Phenylephrine-induced vasoconstriction in TNF KO arteries is not affected by CKI-7. (Fig. 3f) Acute administration of the mTNF-stimulating fusion protein sTNFR1-Fc (100ng/mL) stimulates vasoconstriction that is prevented by CKI-7, indicating that mTNF reverse signaling requires functional CK1. (Fig. 3g) All vessels are healthy prior to experimentation, shown by robust vasoconstriction to phenylephrine (PE) (h) Isolated cremaster resistance arteries underwent pressure myography and subsequent western blotting for ERK1/2 phosphorylation, a molecular readout of mTNF reverse signaling. sTNFR1-Fc stimulates increased ERK1/2 phosphorylation that is inhibited by CKI-7. (i) Representative western blot (j) Mesenteric vascular smooth muscle cells show robust ERK1/2 phosphorylation in response to sTNFR1- Fc that is inhibited by CKI-7 (1 mM).

(Fig. 3a,b,d,e) \*in Fig. 3a, b, c, d and e indicates  $P < 0.05$  by unpaired Student's t- test. (Fig. 3c,f-g) \* in Fig. 3c, f and g indicates  $P < 0.05$  by one-way ANOVA and compared to untreated control responses by Dunnett's post-hoc test. \* indicates  $P < 0.05$  by one-way ANOVA and compared to control (Con) and + indicates  $P < 0.05$  compared to TNFR1-Fc alone (0 mol/L CKI-7) by Bonferroni post-hoc test. The numbers of biological replicates are indicated in parentheses in Fig. 3.

#### **Example 4: Casein kinase 1 delta regulates myogenic responsiveness**

Referring to Fig. 4, mouse cremaster skeletal muscle resistance arteries were isolated and cannulated for pressure myography. Stepwise increases in transmural pressure (20- 100mmHg in 20mmHg steps) elicit myogenic vasoconstriction. (Fig. 4a) The selective CK1 E inhibitor PF-4800567 abrogates myogenic responsiveness (30mM in vitro). (Fig. 4b) PF- 4800567 elicits a dose-dependent reduction in

myogenic responsiveness (n=4-5 at each dose), albeit at concentrations well above the IC<sub>50</sub> (32nM), suggesting that this reduction in tone may be due to off-target effects of the inhibitor. (Fig. 4c) Phenylephrine-induced vasoconstriction is reduced in arteries treated with PF-4800567. (Fig. 4d) The reduction in phenylephrine-induced vasoconstriction remains after correction to baseline tone, indicating a non-specific inhibitory effect of PF-4800567 at the 30mM dose. Together, these data indicate that CK1 E is not likely mediating myogenic responsiveness, since inhibition of responses occurs only at concentrations of PF-4800567 ~1000x greater than the IC<sub>50</sub>, and PF-4800567 inhibits general vessel contractility (as indicated by blunted responses to phenylephrine). (Fig. 4e) The CK1D/E inhibitor PF-670462 reduces myogenic responsiveness (550nM *in vitro*), an effect that is reversible through repeated replacements of the vessel's incubating buffer. (Fig. 4f) PF-670462 elicits dose-dependent decreases in myogenic responsiveness at concentrations approximating the published IC<sub>50</sub> (7.7-14nM), suggesting that the reduction in tone is likely specific to either CK1D or CK1E (n=4-5 at each dose). Since the CK1E-selective inhibitor PF-4800567 was only effective well beyond the range of specificity for CK1E, we suggest that the more potent inhibitory effect of PF-670462 is due to targeting of CK1D. (Fig. 4g) CK1 D signals through TNF as PF-670462 inhibits myogenic responsiveness in wild-type (WT) arteries but not in arteries from TNF knockout (TNF<sup>-/-</sup>) mice. (Fig. 4h) Vessel health is not compromised by PF-670462 as phenylephrine-induced vasoconstriction is intact. (Fig. 4a,c,d) \* in Fig. 4a, c, and d indicates P<0.05 by unpaired Student's t- test. (Fig. 4b,f) \* in Fig. 4b and f indicates P<0.05 by one-way ANOVA and compared to 0 dose by Dunnett's post-hoc test. (Fig. 4e) \* in Fig. 4e indicates P<0.05 by one-way ANOVA and compared to PF-670462 by Dunnett's post-hoc test. (Fig. 4g,h) \* in Fig. 4g and h indicates P<0.05 by one-way ANOVA and compared to WT PF-670462 response by Dunnett's post-hoc test. The numbers of biological replicates are indicated in parentheses in Fig. 4.

### 30 **Example 5: CK1D regulates circadian oscillations in myogenic responsiveness.**

Referring to Fig. 5, mouse cremaster skeletal muscle resistance arteries were isolated at mid-rest phase (ZT7) or mid-active phase (ZT19) and cannulated for pressure myography. One-step increases in transmural pressure (60 to 100mmHg)

elicit myogenic vasoconstriction. (Fig. 5a) PF-670462 (10mM, *in vitro*) reduces myogenic responsiveness only during the rest phase (ZT7) and not during the active phase (ZT19). (Fig. 5b) Vessel dilation is not affected by PF-670462 treatment, indicating that vessel initial tone is consistent prior to pressure stimulation. (Fig. 5c) Cremaster muscle resistance arteries underwent pressure myography and western blotting subsequently assessed ERK1/2 phosphorylation. PF-670462 reduces ERK1/2 phosphorylation to a greater extent during the rest phase (ZT7). (Fig. 5d) Representative western blot. (Fig. 5e) Resting diameter of the vessels is not affected by PF-670462. (Fig. 5f) Prior to administration of PF-670462, vessel health is intact as shown by robust vasoconstriction to phenylephrine. (Fig. 5g) PF-670462 dose-dependently reduces myogenic tone in the mid-rest phase (ZT7) to reach the nadir achieved in the mid-active phase (ZT19) (n=4-7 vessels per dose). (Fig. 5h) Vessel health, indicated by robust phenylephrine-induced vasoconstriction, is not affected by PF-670462 administration.

(Fig. 5a to d) \* in Fig. 5a, b, c, and d indicates  $P < 0.05$  by unpaired Student's t- test within the same time period (ZT7 or ZT19, respectively). (Fig. 5e,f) \* in Fig. 5e and f indicates  $P < 0.05$  by one-way ANOVA. (Fig. 5g) \* in Fig. 5g indicates  $P < 0.05$  by one-way ANOVA and compared to no drug (0 pmol/L) within the same time period (ZT7 or ZT 19, respectively) by Dunnett's post-hoc test. (Fig. 5h) \* in Fig. 5h indicates  $P < 0.05$  by unpaired Student's t- test. The numbers of biological replicates are indicated in parentheses in Fig. 5.

### **Example 6: CK1D inhibition improves microvascular dysfunction and cardiac performance in HF**

Referring to Fig. 6a to c, mice underwent myocardial infarction (ligation of the left-anterior descending coronary artery) or sham surgery. At 8 weeks post myocardial infarction, mice had developed heart failure (HF). Cremaster skeletal muscle resistance arteries were isolated and cannulated for pressure myography. Stepwise increases in transmural pressure (20-100mmHg in 20mmHg steps) elicited myogenic vasoconstriction that was significantly stronger in the HF than in the sham group. (Fig. 6a) In bath treatment with PF-670462 (550nM *in vitro*) normalizes myogenic responsiveness in arteries from HF mice (i.e., the level of myogenic responsiveness is similar to sham values). (Fig. 6b) PF-670462 does not affect myogenic tone in

sham-operated mice. (Fig. 6c) Vessels demonstrate robust function, as phenylephrine-induced vasoconstriction is intact.

Referring to Fig. 6d to f, naive mice were treated with PF-670462 (30-50mg/kg dissolved in 200  $\mu$ L water, intraperitoneal injection) or vehicle (200 $\mu$ l water). 24 hrs later, in mid-rest phase (ZT7), cremaster skeletal muscle resistance arteries underwent pressure myography. (Fig. 6d) Both 30mg/kg and 50mg/kg of PF-670462 reduce myogenic responsiveness, suggesting that the drug is functional in vivo. (Fig. 6e) Phenylephrine-induced vasoconstriction is not altered by in vivo application of PF-670462. (Fig. 6f) In accordance with the reduction in myogenic tone, acute injection of PF-670462 (30mg/kg intraperitoneal) reduces mean arterial blood pressure (MAP) (n=3 per group).

Referring to Fig. 6g to j, following myocardial infarction or sham surgery, mice were chronically treated with PF-670462 (30mg/kg, intraperitoneal injection) or vehicle (DMSO) for 7wks (5days/wk). Cremaster skeletal muscle resistance arteries were isolated for pressure myography (Fig. 6g) mRNA expression of CK1 D and CK1 E in cremaster resistance arteries are not different in HF and sham (n=18 samples per group), suggesting that differences in myogenic tone are driven by post-translational mechanisms. (Fig. 6h) HF-induced elevations in myogenic tone are normalized by chronic treatment with PF-670462. (Fig. 6i) Phenylephrine-induced vasoconstriction is intact with chronic PF-670462 treatment. Reduced tone at lower concentrations of phenylephrine in the PF-670462-treated group is the result of changes in resting myogenic tone. (Fig. 6j) Cardiac output, the quantification of blood outflow from the heart assessed with echocardiography, is elevated with chronic PF-670462 treatment.

(Fig. 6a, b, and g to j) \* in Fig. 6a, b and g to j indicates  $P < 0.05$  by unpaired Student's t- test. (Fig. 6c to e) \* in Fig. 6c to e indicates  $P < 0.05$  by one-way ANOVA and compared to control by Dunnett's post-hoc test. The numbers of biological replicates are indicated in parentheses in Fig. 6.

The present invention shows that CK1 acts as a regulator of mTNF reverse signaling and hence, myogenic responsiveness. The demonstrated ability of CK1 inhibitors to

reduce myogenic responsiveness without affecting agonist-induced vasoconstriction provides a substantial safety margin for clinical applications to diseases where microvascular tone is increased.

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## Claims

1. Use of a casein kinase 1 (CK1) inhibitor having a higher inhibitory effect on CK1 $\delta$  than on CK1 $\epsilon$  for the manufacture of a medicament for the prevention and/or treatment of a vascular and/or cardiovascular disease, wherein the medicament reduces total peripheral resistance in a subject with suffering from said vascular and/or cardiovascular disease.
2. A use as claimed in claim 1, wherein the vascular disease or cardiovascular disease is selected from the group consisting of coronary artery diseases (CAD), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis, subarachnoid haemorrhage and hypertension.
3. A use as claimed in claim 1 or claim 2, wherein the vascular disease or cardiovascular disease is selected from the group consisting of heart failure, subarachnoid haemorrhage and hypertension.
4. A use as claimed in any one of claims 1 to 3, wherein the CK1 inhibitor is 4-[3-cyclohexyl-5-(4-fluoro-phenyl)-3H-imidazol-4-yl]-pyrimidin-2-ylamine or a pharmaceutically acceptable salt thereof.
5. A use as claimed in any one of claims 1 to 4, wherein the CK1 inhibitor is administered to the subject once, twice or thrice daily.
6. A use as claimed in claim 5, wherein the CK1 inhibitor is administered to the subject according to a regime that provides for a maximal pharmacological effect of the at least one CK1 during the resting state of the subject.
7. A use as claimed in claim 6, wherein the CK1 inhibitor is administered to the subject according to a regime that provides for a maximal pharmacological effect of the at least one CK1 at mid rest phase or from -2 to + 2 hours of mid rest phase.

8. A use as claimed in claim 6, wherein the CK1 inhibitor is administered to the subject according to a regime that provides for a maximal pharmacological effect of the at least one CK1 from -1 hours to +1 hour of mid rest phase.
9. A use as claimed in any one of claims 1 to 8 wherein the subject is a mammal.
10. A use as claimed in claim 9, wherein the mammal is a human.
11. A method for the prevention and/or treatment of a vascular and/or cardiovascular disease by reducing total peripheral resistance in a subject suffering from said vascular and/or cardiovascular disease, the method comprising the step of administering an effective amount of at least one CK1 inhibitor having a higher inhibitory effect on CK1 $\delta$  than on CK1 $\epsilon$  to the subject.
12. A method as claimed in claim 11, wherein the vascular disease or cardiovascular disease is selected from the group consisting of coronary artery diseases (CAD), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, abnormal heart rhythms, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, venous thrombosis, subarachnoid haemorrhage and hypertension.
13. A method as claimed in claim 11 or claim 12, wherein the vascular disease or cardiovascular disease is selected from the group consisting of heart failure, subarachnoid haemorrhage and hypertension.
14. A method as claimed in any one of claims 11 to 13, wherein the CK1 inhibitor is 4-[3-cyclohexyl-5-(4-fluoro-phenyl)-3H-imidazol-4-yl]-pyrimidin-2-ylamine or a pharmaceutically acceptable salt thereof.
15. A method as claimed in any one of claims 1 to 14, wherein the CK1 inhibitor is administered to the subject once, twice or thrice daily.
16. A method as claimed in claim 15, wherein the CK1 inhibitor is administered to the subject according to a regime that provides for a maximal pharmacological effect of the at least one CK1 during the resting state of the subject.

17. A method as claimed in claim 16, wherein the CK1 inhibitor is administered to the subject according to a regime that provides for a maximal pharmacological effect of the at least one CK1 at mid rest phase or from - 2 to about + 2 hours of mid rest phase.
18. A method as claimed in claim 16, wherein the CK1 inhibitor is administered to the subject according to a regime that provides for a maximal pharmacological effect of the at least one CK1 from about - 1 to about + 1hour of mid rest phase.
19. A method as claimed in any one claims 11 to 18, wherein the subject is a mammal.
20. A method as claimed in claim 19, wherein the mammal is a human.

Fig. 1A

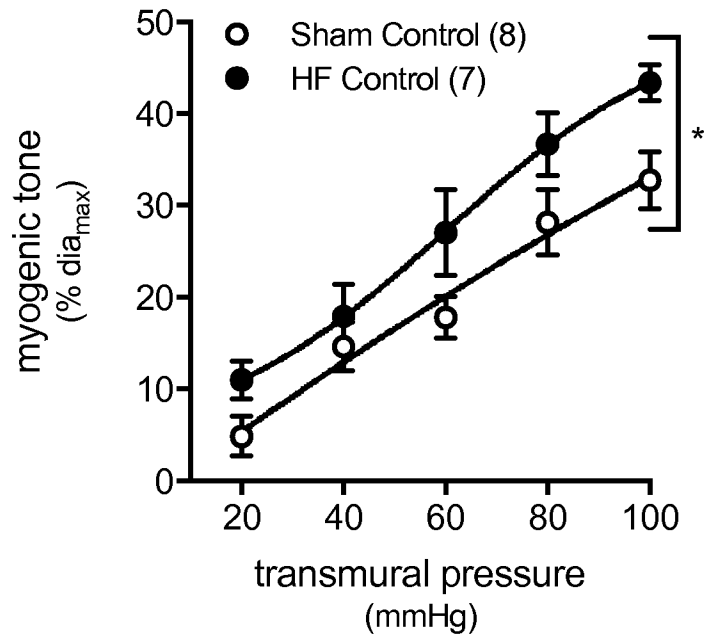


Fig. 1B

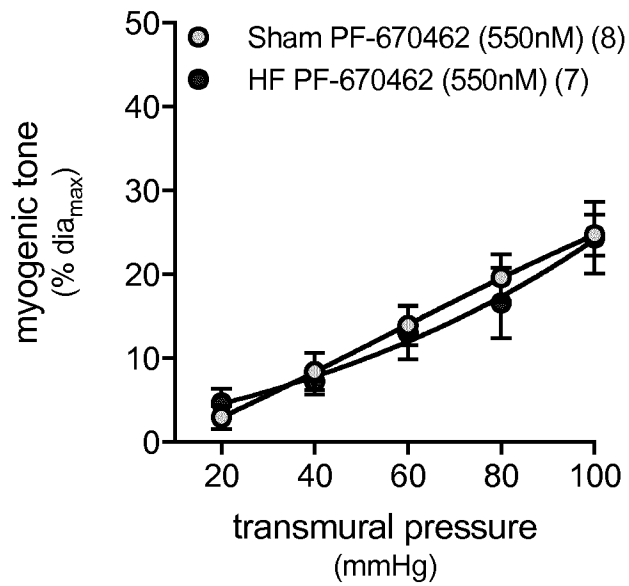
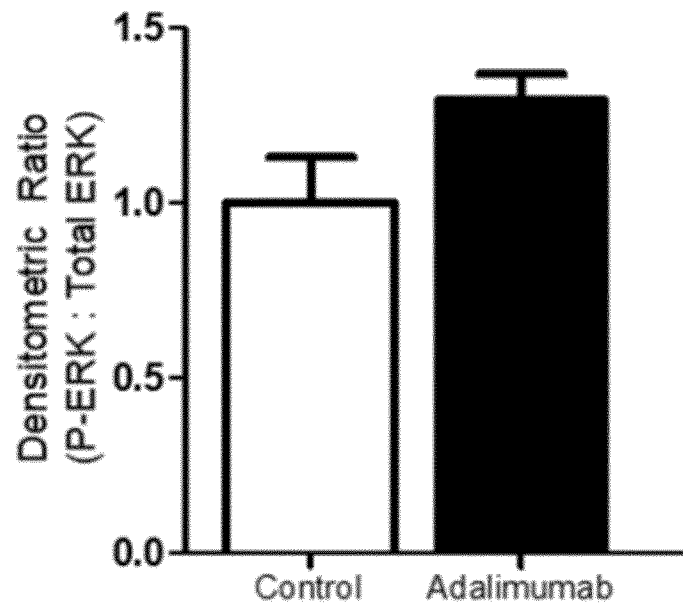


Fig. 2

a.



b.

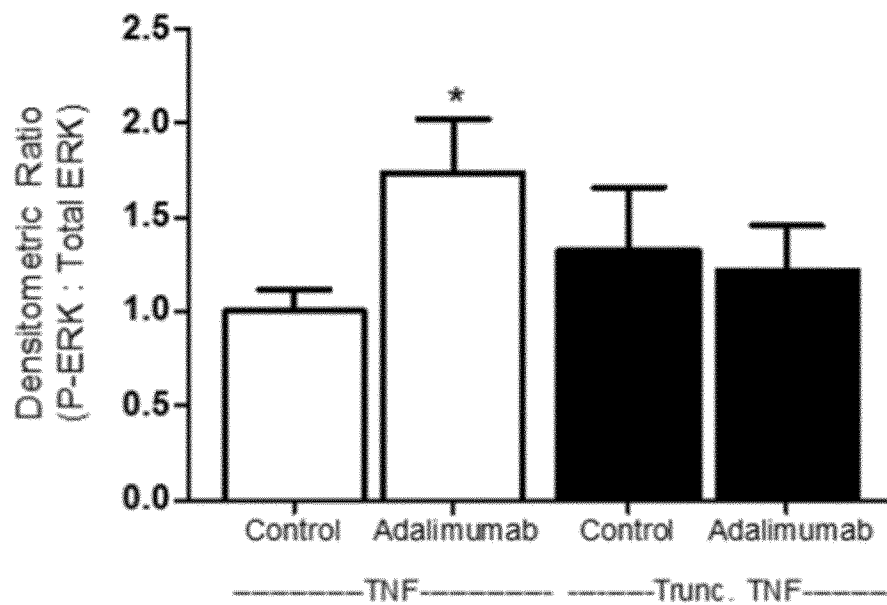


Fig. 3

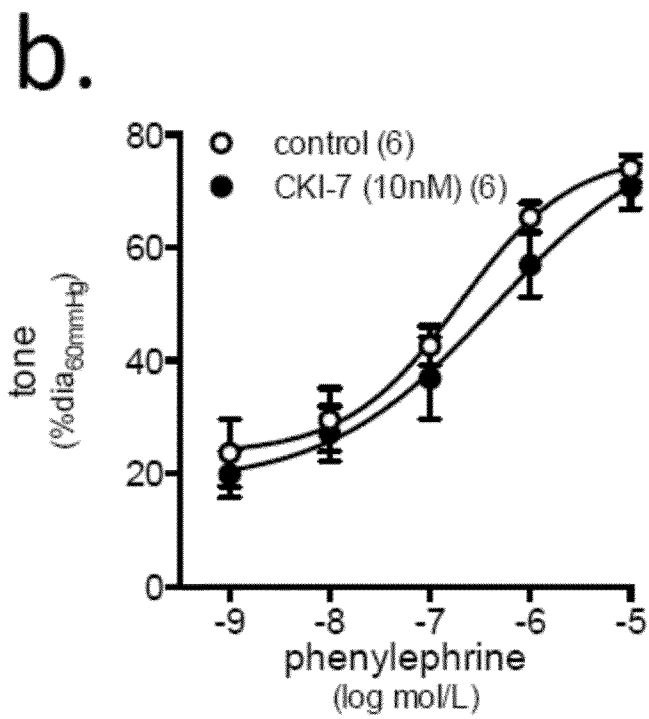
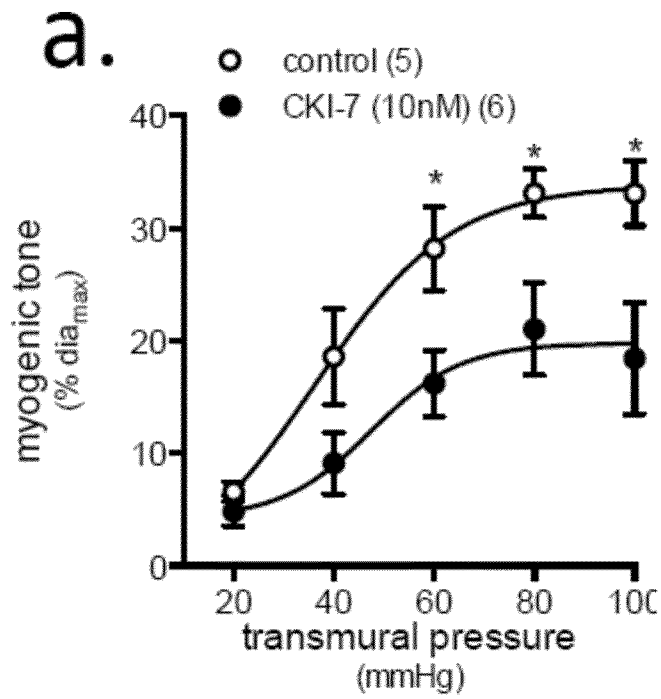


Fig. 3 (continued)

C.

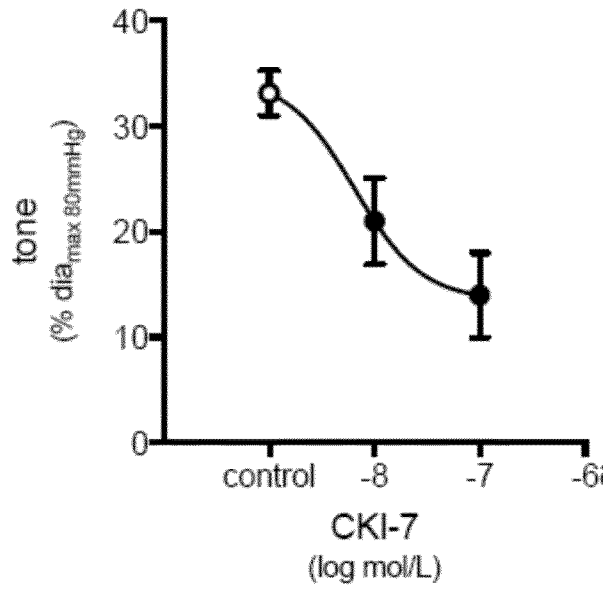


Fig. 3d

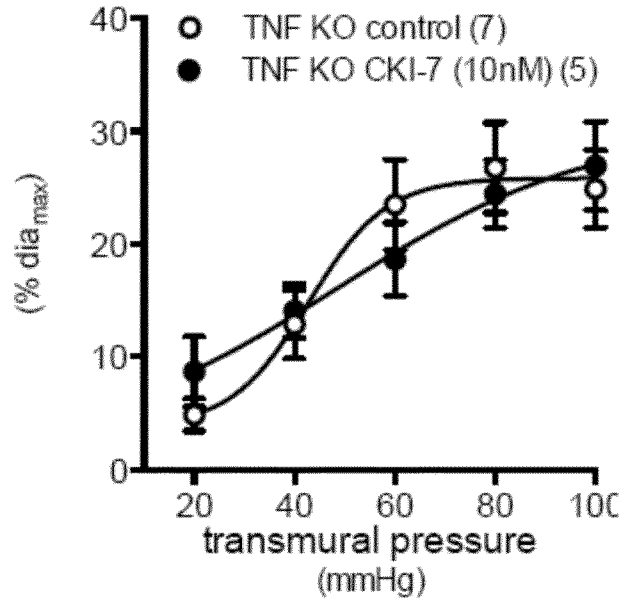


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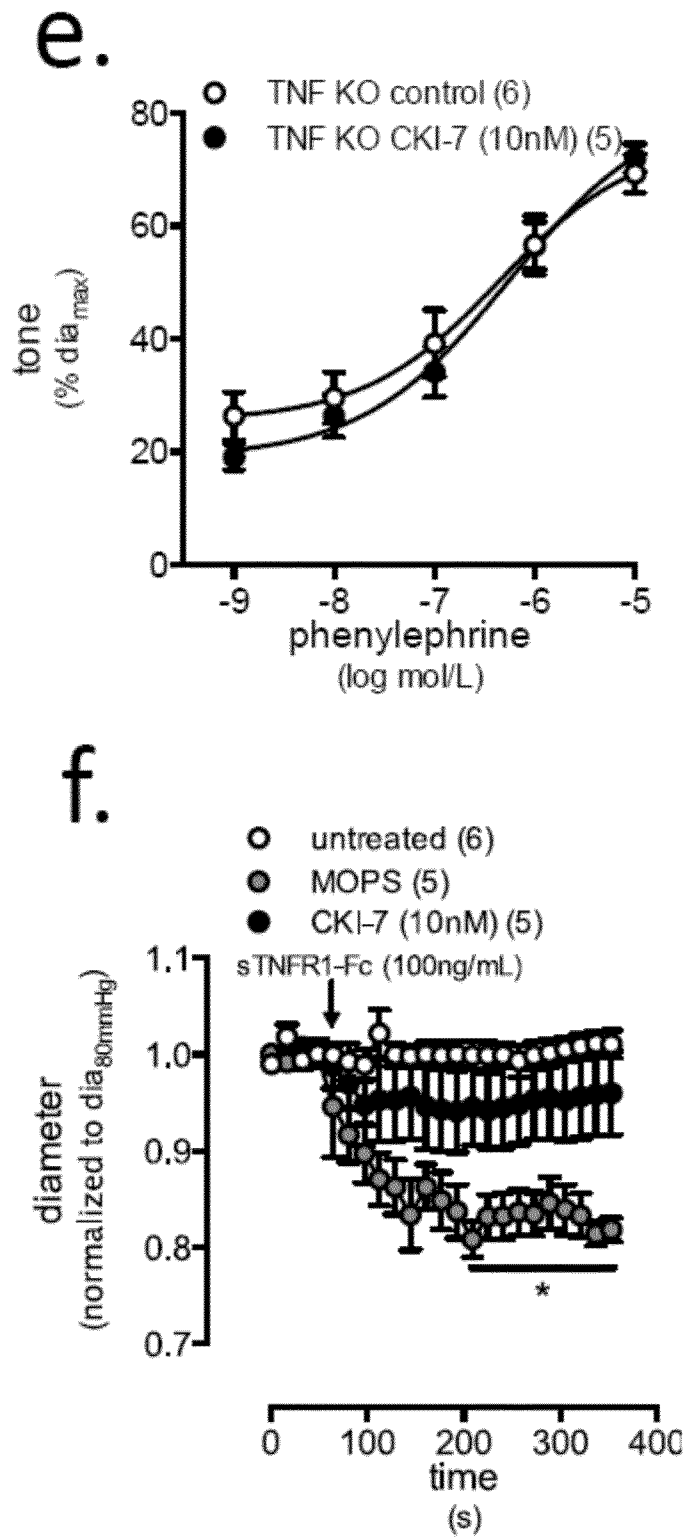


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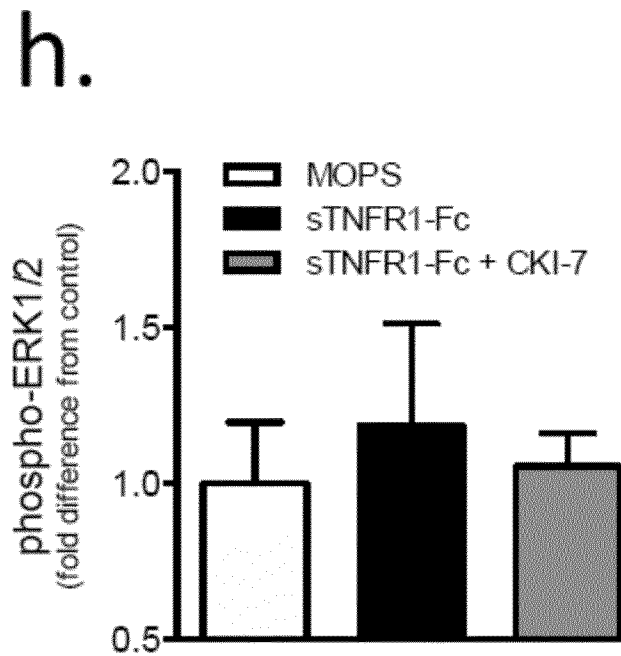
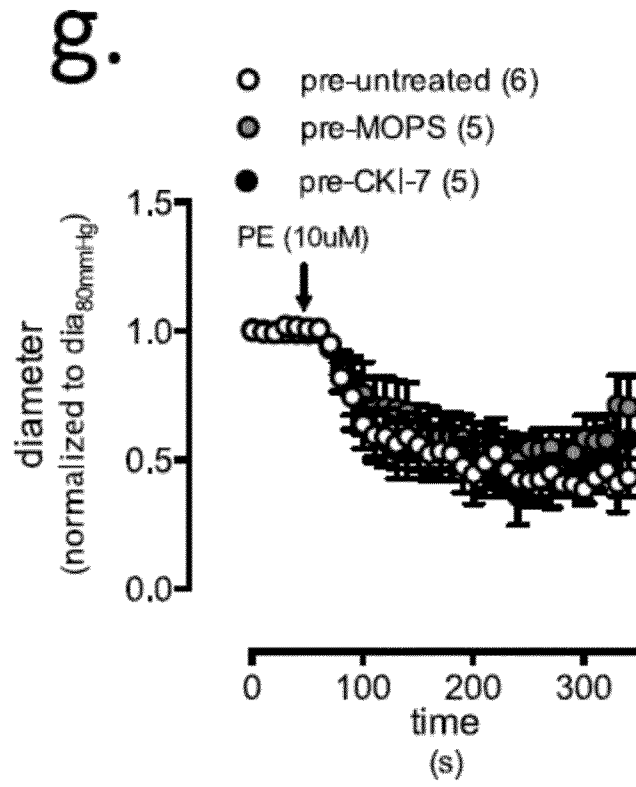


Fig. 3 (continued)

Fig. 3i

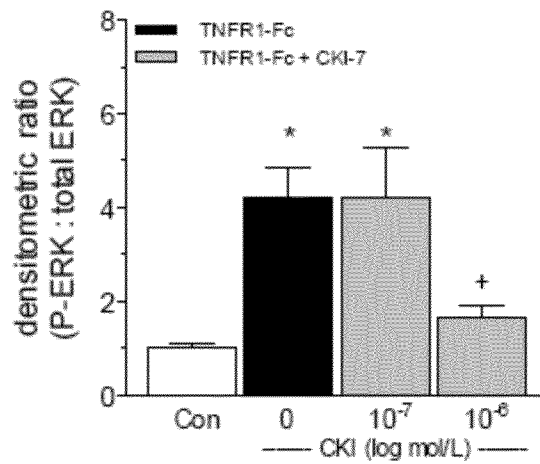
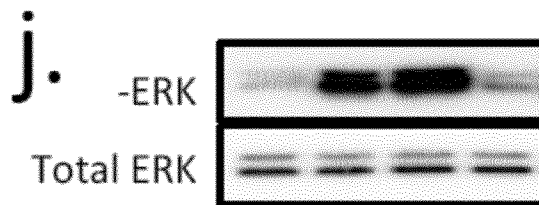
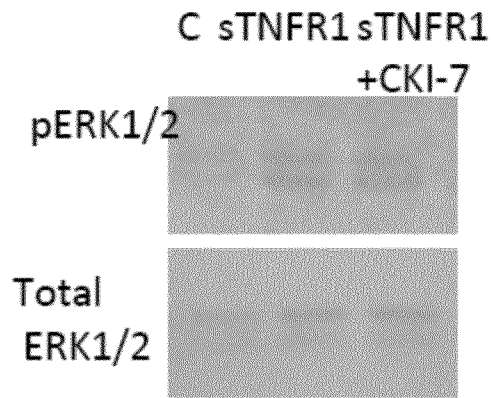


Fig. 4

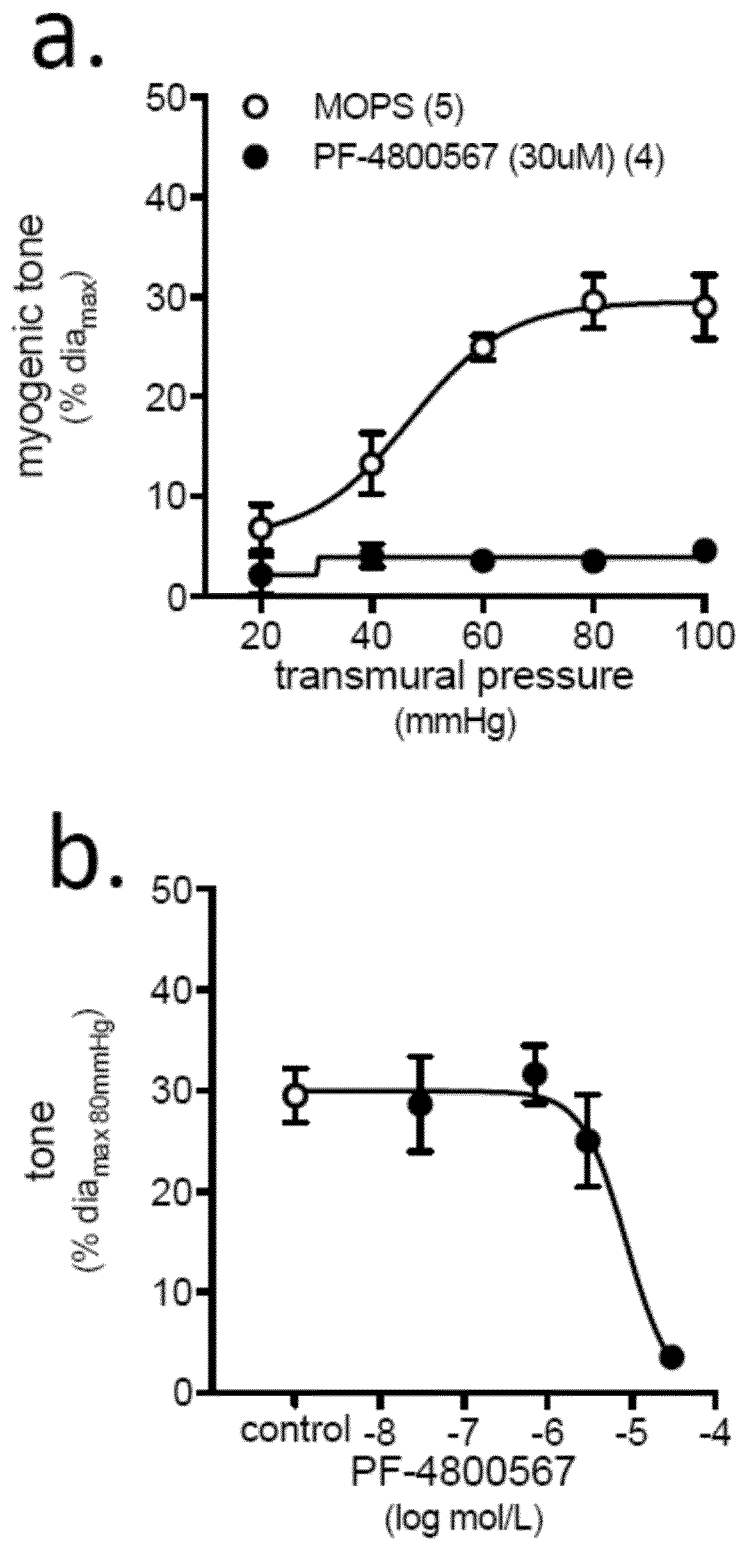


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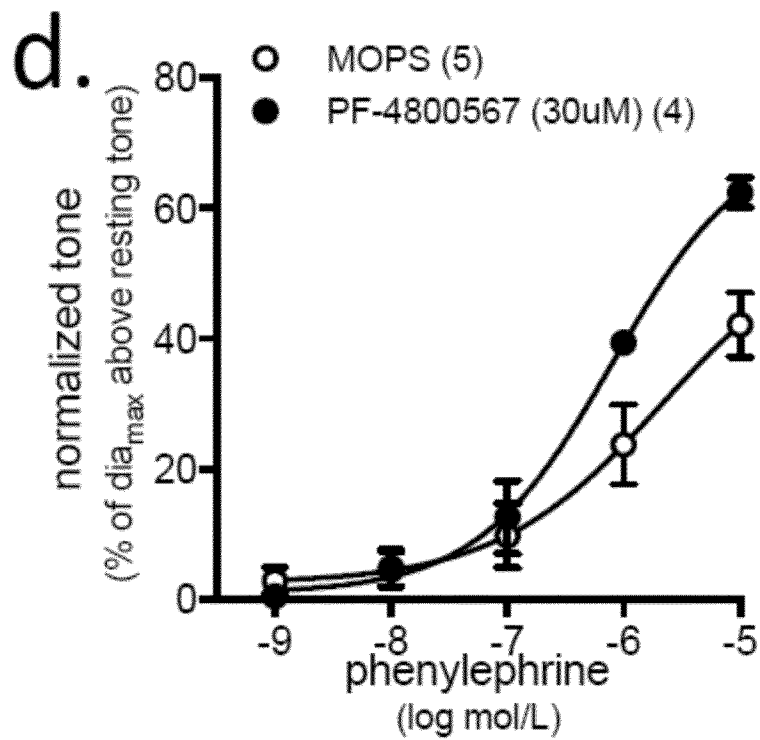
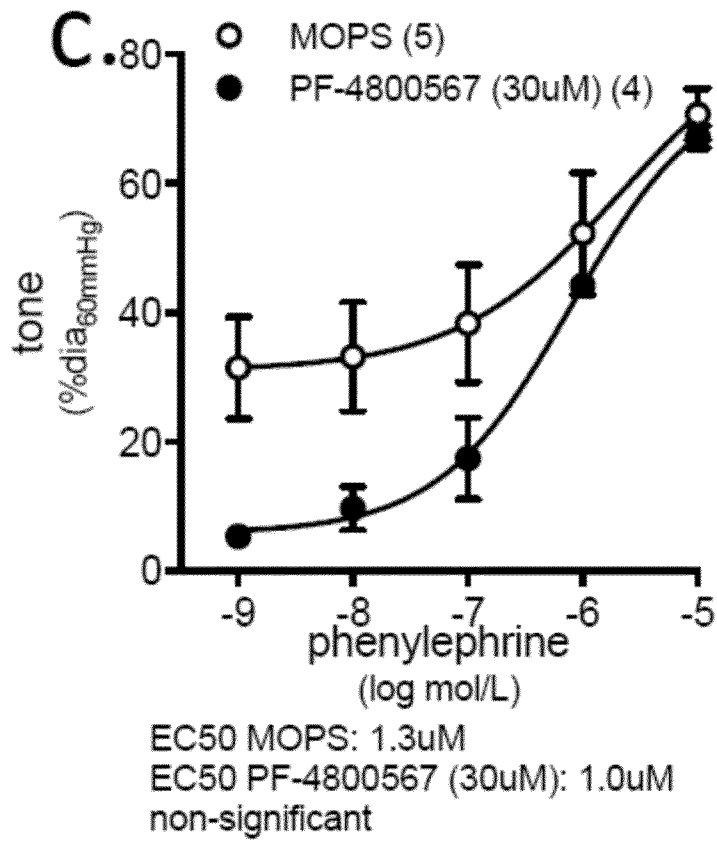


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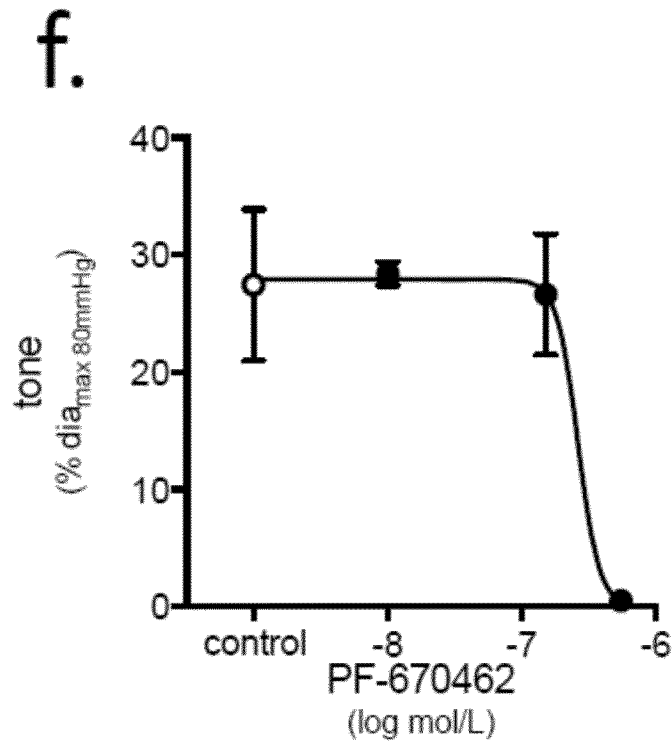
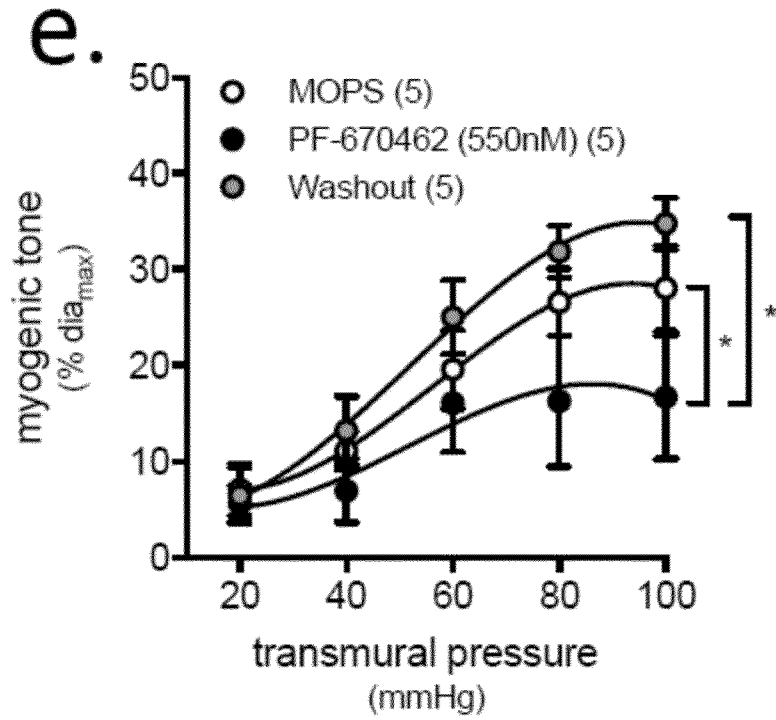


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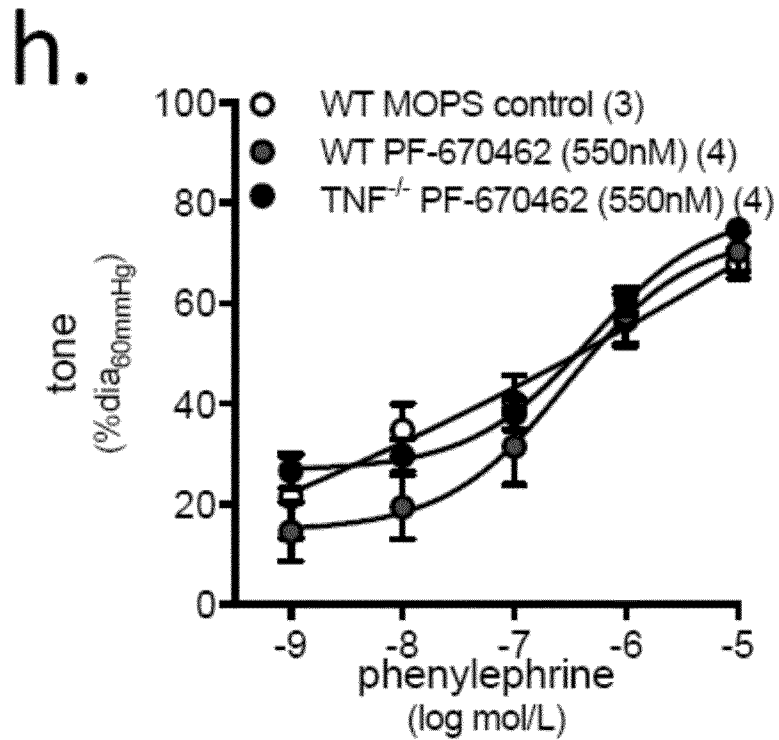
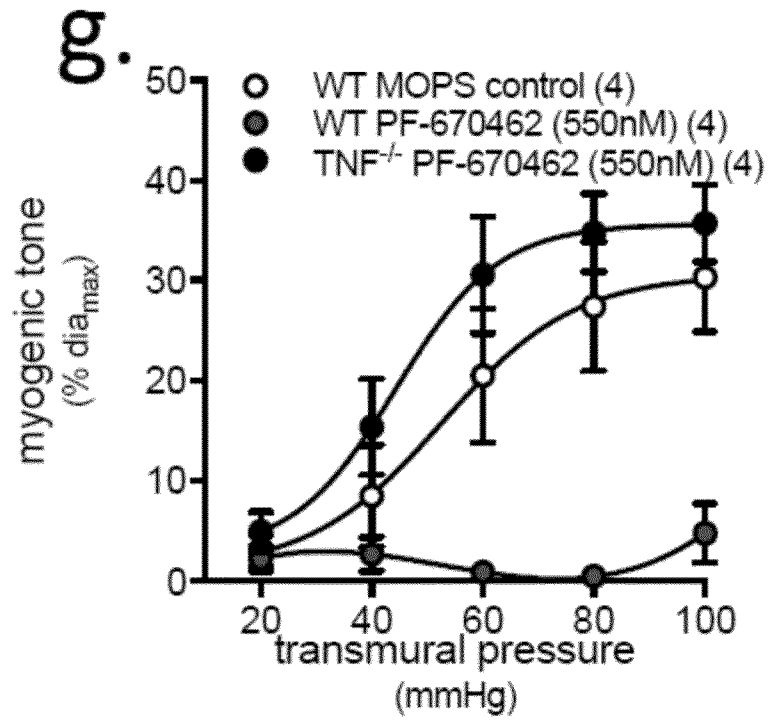


Fig. 5

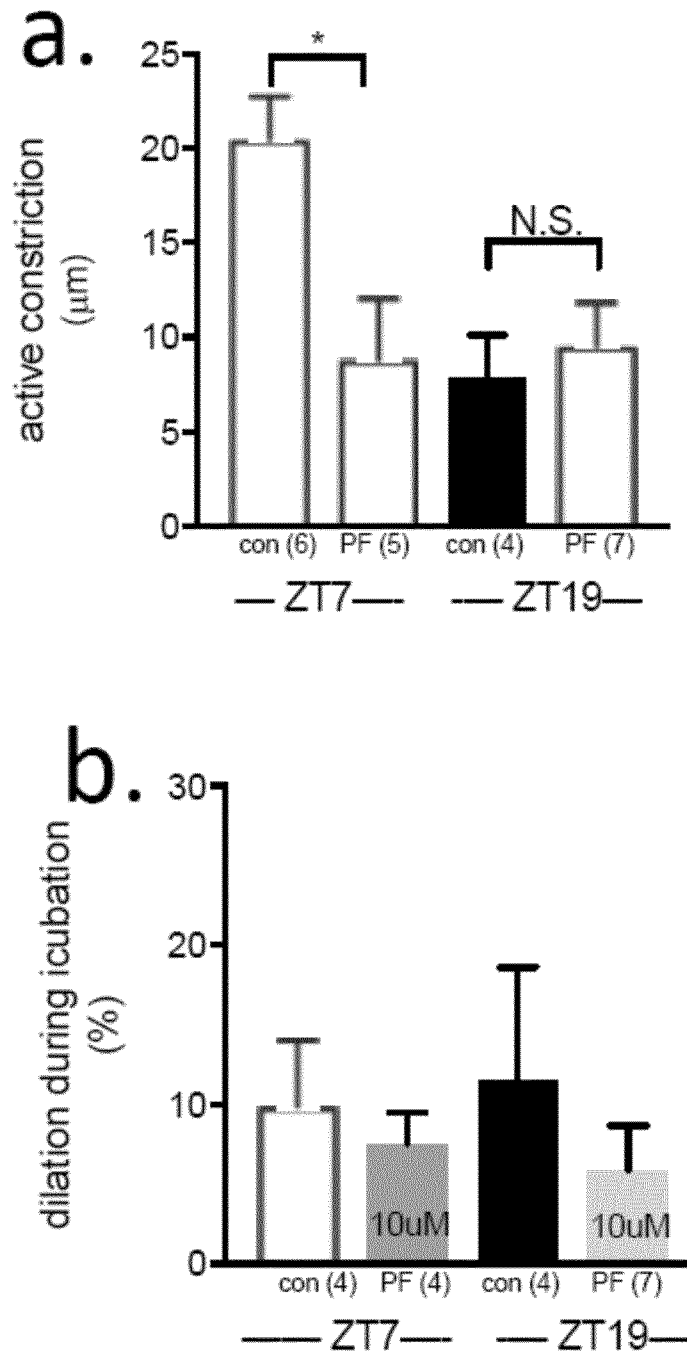
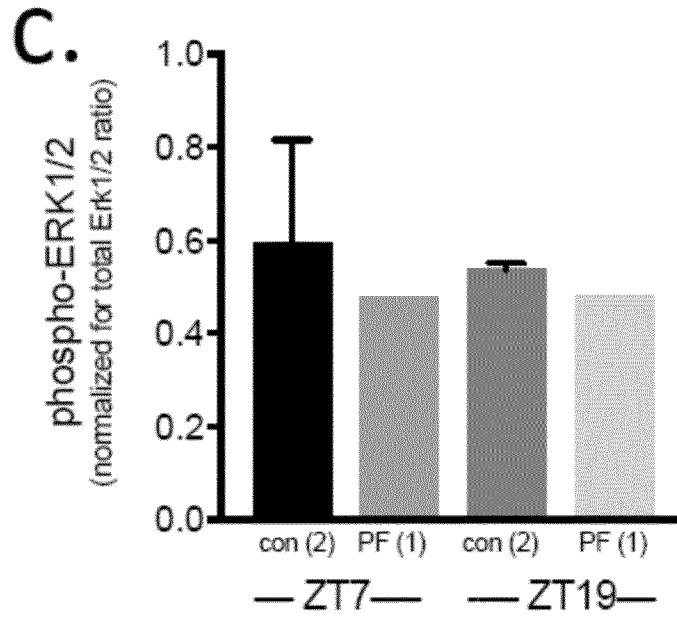


Fig. 5 (continued)



**d.**

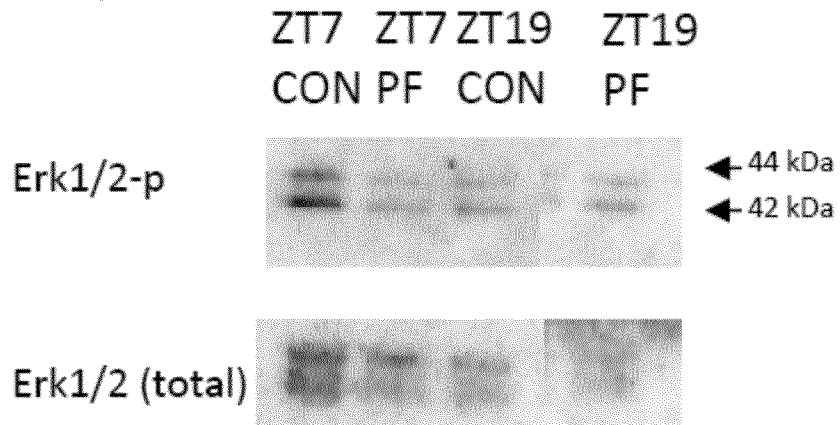
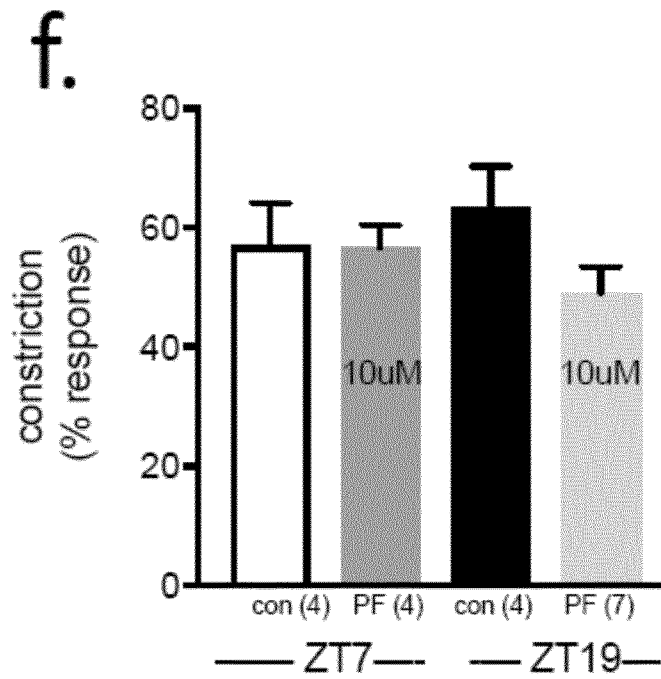
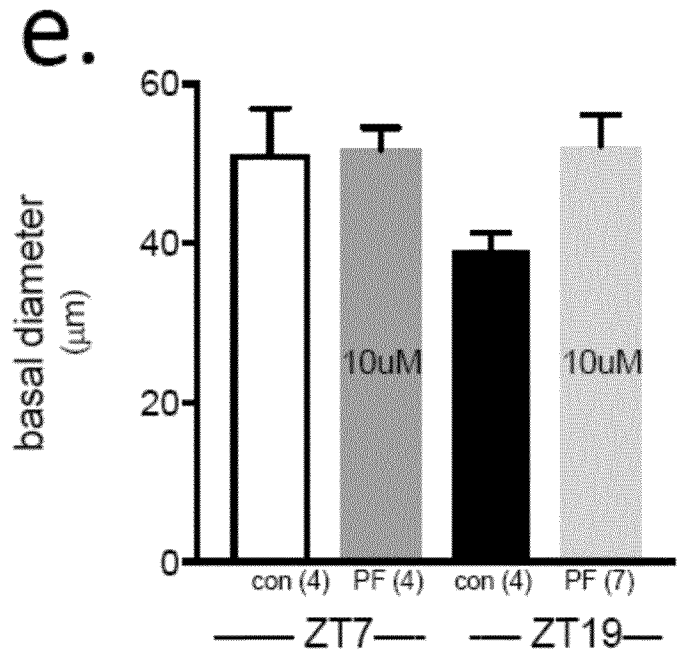


Fig. 5 (continued)



10uM PE

Fig. 5 (continued)

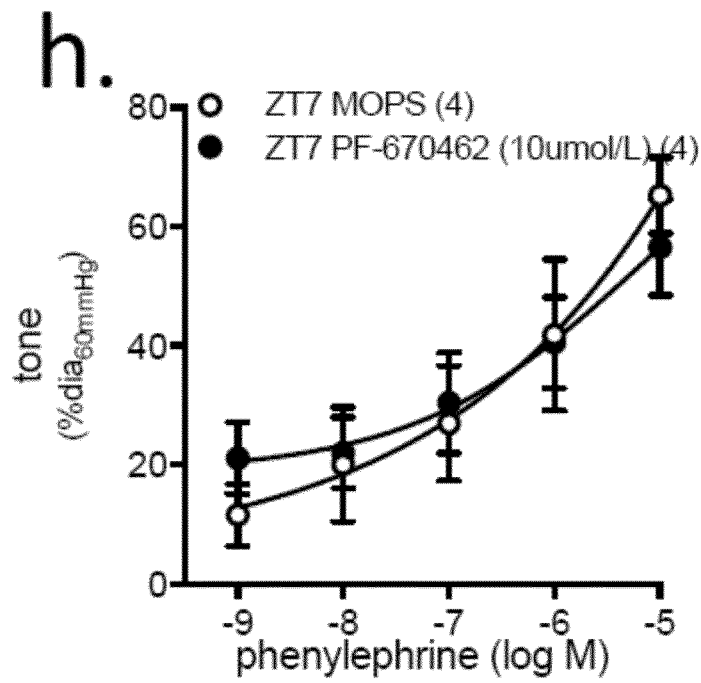
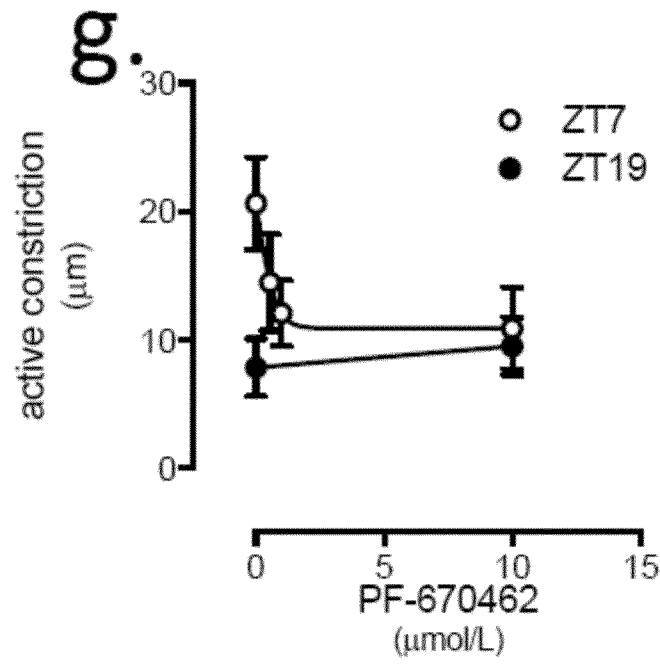


Fig. 6

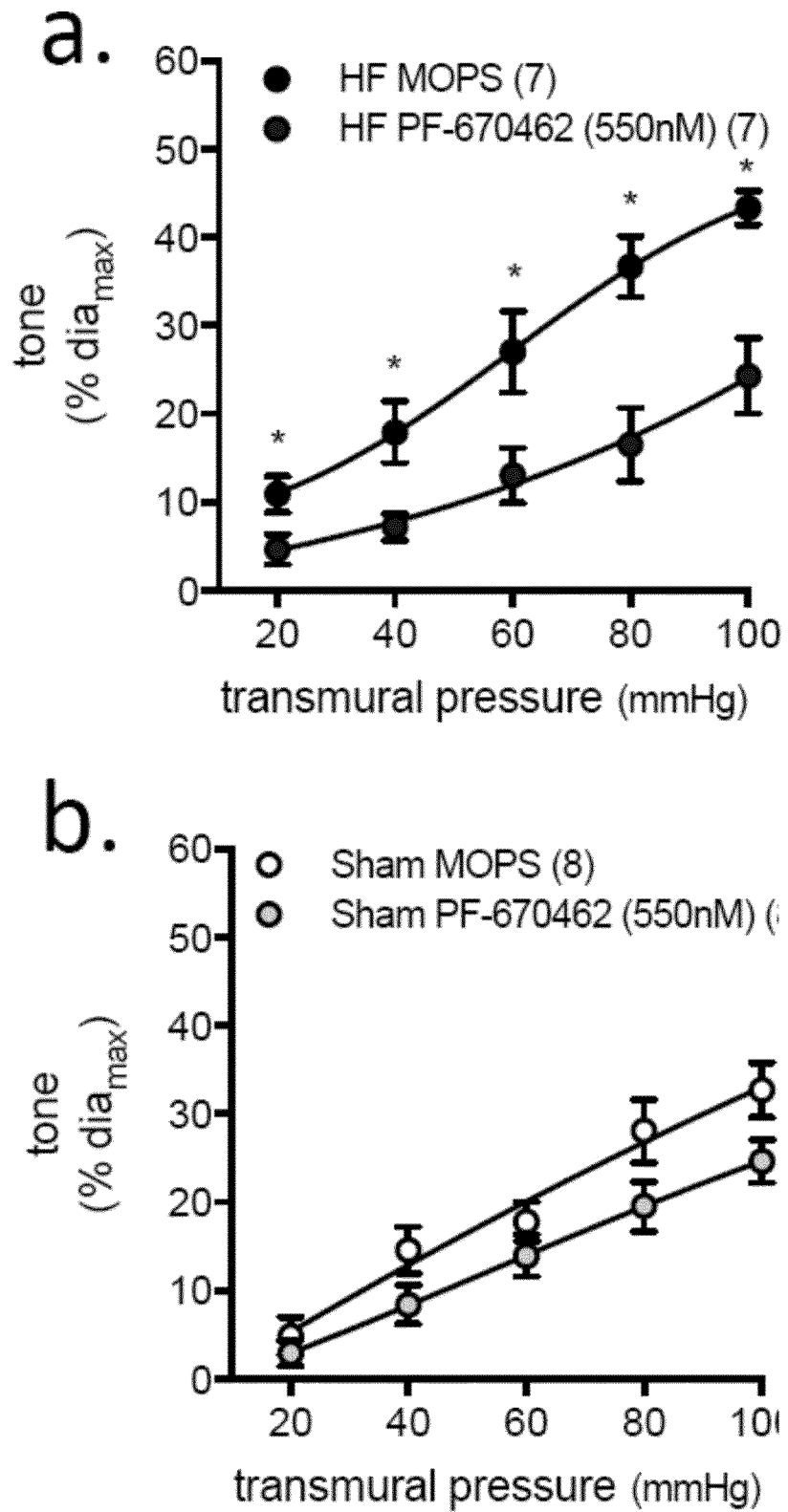


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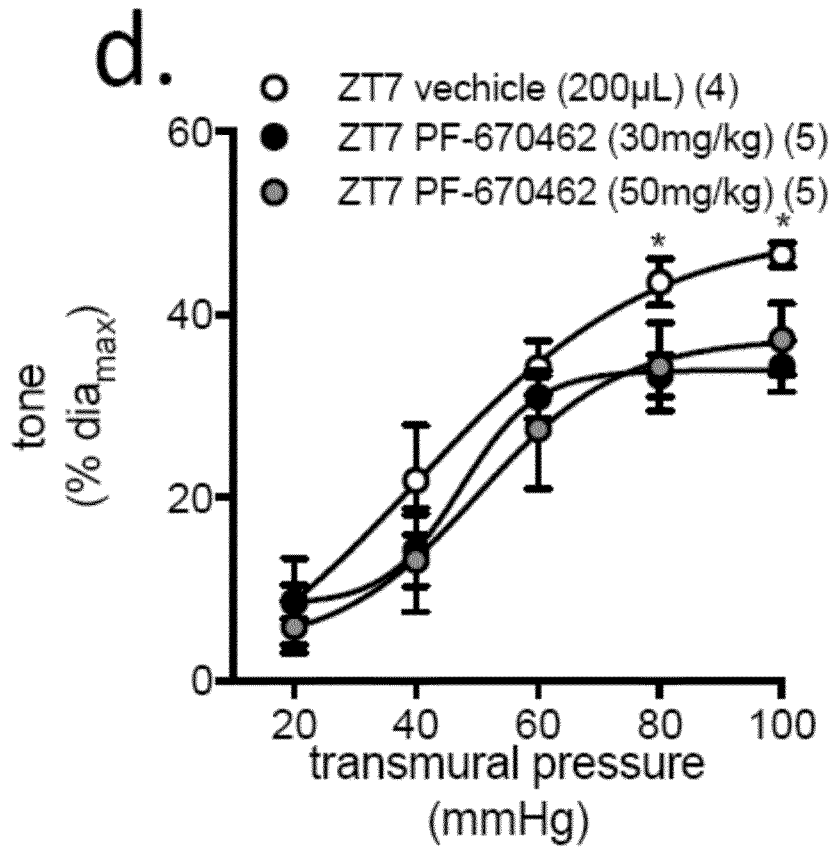
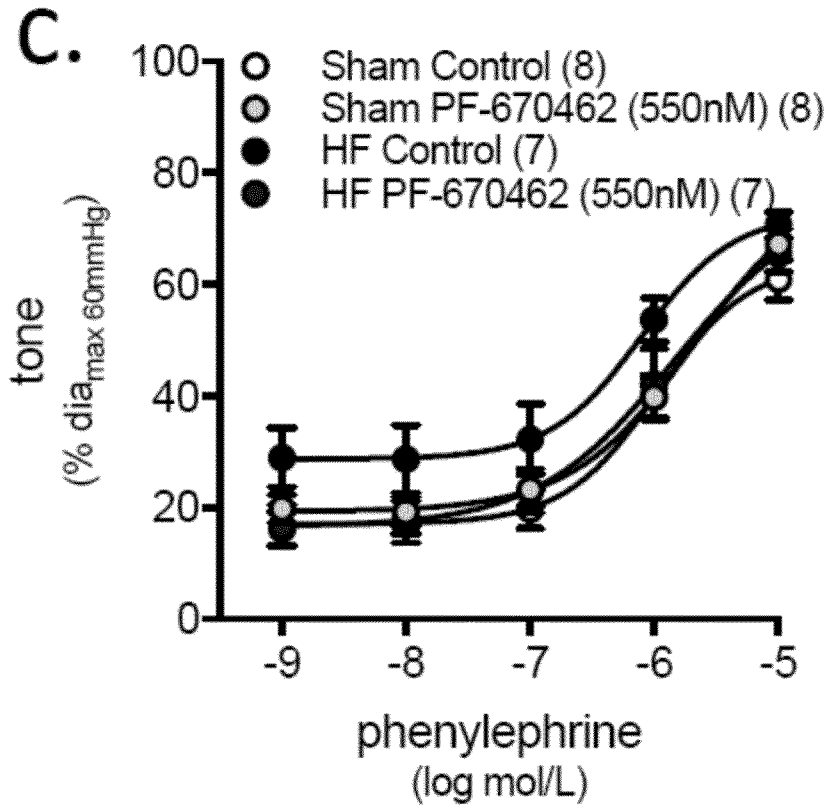


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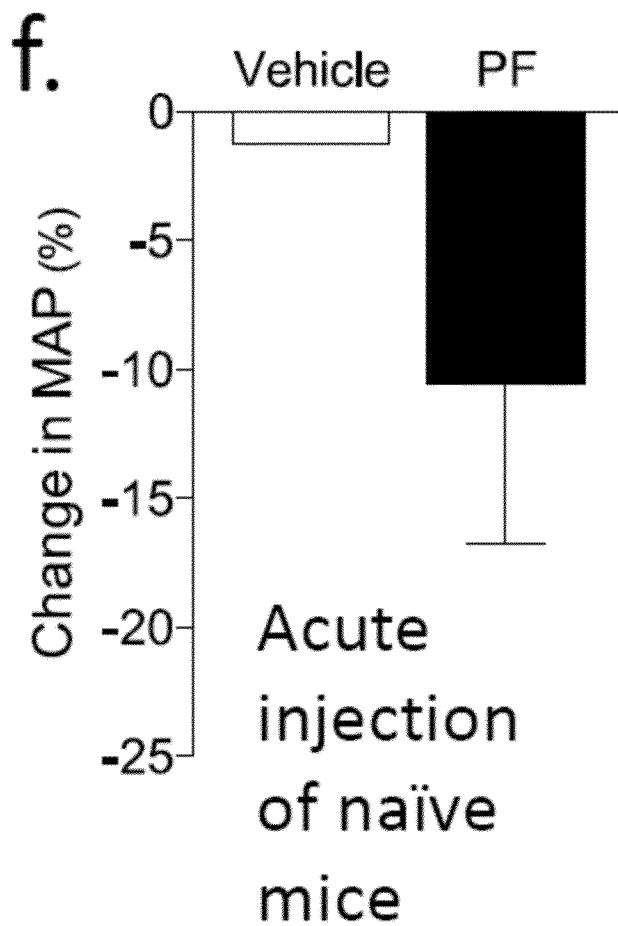
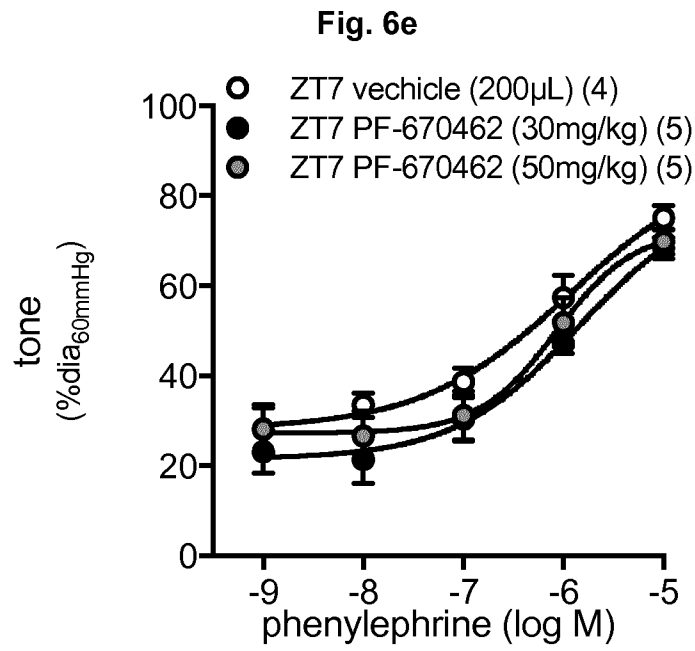


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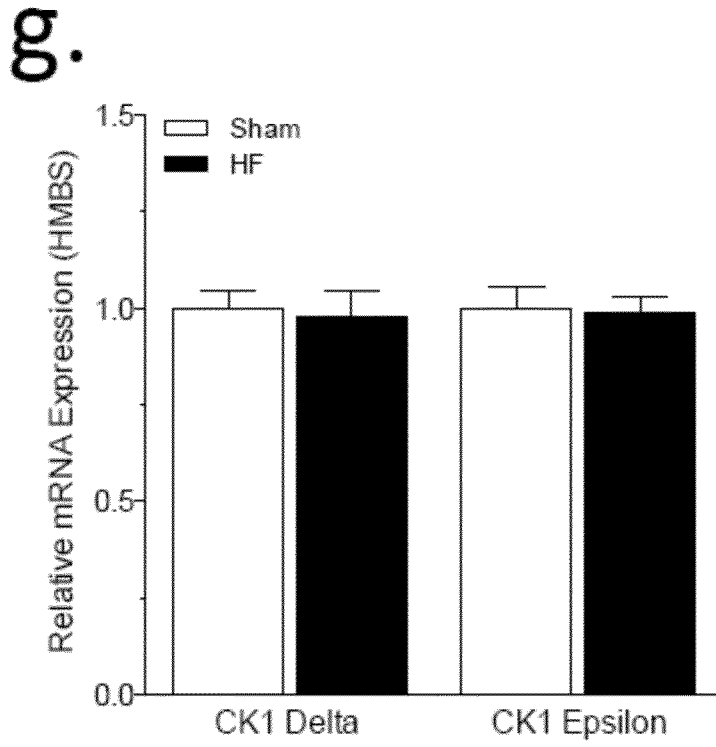


Fig. 6h

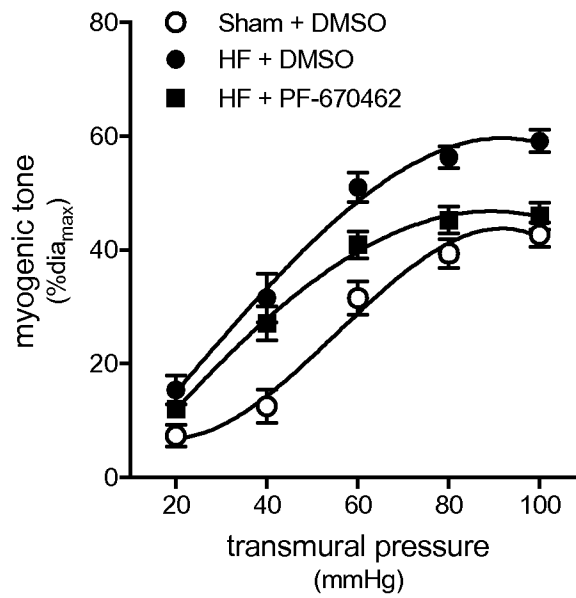
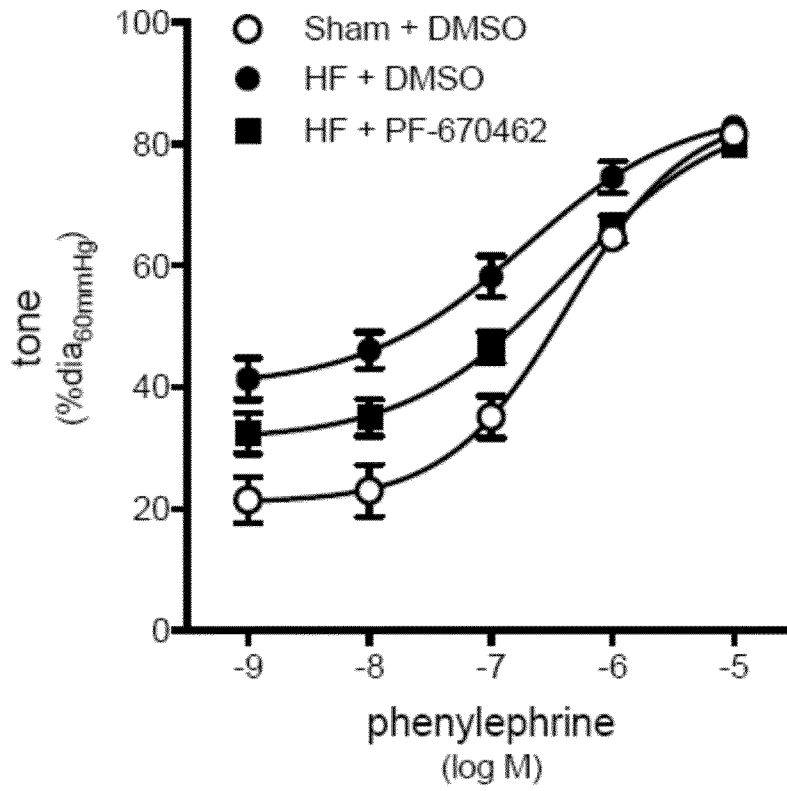


Fig. 6 (continued)

i.



j.

