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The protein acyl transferase ZDHHC21 modulates α1 adrenergic receptor function and regulates hemodynamics

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Abstract

Objective—Palmitoylation, the reversible addition of the lipid palmitate to a cysteine, can alter protein localization, stability, and function. The ZDHHC family of protein acyl transferases catalyzes palmitoylation of numerous proteins. The role of ZDHHC enzymes in intact tissue and *in vivo* is largely unknown. Herein, we characterize vascular functions in a mouse that expresses a nonfunctional ZDHHC21 ("F233Δ").

Approach and Results—Physiological studies of isolated aortae and mesenteric arteries from F233Δ mice revealed an unexpected defect in responsiveness to phenylephrine, an α1 adrenergic receptor agonist. In vivo, F233Δ mice displayed a blunted response to infusion of phenylephrine and were found to have elevated catecholamine levels and elevated vascular α1 adrenergic receptor gene expression. Telemetry studies showed that the F233Δ mice were tachycardic and hypotensive at baseline, consistent with diminished vascular tone. In biochemical studies, ZDHHC21 was shown to palmitoylate the α1D adrenoceptor, and to interact with it in a molecular complex, thus suggesting a possible molecular mechanism by which the receptor can be regulated by ZDHHC21.

Conclusions—Together the data support a model in which ZDHHC21 F233Δ diminishes the function of vascular α1 adrenergic receptors, leading to reduced vascular tone which manifests *in vivo* as hypotension and tachycardia. This is to our knowledge the first demonstration of a ZDHHC isoform affecting vascular function *in vivo* and identifies a novel molecular mode of regulation of vascular tone and blood pressure.

Keywords

palmitoylation; blood pressure; alpha adrenergic signaling

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Disclosures

None.

Introduction

The maintenance of vascular tone is of paramount importance to the health and survival of humans and other mammals. Multiple integrated signaling pathways coordinate to control vascular tone, which in turn determine blood flow distribution and blood pressure via alteration of peripheral vascular resistance. The α1 adrenergic receptor (AR) is a key determinant of vascular tone and its signaling has been the subject of intensive study over many decades. It is the target of several drugs that are used clinically to modify blood pressure[1]. The identification of novel molecular pathways that may regulate α1 adrenergic signaling and blood pressure is of great importance in further developing our understanding of these critical pathways and in identifying novel drug targets.

Protein thioacylation is the post-translational attachment of a lipid, generally the saturated 16 carbon palmitate, to a cysteine sidechain via a labile thioester bond. Commonly referred to as palmitoylation, this modification typically affects the function of the substrate proteins by altering trafficking, localization, and/or stability[2]. Unlike other lipid modifications such as prenylation or myristolylation, palmitoylation is reversible and thus may be regulated. Novel proteomic studies performed by us and others have shown that hundreds of cellular proteins in diverse tissues and cell types are palmitoylated and that palmitoylation can regulate cellular functions[3–6].

The ZDHHC family includes more than 20 genes in mice and humans which encode enzymes that catalyze palmitoylation of substrate palmitoylproteins[7]. In yeast, knockdown of one or more ZDHHC isoforms attenuated palmitoylation of nearly all known palmitoylproteins, highlighting the importance of these enzymes in regulating cellular protein palmitoylation[4]. Historically, protein palmitoylation has been studied largely in biochemical or cellular assays. However, the recent identification of the ZDHHC family of proteins has allowed for *in vivo* studies on the role of protein palmitoylation via genetic approaches to alter enzyme expression and consequently modify cellular palmitoylprotein content. Several mouse models of ZDHHC deficiency have been generated to examine the function of this family of enzymes. For example, a mouse with a hypomorphic ZDHHC5 allele displayed evidence of defective hippocampal dependent learning[8] while knockout of ZDHHC17[9] and ZDHHC13[10] each led to a phenotype that mimics Huntington's disease, a progressive neurodegenerative disorder. In addition, ZDHHC enzymes have been associated with human diseases such as schizophrenia[11] and certain cancers[12, 13].

ZDHHC21 is a protein acyl transferase known to be expressed in endothelial cells and elsewhere[14, 15]. We have previously shown that in cultured endothelial cells ZDHHC21 supports the palmitoylation of important functional proteins including endothelial nitric oxide synthase (eNOS)[15], which produces the pleiotropic gaseous second messenger nitric oxide, and PECAM1, a cell adhesion molecule that may play a role in angiogenesis and transendothelial cell migration[3]. Others have identified additional substrates for ZDHHC21 including sex steroid receptors[16] and the nonreceptor tyrosine kinases Fyn[17] and Lck[18].

In the present work, we sought to characterize the role of ZDHHC21 in vascular function *in vivo*. We used the depilated mouse[19] (MGI: 94884) which harbors a spontaneous mutation

in the gene for ZDHHC21. The resulting mutant protein has a deletion of phenylalanine 233 ("F233Δ") and lacks acyl transferase activity toward established substrates[17]. For example, ZDHHC21 F233Δ cannot palmitoylate eNOS nor Fyn in *in vitro* assays. Unexpectedly, we find that the F233Δ homozygous mice as well as derived cells and tissues display reduced responsiveness to α1 adrenergic receptor (αAR) agonists. In vivo, tachycardia and hypotension were observed in the F233Δ mice, findings consistent with reduced peripheral vascular resistance as the result of impaired vascular action of α1 AR. Molecularly, ZDHHC21 is found to from a complex with the α1D AR, and increase its palmitoylation. These results suggest a molecular mechanism for the action of ZDHHC21, since palmitoylation has been shown to alter signaling and function of many related G protein coupled receptors. These data are to our knowledge the first demonstration of the *in vivo* role of a particular protein acyl transferase in vascular function and reveal a novel mode of regulation of vascular responsiveness to α1 AR agonists. These data may pave the way to novel therapeutics that modulate vascular tone by altering protein acyl transferase activity.

Material and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Vessels isolated from F233Δ mice display impaired responsiveness to phenylephrine, an α1 adrenergic receptor agonist

In order to evaluate vascular function in F233Δ mice, we studied aortic rings isolated from WT and mutant mice by wire myography (Fig. 1). When contraction was induced by phenylephrine, an α1 adrenergic receptor (AR) agonist, maximal increases in tension were reduced by ~30% in F233Δ rings relative to WT (Fig. 1A; WT, 0.92 ± 0.08 g; F233Δ, 0.64 ± 0.05 g; $p<0.001$). Log(EC_{50}) for phenylephrine were similar (−7.0 ± 0.3 vs −6.9 ± 0.3). Contraction induced by a distinct receptor agonist, serotonin, was not impaired (Fig. 1B), nor was contraction induced by high concentrations of potassium, a receptor-independent stimulus (Fig. 1C).

In addition, endothelial-dependent relaxations were studied in response to acetylcholine. Regardless of whether vessels were preconstricted with phenylephrine or serotonin, endothelial dependent relaxations were indistinguishable in WT and F233Δ mice (Supplemental Fig. 1).

Since the aorta is a conduit vessel and does not contribute significantly to peripheral vascular resistance *in vivo*, we sought to evaluate function of the mesenteric artery. Using pressure myography in cannulated vessels, we found that high concentrations of potassium in both WT and F233Δ third order mesenteric arteries induced similar contractions (not shown). However, as in the aortae, vessels derived from F233Δ mice displayed significantly reduced maximal contraction to phenylephrine (Fig. 1D; WT, 109 ± 7 %; F233Δ, 49 ± 5 %, $P<0.0001$). In addition, Log(EC_{50}) for phenylephrine in the mutant was similar to WT (WT, −5.9 ± 0.2; F233Δ, −5.4 ± 0.3).

The above data suggest defective functioning of the α1 AR in the vascular smooth muscle cells. We have previously demonstrated expression of ZDHHC21 using quantitative PCR (qPCR) approaches in cultured endothelial cells[3], but expression has not been documented in vascular smooth muscle. We thus performed qPCR experiments on a variety of mouse tissues and cultured mouse cells (Suppl. Fig. 2). ZDHHC21 transcripts were confirmed in aorta, heart, as well as cultured mouse aortic smooth muscle cells. Overall, expression was noted in all tissues tested at fairly similar levels: the highest expressing tissue was only threefold higher than the lowest. Similar widespread expression of ZDHHC21 has been reported elsewhere[14].

F233Δ mice display impaired response to infusion of phenylephrine

In order to determine whether the hyporesponsiveness to phenylephrine that was observed in isolated vessels was also present in intact mice, phenylephrine was infused into WT and F233Δ mice while blood pressure was monitored. Mice were maintained under inhaled isoflurane anesthesia while blood pressure was measured directly via a carotid artery catheter. Pre-infusion blood pressures were similar in WT and F233Δ mice under these conditions. Phenylephrine was infused at 30ug/kg/min for 15 min, and the steady state blood pressure was determined at the conclusion of the infusion period. As shown in Fig. 2, SBP increased substantially within 2–3 min following initiation of the phenylephrine infusion. At 15 min, the steady state SBP had increased by 15.6 +/− 1.8 mmHg in WT versus only 8.2 +/− 0.7 mmHg in F233Δ mice (p=0.003; n=6).

F233Δ mice display reduced blood pressure and tachycardia

The impaired response to α1 AR agonists observed in isolated vessels and in intact mice suggest that F233Δ mice might exhibit evidence of diminished peripheral vascular resistance *in vivo*. In order to explore this possibility in awake, unrestrained mice, we used a telemetry system to characterize the hemodynamics of the F233Δ and WT mice. The averaged results from data collected continuously over 72h showed that all the mice displayed characteristic diurnal variations in blood pressure (BP) and heart rate (HR) (Fig. 3). The F233Δ mice were hypotensive, particularly at peak nighttime hours (Fig. 3A, Supplemental Table 1), which in nocturnal rodents is a period of increased activity, HR, and BP. Mean arterial pressure (MAP) at night was reduced in F233Δ mice relative to WT by 5.1 +/− 2.1 mmHg (n=7, p=0.03), but was not statistically different during the day (Fig. 3A, 3B; Supplemental Table 1).

The F233Δ consistently displayed increased HR relative to WT (Fig. 3G, H), however the pattern was opposite that of BP in that differences were greater during the day (66 +/− 20 beats/min, p=0.007) and smaller at night (49+/−19 beats/min, p = 0.02). The tachycardia was not the result of increased physical activity on the part of the F233Δ mice as measurements of activity collected using the telemetry system revealed that the F233Δ mice were less active at night that the WT mice (WT, 13.9 ± 2.0 vs. F233Δ, 5.2 ± 0.4 arbitrary units, p =0.001).

Further analysis of relationships between HR and BP suggest that the tachycardia in the F233Δ mice served to attenuate differences in blood pressure between the strains. For

example, the differences in blood pressure were smallest during the day, when greatest relative increase in HR in the F233Δ mice was observed (Fig. 3; Supplemental Table 1). At night, the differences in blood pressure were largest, and differences in HR were smallest. A plot of the difference in mean HR between WT and F233Δ versus the difference in MAP (Suppl Fig. 3A) suggests that MAP would be ~13 mmHg less in F233Δ mice if HRs were equivalent. The plot also reveals that when blood pressure was similar between the strains, the HR was ~70 bpm greater in the F233Δ mice. A plot of the same data set as mean HR vs. mean MAP for each strains clearly indicate the rightward shift of the curve in the F233Δ mice, illustrating the increased HR required to attain the same BP in this strain (Suppl Fig. 3B).

Variability in heart rate was assessed by determining the standard deviation of inter-beat intervals (SDIBI) in WT and F233Δ mice. This variability may increase with increased cholinergic input[20]. Although the SDIBI was less in F233Δ mice, the difference was not statistically significant (WT, 6.5±1.5 msec; F233Δ 4.7±1.0 msec; p= 0.32; Supplemental Table II).

F233Δ mice have elevated catecholamine levels and expression of α1 AR

Since mutant mice and isolated arteries were found to be hyporesponsive to pharmacologic α1 adrenergic agonist, we hypothesized that levels of endogenous expression of α1 AR and its agonists might be altered in the mutant mice. In order to assess this possibility, we measured catecholamine levels in overnight urine collections (Fig. 4). Overnight collections reflect levels present when BP differences were greatest. Both adrenaline and noradrenaline levels were found to be significantly elevated in the F233Δ mice, suggesting increased average plasma levels during this time period. Further, qPCR analysis of aortae showed that mRNA levels for α 1A AR was significantly increased in mutant vessels, while subtypes 1B and 1D were not statistically different (Fig. 4C). In contrast, mRNA levels for contractile proteins, MYH11 and SMA2, were not significantly different in F233Δ as compared to WT.

F233Δ mice do not display evidence of cardiac abnormalities

The combination of hypotension and tachycardia seen in the F233Δ mice, while consistent with diminished vascular tone, might also be the result of cardiac dysfunction. In order to assess this possibility, we performed transthoracic echocardiography to examine cardiac structure and function. There were no significant differences in stroke volume, ejection fraction, or left ventricular volume at systole or diastole between WT and F233Δ mice (Table 1). Further, H+E stained sections of F233Δ myocardium did not reveal any obvious abnormalities (not shown). These data together suggest the hemodynamic phenotype does not relate to abnormal cardiac function.

F233Δ mice do not have evidence of abnormal fluid losses

The combination of tachycardia and hypotension might arise from a reduction in intravascular volume, which would contribute to reduced cardiac output by reducing preload. The normal stroke volume and left ventricular diastolic volume observed by echocardiography argues against this possibility (Table 1), but we nonetheless further evaluated fluid losses from skin and kidney. The F233Δ mice have abnormal skin

characterized by thickened epidermis and thinned coat raising the possibility of a defect in cutaneous barrier function, though none was detected in published investigations[17]. To confirm this, we directly measured trans-epidermal water losses in homozygous mutant and heterozygous control mice at age 4–6d, before the onset of hair growth which interferes with the measurements. The skin and fur of heterozygous mice are grossly indistinguishable from WT, thus water flux is the heterozygotes is expected to reflect that in the WT. In this assay, no significant difference between F233Δ and heterozygous mice was noted (Fig. 5).

In order to evaluate whether the F233Δ mice displayed abnormal handling of salt and water, serum analyses from mice on standard chow were performed (Table 2). They revealed no differences in blood urea nitrogen, sodium, nor potassium, suggesting no significant differences in renal function, renal handling of water and potassium, nor in the renin-angiotensin-aldosterone pathway. H+E stained sections of renal tissue did not reveal any obvious significant abnormalities in F233Δ mice (not shown). In order to further test for hemodynamically significant renal salt (and water) wasting, mice were placed on a very low salt diet (0.01%) while simultaneously monitoring hemodynamic status using a telemetry system. If the mutant mice were unable to appropriately retain sodium under conditions of dietary deprivation, they would be expected to demonstrate worsening tachycardia and hypotension associated with progressive fluid losses and intravascular depletion. However, both F233Δ and WT mice were able to tolerate the low sodium diet for two weeks with no discernible change in BP or HR (not shown). These data argue against significant salt wasting, but do not rule out a lowered “set point” for sodium retention under conditions of normal dietary intake.

Fibroblasts derived from F233Δ mice display reduced responses to phenylephrine

In order to further explore the mechanisms underlying the observed hyporesponsiveness to α1 AR agonism in F233Δ mice, embryonic fibroblast cell lines were derived from WT and F233Δ mice. Signaling induced by phenylephrine was assessed by activation of the ERK1/2 pathway, a common pathway activated downstream of a variety of GPCRs. Signaling by F233Δ MEFs in response to phenylephrine (10 μM) was markedly diminished (Fig. 6). At 5 min, the maximal increase in the ratio of phosphoERK to total ERK relative to time 0 was 2.5 +/− 0.3 for WT vs. 1.3 +/− 0.1 for F233Δ, $p<0.001$, n= 7) However, signaling by serotonin (10uM) was no different at 5 min (2.6 +/− 0.2 vs. 2.7 +/− 0.1, p= NS), mimicking the results observed with serotonin in isolated aortic rings. These results corroborate those obtained in intact aortic rings, and suggest that the molecular mechanism underlying this defect is preserved in cultured cells, and does not depend on the presence of differentiated smooth muscle cells nor of intact vascular tissue.

ZDHHC21 palmitoylates and interacts with α1D AR *in vitro*

In order to examine the molecular basis of diminished α1 AR function in F233Δ vessels, we hypothesized that ZDHHC21 may palmitoylate key proteins involved in the α1 AR signaling pathway, and that loss of palmitoylation of those proteins might lead to disruption of α1 AR signaling in the F233Δ mouse. Several proteins involved in canonical signaling by α1ARs are known to be palmitoylated. Most Class A G protein coupled receptors are palmitoylated at one or more cysteines in the C terminal tail distal to the seventh (i.e., last)

transmembrane helix, thus creating a “fourth” cytoplasmic loop [21]. Palmitoylation of the α1B AR has been reported at such a position [22]. Further, Gαq, the heterotrimeric G protein activated by α1 AR, is known to be palmitoylated, and to require palmitoylation for proper interaction with receptors[22, 23].

We tested for interactions between ZDHHC21 and α1D AR and Gαq using a standard co-expression strategy [24] and biosynthetic incorporation of a palmitate analogue, 17-ODYA, into the palmitoylproteins [6]. ODYA can be derivatized with a fluorophore using click chemistry to allow for visualization and quantitation of protein palmitoylation.[6] Since the α1D subtype mediates vascular contraction in response to phenylephrine and catecholamines[25, 26] we assessed whether α1D AR was a substrate for ZDHHC21. As seen in Fig. 7A and quantified in 7B, palmitoylation of α1D AR (myc tagged) was increased ~2.5 fold in the presence of WT ZDHHC21 (HA-tagged), but not when co-expressed with either ZDHHC21 F233Δ or a mutant in which the key catalytic cysteine is mutated to serine (C120S). Of note, α1D AR was detected as 3 different bands, consistent with the presence of monomers, dimers and higher order aggregates. This multimerization of heterologously expressed α1 AR has been previously reported [27]. Total expression of α1D AR was increased by expression of WT, but not mutant ZDHHC21 (Fig 7A, 7B).

In addition to the receptor, the ability of ZDHHC21 to palmitoylate Gαq was tested, but no interaction was detected, in agreement with previously published results [18]. In contrast, both ZDHHC3 and ZDHHC7 increased Gaq palmitoylation, as previously reported[18] (Fig. 7C and quantified in 7D).

As has been pointed out previously [28], a limitation of metabolic labeling studies in the assessment of palmitoylation is that it may reflect increased steady state palmitoylation, or merely increased turnover with either unchanged or even decreased steady state palmitoylation. In order to distinguish between these possibilities, we employed acyl-RAC methodology to purify palmitoylated receptors from cell lysates [29, 30]. This label-free method purifies palmitoylated proteins from a mixture in the presence (but not absence) of hydroxylamine, and thus reflects the total pool of palmitoylated protein present. Using this analysis, we found that palmitoylation of α1D AR was increased in the presence of ZDHHC21, albeit to a smaller degree than was observed using ODYA technique. These data suggest that ZDHHC21 does in fact increase steady state palmitoylation of α1D AR, as well as total expression of α1D AR. The smaller magnitude of the effect in this assay as compared with the ODYA technique is not unexpected, as it only distinguishes between protein that is not palmitoylated at all from that with at least one palmitoyl group. The addition of more palmitoyl groups beyond the first will not increase signal in the RAC assay, as it could in the ODYA/click experiment (Fig. 7A).

We additionally undertook analysis of α1A and α1B AR palmitoylation using ODYA labeling. However, extensive aggregation observed with these receptors precluded quantitative interpretation of the experiments (not shown). Aggregations seemed to be worse after immunoprecipitation, and exacerbated further by the click chemistry labeling reaction, perhaps due to the use of organic solvents in the reaction We tried various detergent, buffer, and reducing conditions without satisfactory results. Experiments using the RAC technique

(which is less sensitive overall but less susceptible to aggregation due to use of SDS denaturation) failed to demonstrate increased palmitoylation of either α1A or α1B (Supplemental Fig. 4). These data do not rule out an interaction, but suggest that the effects on α1D are the most robust.

As further evidence for interaction between α1D AR and ZDHHC21 co-immunoprecipitation studies were performed. In several cases, ZDHHC enzymes have been found to co-immunoprecipitate with substrates [15, 31]. Indeed, ZDHHC21 could be shown to co-immunoprecipitate with α1D AR when co-expressed in HEK cells (Fig. 7G). Unfortunately, such experiments could not be performed on native tissues owning to lack of specific antibodies for α1 AR [32]. Together, these data suggest the ZDHHC21 could affect the function of α1 AR in VSMC by directly palmitoylating the α1D receptor.

Discussion

This report documents the first demonstration that a particular ZDHHC palmitoyltransferase can regulate vascular function *in vivo*. Collectively, the data support a model (Fig. 8) in which inactivation of ZDHHC21 causes reduced responses to α1 AR agonists by diminishing palmitoylation of the α1D AR, which manifests in vivo as diminished vascular tone (i.e, hypotension and tachycardia). Reduced responsiveness to phenylephrine was observed in intact mice (Fig. 2), isolated vessels (Fig. 1), as well as embryonic fibroblasts (Fig. 6). In contrast, responses to other stimuli for vascular contraction remained intact, such as serotonin and high [K+] in both isolated aortic rings (Fig. 1) and in MEFs (Fig 6), which suggests that the F233Δ vessels are not globally disabled. In further support of this model, in which disruption of vascular responsiveness to α1 AR plays a central role, we found increased levels of endogenous catecholamines and vascular α1 AR expression in F233Δ mice (Fig. 4).

The F233Δ mice were found to be hypotensive and tachycardic, which is consistent with diminished vascular tone. Diminished vascular tone is the expected phenotype in the setting of reduced α1 AR activity. Other explanations for the tachycardia and hypotension, such as cardiac abnormalities or loss of fluids via skin or kidney, were sought but not observed (Fig. 5, Table 1). Abnormalities of α1 AR function can clearly give rise to phenotypes similar to the one identified here for the ZDHHC21 mutant mouse. The most convincing data come from studies of α1D KO mice, which display impaired phenylephrine induced contraction in aortic rings [25, 33], dimished pressor reponse to infused phenylephrine in intact mice[25], and hypotension[25, 33, 34]. A separate study also found hypotension in α1D KO mice relative to WT, but the difference (~3mm Hg) did not reach statistical significance[35]. Further, two papers have shown that α1D KO mice are resistant to the development of hypertension in a model employing salt loading and subtotal nephrectomy[33, 35].

The reduction in blood pressure in the F233Δ mice was relatively modest, in part due to masking by relative tachycardia (Suppl. Fig. 3). The tachycardia may be a “compensatory” response to the diminished vascular tone, but it could also result from other molecular defects not identified here, such as hyperactivity of the β1 AR. The differences in blood pressure in F233Δ mice were largest, and statistically significant, only at night. This result

makes sense in light of our model which posits a role for diminished α1 AR pressor function, in that night is the time at which catecholamine levels are highest in rodents, and thus the time at which deficiency in α1 AR signaling would be expected to be most pronounced. A similar diurnal variation in blood pressure abnormalities was reported in the mouse knockout of TRIC-A, a monovalent cation channel. These mice displayed hypertension, but only during the daytime, as the high sympathetic output of nighttime hours masked the effect [36].

The Guytonian model of long term BP regulation would predict that in response to hypotension, the kidney would minimize sodium excretion so as to expand intravascular volume and normalize blood pressure[37]. However, such renal compensation likely does not occur in the setting of vascular α1 AR dysfunction due to the involvement of the intrarenal vasculature in addition to other peripheral beds. Thus, renal blood flow is predicted to be preserved even at lower arterial blood pressures so that sodium retention is not triggered. Further, since α1 AR expressed in renal tubular epithelial cells may be involved in promoting renal tubular sodium reabsorption[38], its dysfunction may thwart the normal increase in tubular sodium reabsorption anticipated in the setting of hypotension. Regardless, the similarity of the phenotype between the F233Δ mice and several of the α1 AR KO mice suggests that defects in α1 AR are indeed sufficient to cause hypotension and tachycardia.

Regarding the molecular mechanisms underlying the defect in α1 AR signaling in the F233Δ mice, our results suggest that ZDHHC21 can directly interact with and palmitoylate the α1D AR (Fig. 7), a subtype important for vasoconstriction in various vascular beds [26, 39]. The palmitoylation of α1D was specific, as ZDHHC21 could not palmitoylate Gaq (Fig 7C). Palmitoylation of the α1A and the α1B receptors could not be demonstrated, though the data do not rule out an interaction (Suppl Fig 4). Unfortunately, direct demonstration of altered palmitoylation levels of α1D receptors *in vivo* is technically quite difficult, owing to lack of availability of a suitable antibody [32], and low receptor expression levels.

Numerous G protein coupled receptors have been found to be regulated in different ways by palmitoylation, which affects nearly all Family A receptors. Palmitoylation has been found in different studies to affect ligand binding, G protein coupling, and desensitization, though the details have varied depending on the particular receptor studied (reviewed in [21, 40]). In addition, complexity related to GPCR palmitoylation and its functional role may arise from the fact that many GPCRs are palmitoylated at more than one site—and different sites may have different kinetics and play different functional roles, as was recently reported it the β1 AR [41]. There are no previous reports regarding palmitoylation of α1D AR, however, the α1B AR was found to be palmitoylated at two cysteines in the C terminal tail in a ligand dependent fashion[22]. The significance of this palmitoylation was not determined and the catalytic enzymes(s) were not identified [22, 23].

Here, we find evidence that stability of α1D AR are affected by palmitoylation, since co-expression of WT ZDHHC21 (but not the inactive mutants F233Δ or C120S) increased the expression levels (Fig 7B, 7F). An interrelationship between palmitoylation and protein

abundance has been observed in multiple proteins, and more recently was found to be a nearly universal phenomenon in proteomic analyses of hypomorph of ZDHHC5 [42] and hypomorph of ZDHHC 17 [43]. In both cases, nearly all proteins found to be in less abundance after purification of palmitoylproteins were also found to be in less abundance in total pools. These results, using two unrelated systems, suggests that palmitoylation may commonly affect protein abundance and/or stability.

Investigations in the area of GPCR palmitoylation have nearly all been done in vitro, since creation of in vivo models of GPCRs that lack palmitoylation with traditional methods is difficult. Further, manipulation of GPCR palmitoylation via genetic alteration of ZDHHC enzyme expression has not been possible owing to lack of information on ZDHHC-substrate interactions, though recent data on GPCR palmitoylation has addressed this [44, 45]. In the one in vivo study on GPCR palmitoylation published, on the photoreceptor rhodopsin, a novel phenotype was discovered involving lack of stability of the unliganded opsin that is formed following bleaching of the receptor. Functionally, this instability caused retinal degeneration following exposure to bright light. This phenotype was not previously appreciated, despite multiple studies of rhodopsin palmitoylation that had been done in vitro. The F233Δ mouse thus represents an opportunity to explore the consequences of GPCR palmitoylation in vivo using genetic approaches.

Identifying substrates of ZDHHC enzymes that mediate in vivo phenotypes has been a difficult question [42, 43]. Although several mouse models of different ZDHHC isoform mutants, hypomorphs and knockouts have been reported, each with striking phenotypes ranging from neurodegeneration [10, 46] to osteoporosis[47], the identification of the exact molecular mechanism (i.e,, the specific protein substrate in which lack of palmitoylation results in the observed phenotype) has been generally elusive. Broadly, investigators have tried unbiased proteomic approaches [42, 43] as well as more focused candidate gene approaches. The former has involved purification of palmitoyl proteins from tissues or cells of WT and mutant animals coupled with quantitative proteomic techniques. So far, relatively modest changes in the palmitoylome have been detected using these approaches, though a number of interesting candidates have been identified whose relevance to observed phenotypes awaits confirmation[42, 43]. Here, we used a candidate gene approach, which has the advantage of focusing on proteins with a known role in the observed phenotype. Further, this approach allows for direct investigation of proteins that may be too low in abundance to be readily detected with proteomic methods, such as GPCRs. A similar approach led to the identification of MT1-MMP as a possible substrate of ZDHHC13 that causes the osteoporosis that occurs in mice with a nonsense mutation in *Zdhhc13* [48].

Of course the candidate gene approach will not identify every substrate of a given ZDHHC enzyme, and available data suggest that most enzymes have multiple substrates [4]. For example, several additional substrates of ZDHHC21 have been reported, including eNOS, PECAM1, fyn [49], and sex steroid receptors [16]. It is likely that multiple proteins are differently palmitoylated in F233Δ mice besides α1D AR. Thus the hemodynamic phenotypes described herein may not be (solely) due to nor fully explained by the identified interaction between ZDHHC21 and α1D AR.

Of note, although previously published *in vitro* experiments had shown the knockdown of ZDHHC21 led to diminished palmitoylation of eNOS and reduced NO production[50], experiments with the F233Δ mice did not show any evidence of diminished eNOS function *in vivo*. For example, the mice were hypotensive (Fig. 3), not hypertensive as would be expected if eNOS function were diminished[51]. Further, endothelial function, as judged by acetylcholine-induced relaxations in isolated vessels (which depends on stimulated NO production), was normal (Supplemental Fig. 1). Thus, either the eNOS effects are overwhelmed by defective palmitoylation of other proteins, or eNOS is palmitoylated normally in the F233Δ mice, perhaps due to upregulation of other ZDHHC isoforms with reported activity toward eNOS[50].

In sum, this report shows for the first time that a ZDHHC protein acyl transferase enzyme, ZDHHC21, can affect vascular function in vivo, possibly by disrupting α1 AR mediated vascular contraction. Several chemical inhibitors of both protein acyl transfersases[52, 53] and acyl protein thioesterases[54, 55], enyzmes which catalyze palmitate removal, have been described. Thus, pharmacological manipulation of palmitoylation may soon be possible. ZDHHC21 and related family members may present novel targets for drug therapy to modulate vascular function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and acronyms

AR	adrenergic receptor
F233Δ	mutant of ZDHHC21 protein in which phenylalanine 233 is deleted
ODYA	17-octadecynoic acid
5-HT	serotonin
SDIBI	standard deviation of inter-beat intervals

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Significance

This study identifies the protein acyl transferase enzyme ZDHHC21 as a novel regulator of alpha1 adrenergic receptor (AR) signaling. Mice with nonfunctional mutants of ZDHHC21 display defective reponses to infusions of α1 AR agonist, as do blood vessels and cells derived from these mice. The mice also display tachycardia and hypotension, despite elevated catecholamine levels. These findings are consistent with defective α1AR signaling. Further, ZDHHC21 is found to complex with and to increase the attachment of the saturated lipid palmitate to α1 AR. Since palmitoylation commonly affects the function of G protein coupled receptors like the α1 AR, these findings suggest a novel molecular mechanism by which α1 adrenergic signaling, vascular tone and blood pressure may be regulated.

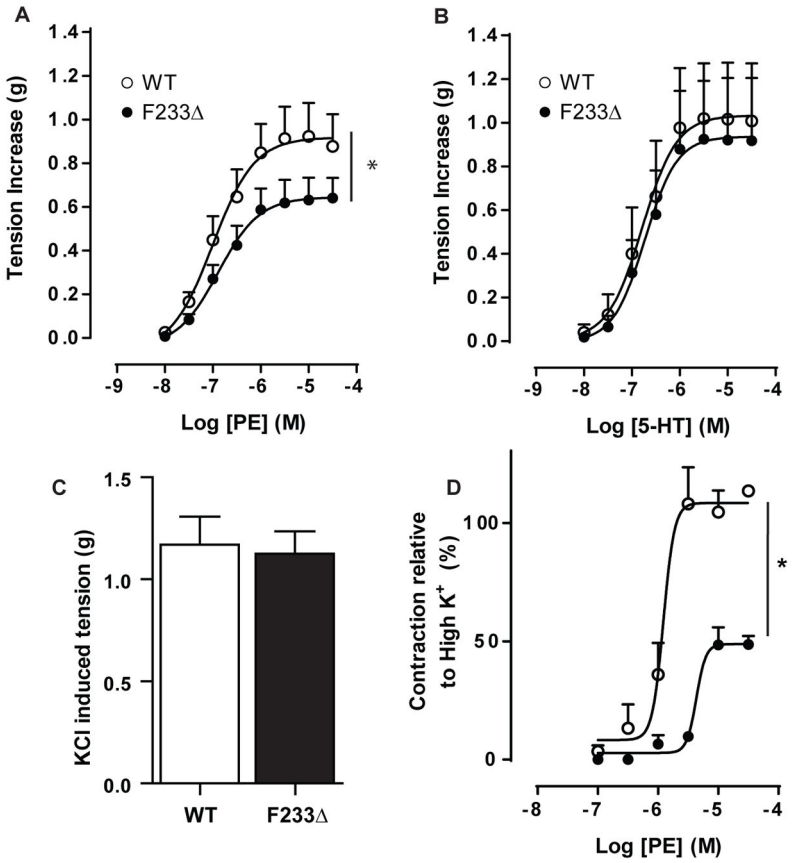

Fig. 1. Myographic measurements on isolated vessels show selective defect in response of F233Δ vessels to phenylephrine, an α1 adrenergic receptor agonist

A–C, Wire myography of aortic rings. **A, B**, Tension induced in F233Δ vessels by phenylephrine (PE) is reduced, whereas serotonin (5-HT) induced tension is normal. **C**, Contraction of aortic rings induced by high potassium (60mM), which is receptor independent, is normal. **D**, Pressure myography studies of isolated 3rd order mesenteric arteries shows reduced responsiveness of F233Δ vessels to phenylephrine. (*, $p<0.05$ for difference in maximal response as judged by extra sum-of-squares F test. n=3 mice (with 2–3 rings per mouse averaged) for A–C; n=3 mice (with 1–2 vessels per mouse averaged) in D).

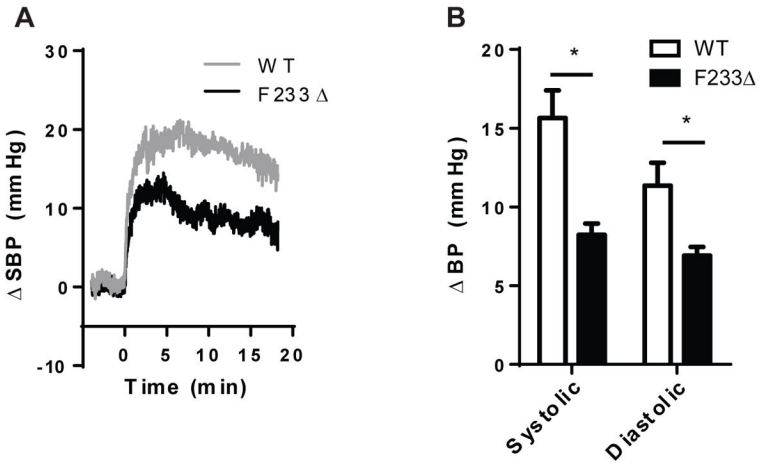

Fig. 2. F233Δ mice show impaired responses to phenylephrine infusions

A, Phenylephrine was infused intravenously at 30 μg/kg/min starting at time zero while hemodynamics were monitored via an intraarterial catheter in anesthetized mice. Changes in SBP over time are shown for WT and F233Δ mice from a representative experiment. **B**, Mean plateau changes in SBP and DBP after 15 min of phenylephrine infusion are plotted; n=6. *, $p < 0.05$ by two-tailed t test.

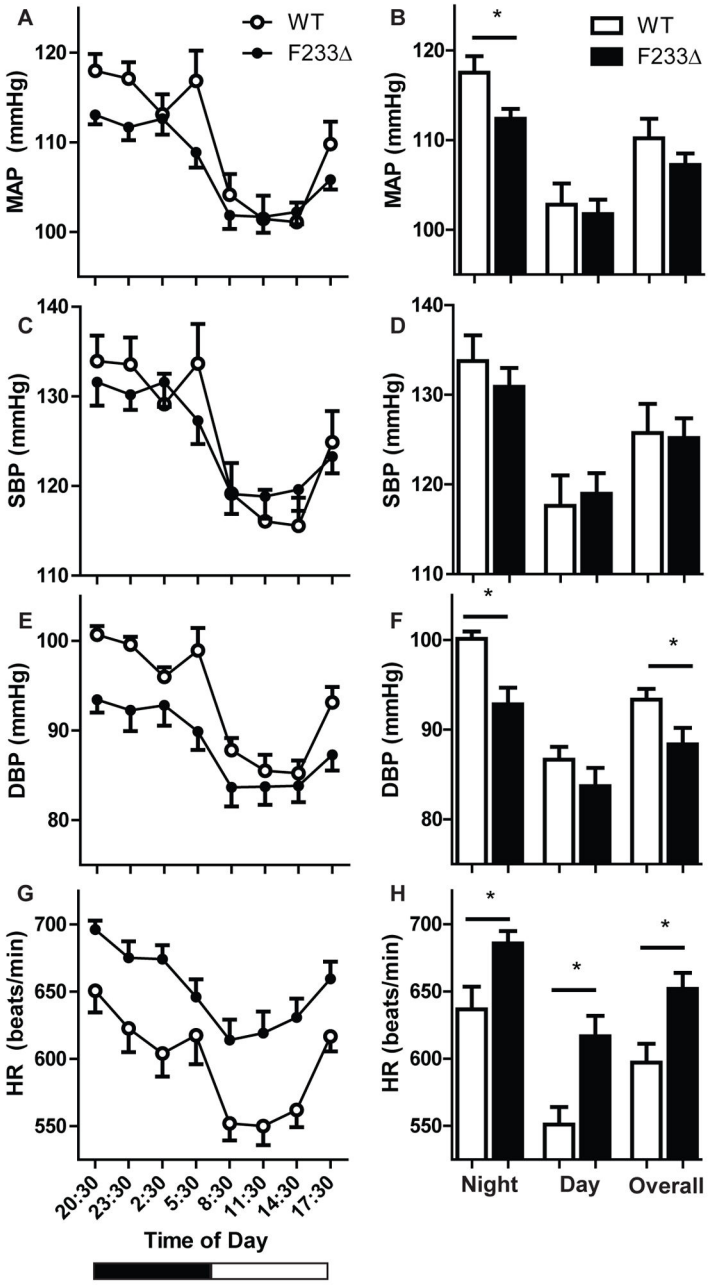

Fig. 3. F233Δ mice are tachycardic and hypotensive

Blood pressure (BP) and heart rates (HR) were determined in unrestrained, unanesthetized mice using an implantable telemetry system. **A, C, E, G**, 24 hours of data are shown at times indicated on a 24 hour scale; night is indicated by a black bar and day by white. Data shown at each point represent 3 hour rolling averages of data sampled once each minute. Further, data at each time point are averaged from 3 consecutive days of monitoring for each mouse. MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure. The data show decreased MAP and DBP during the night in F233Δ mice. In addition, the F233Δ mice are tachycardic at all time points relative to WT. **B, D, F, H**, Mean values for

MAP and HR were calculated at peak night (19:00–1:00) and day (7:00–13:00) times and displayed as bar graphs. *, $p<0.05$ by two tailed t test, n=7 mice per group.

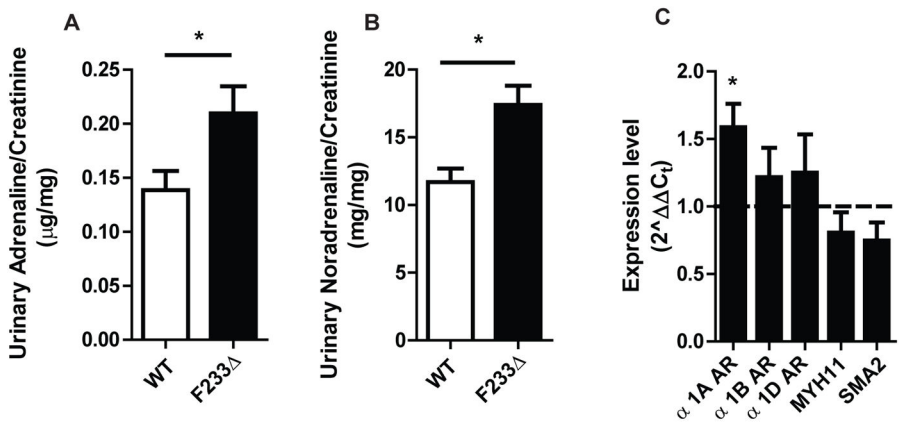

Fig. 4. Altered levels of catecholamines and α1 adrenergic receptor transcripts in F233Δ mice

A, B. Ratios of urinary adrenaline and noradrenaline to creatinine were elevated in F233Δ mice. n=9–14 mice per group. *, $p<0.05$ by two tailed unpaired t test. **C**, Aortae from F233Δ mice displayed increased expression of α adrenergic receptor 1A gene but not genes encoding the structural proteins MYH11 or SMA2 as judged by qPCR. *, $p<0.05$ for F233Δ compared to WT.

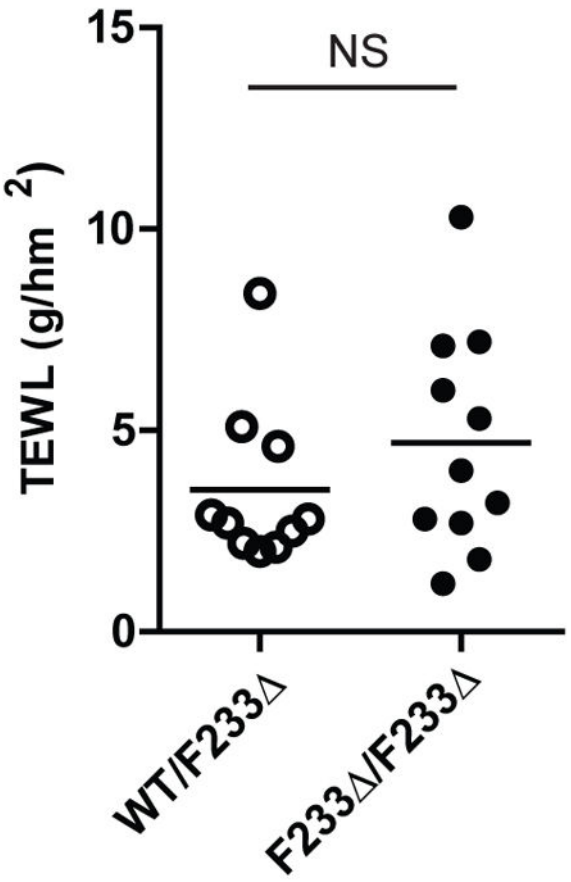

Fig. 5. F233Δ mice do not lose fluid via skin

Transepidermal water losses (TEWL) were measured to determine whether fluid losses might occur via the skin, given the abnormal fur and skin in F233Δ mice[17]. TEWL in the F233Δ mice was not significantly different from that in the WT mice ($p = 0.29$ by two tailed t test).

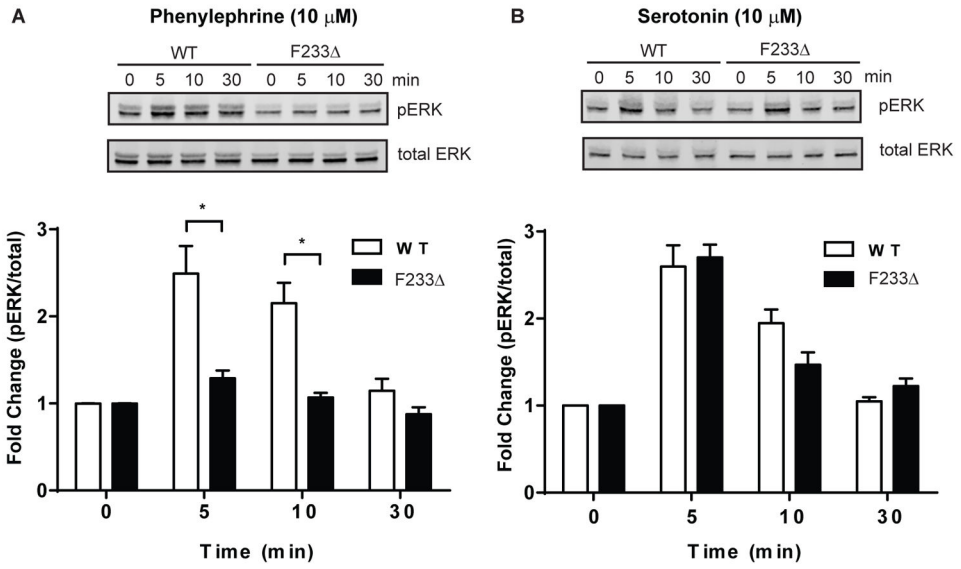

Fig. 6. Phenylephrine signaling is impaired in embryonic fibroblasts from F233Δ mice

The phosphorylation of ERK was measured by Western blot at indicated time points after exposure to 10 μM phenylephrine (**A**) or 10 μM serotonin (**B**). Phospho-ERK signal was normalized by total ERK, and adjusted to 1 at time zero. Phenylephrine signaling was impaired but serotonin signaling was normal. *, $p<0.05$ by 2-way ANOVA with Bonferroni post-test; n=7.

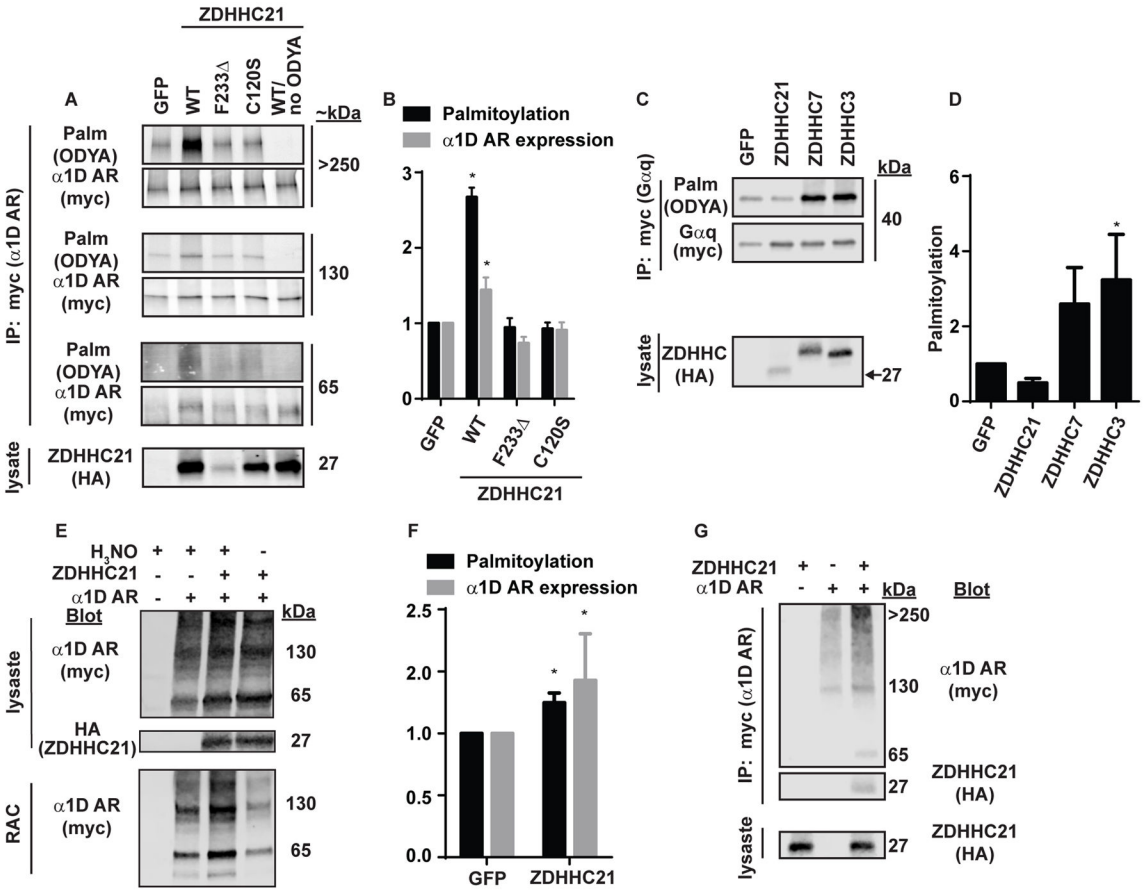

Fig. 7. ZDHHC21 palmitoylates α1D AR but not Gαq

A. α1D AR (myc-tagged) was expressed in HEK293 cells along with GFP (control), WT ZDHHC21, or nonfunctional ZDHHC21 mutants (HA-tagged). The degree of palmitoylation was determined by metabolic labeling with the palmitate analog ODYA, followed by immunoprecipiation of α1D AR, and click chemistry to attach a fluorophore to incorporated ODYA. The intensity of the ODYA signal was normalized to the total amount of α1D AR as determined by Western blot. The α1D AR tends to aggregate when expressed in HEK293 cells, and can be detected as monomers, dimers, and higher order aggregates at mol wt of ~65, ~130, and >250kDa. **B**, Quantitation of palmitoylation (i.e., normalized ODYA incorporation) and total α1D AR expression, n=3, *, $p<0.05$ compared to GFP, by one-way ANOVA with Dunnett's post test. **C**. ZDHHC21 does not palmitoylate Gαq, whereas ZDHHC3 does. **D**. Quantitation of Gαq palmitoylation. n=3, *, $p<0.05$ compared to GFP, by one-way ANOVA with Dunnett's post test. **E**. ZDHHC21 increases α1D AR palmitoylation as judge by acyl-RAC methodology. Palmitoylated alpha1D AR is isolated in presence, but not absence of hydroxylamine (H_3NO). **F**. Quantitation of palmitoylation (normalized to total expression) and total expression of α1D AR; both are increased by ZDHHC21. n=5 *, $p<0.05$ compared to GFP by two tailed t test. **G**, ZDHHC21 can be coimmunoprecipitated by α1D AR, suggesting the two proteins interact in a molecular complex. The data are representative of three experiments.

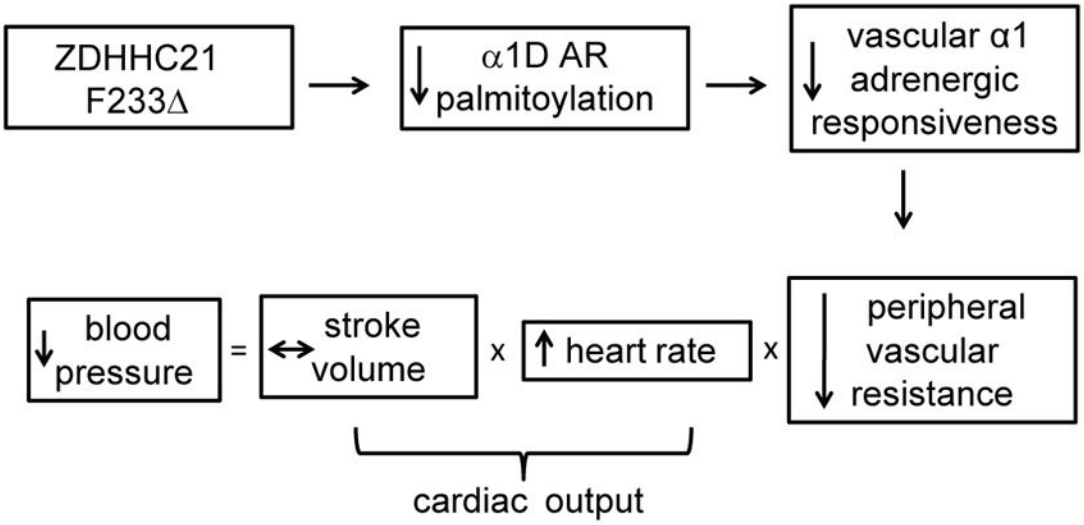

Fig. 8. Proposed model of disturbed physiology in F233Δ mice

Mutation of ZDHHC21 causes decreased α1D AR function (possibly due to reduced vascular α1D AR palmitoylation) which results in diminished peripheral vascular resistance (PVR). The reduced PVR manifests as reduced BP as observed in the telemetry studies, although the reduction is mitigated by increased HR (Fig. 3).

Table 1

Echocardiographic parameters

Anesthetized mice were analyzed with a Vevo 2100 ultrasound system. Parameters shown were derived by tracing the images of the left ventricle at systole and diastole. F233Δ mice showed no significant difference from WT in any of the parameters shown; n= 9, p>0.05 for all comparisons by unpaired two-tailed t test.

	WT	F233Δ
Stroke Vol, μL	41.7 ± 1.2	40.8 ± 2.1
Ejection Fraction, %	53 ± 3	55 ± 2
Left Ventricular (LV) Mass, mg	114.0 ± 4.7	111.5 ± 8.2
LV volume, μL (diastole)	79.4 ± 2.8	76.3 ± 5.5
LV volume, μL (systole)	38.1 ± 3.2	35.6 ± 3.9

Table 2

Blood analyses

Blood was collected via retro-orbital bleeds from anesthetized mice and analyzed using an iStat instrument and EC8+ cartridges. Data are the mean +/− SEM; n=11 for each group. P values are calculated by two tailed unpaired Student's t test.

	WT	F233Δ	p
Na, mEq/L	145.0 3.0	145.5 ± 1.3	0.695
K, mEq/L	4.3 ± 0.8	4.5 ± 0.5	0.309
Cl, mEq/L	113.9 ± 1.9	110.5 ± 1.5	0.003
HCO3, mEq/L	22.8 ± 1.9	26.5 ± 1.9	0.001
BUN, mg/dL	23.1 ± 3.9	19.0 ± 1.3	0.116
Glu, mg/dL	206.5 ± 24.8	204.9 ± 36.5	0.911
Hct, %	42.5 ± 2.3	43.3 ± 1.7	0.496
pH	7.2 ± 0.1	7.2 ± 0.1	0.426
pCO2, mmHg	59.4 ± 9.6	65.1 ± 6.0	0.096
AG	12.5 ± 2.5	13.0 ± 2.2	0.630
Hgb, g/dL	14.5 ± 0.8	14.7 ± 0.6	0.534