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CRISPR-Mediated SNP Modeling in Rats Reveals Insight into Reduced Cardiovascular Risk Associated with Mediterranean *G6PD* Variant

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Abstract

Epidemiological studies suggest that individuals in the Mediterranean region with a loss-of-function, non-synonymous single nucleotide polymorphism (SNP; S188F), in glucose-6-phosphate dehydrogenase (*G6pd*) are less susceptible to vascular diseases. However, this association has not yet been experimentally proven. Here, we set out to determine whether the Mediterranean mutation confers protection from vascular diseases and to discover the underlying protective mechanism. We generated a rat model with the Mediterranean SNP ($G6PD^{S188F}$) using CRISPR-Cas9 genome editing. In rats homozygous for the mutation, G6PD activity, but not expression, was reduced to 20% of wild-type (WT) littermates. Additionally, unbiased metabolomics analysis revealed that the pentose phosphate pathway (PPP) and other ancillary metabolic pathways connected to the PPP were reduced ($P<0.05$) in the arteries of $G6PD^{S188F}$ versus WT rats. Intriguingly, $G6PD^{S188F}$ mutants, as compared to WT rats, developed less large arterial stiffness and hypertension evoked by high fat diet and nitric oxide synthase inhibition with L-N^G-nitroarginine methyl ester (L-NAME). Intravenous injection of a voltage-gated L-type Ca^{2+} channel agonist (methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropyridine-3-carboxylate; Bay K8644) acutely increased blood pressure in WT but not in $G6PD^{S188F}$ rats. Finally, our results suggested that: 1) lower resting membrane potential of smooth muscle caused by increased expression of K^+ channel proteins and 2) decreased voltage-gated Ca^{2+} channel activity in smooth muscle contributed to reduced hypertension and arterial stiffness evoked by L-NAME and high fat diet to $G6PD^{S188F}$ mutants as compared to WT rats. In summary,

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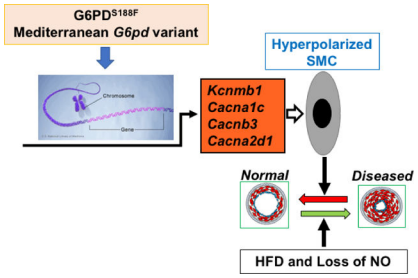
a mutation resulting in the replacement of a single amino acid (S188F) in G6PD, the rate-limiting enzyme in the PPP, ascribed properties to the vascular smooth muscle that shields the organism from risk factors associated with vascular diseases.

Graphical Abstract



Keywords

NADPH; Pentose Phosphate Pathway; Glycolysis; Vascular; Hypertension; Stiffness

Introduction

G6PD is the rate-limiting enzyme in the pentose phosphate pathway (PPP) – a fundamental component of cellular metabolism of glucose.[1] The biochemical reactions that constitute the PPP are found in most life forms highlighting their essential role in metabolism. It is well established that G6PD and the PPP are a significant source of ribose sugar and NADPH, the reducing potential, in almost all cell types. Additionally, G6PD and the PPP are connected to ancillary metabolic pathways, mostly through the regulation of the levels of the reducing equivalent NADPH.[2] Deficiency of G6PD is the most common enzymopathy in humans with over 400 million people worldwide suffering from different degrees of enzymopathy.[3, 4] G6PD deficiency increases the susceptibility of red blood cells (RBCs) to oxidative hemolysis – a factor complicating anti-malaria treatment with pro-oxidant quinone drugs.[5] However, although the PPP has been implicated in neurodegenerative disease,[1, 6] cardiovascular disease,[1, 7, 8] cancer,[1, 9, 10] and aging,[11] very little is known regarding the impact of G6PD deficiency on cardiovascular physiology and pathophysiology.

Epidemiological studies suggest that individuals in the Mediterranean region with a loss-of-function, non-synonymous single nucleotide polymorphism (SNP; S188F), in *G6PD* are less susceptible to vascular diseases.[12–14] If this is true, then understanding the fundamental molecular basis for the cardiovascular protection afforded by the Mediterranean G6PD mutant will be of paramount importance in developing novel strategies that reduce the risk of coronary artery disease, peripheral vascular disease, and stroke – the leading causes of morbidity and mortality in the USA and worldwide.[15]

G6PD-derived NADPH is an essential cofactor for the generation of nitric oxide, the most potent vasodilator, by endothelial nitric oxide synthase.[16] How individuals in the Mediterranean region with a G6PD deficiency are protected from vascular diseases is unknown, and there is no experimental proof of the association between the loss-of-function

SNP (S188F) in G6PD and protection from vascular diseases. Therefore, to directly address whether the Mediterranean SNP is or is not protective in the cardiovascular system, we generated a rat carrying the Mediterranean SNP ($G6PD^{S188F}$) using CRISPR-Cas9 gene editing and determined the effects of high fat diet and loss of nitric oxide on vascular function in $G6PD^{S188F}$ rats. We found that replacement of a single amino acid (S188F) in G6PD ascribed properties to the vascular smooth muscle that shielded the rats from some vascular diseases.

Methods

Detail methods, experimental protocols, and additional results are available in online Supplement.

Experimental protocols using animals had approval (A3362–01) from the New York Medical College and Medical College of Wisconsin Institutional Animal Care and Use Committees and experiments were performed in blinded fashion. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

CRISPR-Cas9 genome editing:

A rat model harboring a non-synonymous (S188F) substitution in the coding region of the rat *G6pd* gene, close to the G6PD catalytic domain, was developed using a 3-component CRISPR editing approach[17] in fertilized Sprague Dawley (Crl:SD, Charles River Laboratories) rat embryos.

CRISPOR[18] was used to design a CRISPR RNA (crRNA) located in close proximity to the SNP variant. The sequence of this crRNA (CUGGUCCUCACGAAACAGAG) was submitted for synthesis (Synthego, Redwood City, CA) and used with a transactivating crRNA (tracrRNA) and a single-strand oligonucleotide (Ultramer, IDT: 5'-GAACCGCATCATAGTGGAGAAGCCCTTCGGGAGAGACCTGCAGAGCTCCAATCAACTGTCGAACCACATCT**TT**TCTCTGTTTCGTGAGGACCAGATCTACCGCATTGACCACTACCTGGGCAAAGAGATGGTCCAGAACCTCATGG −3') serving as a homology-directed repair template carrying the SNP variant (bold underlined sequence, includes the SNP and a second mutation to disrupt the protospacer adjacent motif). Ribonucleoprotein complex (crRNA/tracrRNA/Cas9; 50 ng/ul) and ssODN (50 ng/ul) were co-injected by pronuclear injection into Crl:SD embryos. Genotyping rat pups was performed by PCR and Sanger sequencing. Founders were bred for germline transmission of the SNP allele. Predicted off-targeting events were determined with the CRISPOR tool;[18] two predicted noncoding off-target sites were found to be > 4 Mb away from the SNP suggesting probable segregation upon breeding. These $G6PD^{S188F}$ mutants and their wild type (WT) age-matched littermates were used in this study.

Echocardiography, hemodynamics measurements, force measurements, membrane potential and intracellular Ca^{2+} levels, metabolomics, G6PD activity, nitrite measurements, histology, qPCR, and Western blotting, were performed as described previously[19–27] and detail protocols are given in the online supplement.

Statistical Analysis.

Graphs and statistical analyses were prepared with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA) and Metaboanalyst 3.0. Values are presented as the mean ± SEM of the number of samples (n) from different animals. Two-way ANOVA with *post-hoc* Bonferroni correction was used to compare multiple groups, and unpaired Student's t-test was used to compare two groups. Values of $p < 0.05$ were considered significant.

Results

Activity of PPP and other ancillary pathways was reduced in CRISPR-Cas9-edited G6PD^{S188F} rats:

Sanger sequencing data demonstrate two nucleotide substitutions in *G6pd* (Fig. 1A). We substituted two nucleotides, preserving the S188F nonsynonymous substitution to simplify genotyping and destroy the PAM site (CCT). Although we did not perform whole genome sequencing for off-targets, the Cutting Frequency Determination specificity score of the crRNA was high (84) with only two predicted off-target sites on the X-chromosome and both noncoding sites are more than 4 Mb away from the on-target site based on the CRISPOR tool.[18] Thus, we deem relevant off-targeting to be very low, if at all. There were no detectable differences in the baseline morphological (structure) and functional (physiological) phenotype between G6PD^{S188F} and wild-type (WT) rats. Moreover, there was no difference in body weight and hematocrit between six-month-old G6PD^{S188F} and WT rats. However, while the expression of G6PD was not different, activity was reduced by 80% in liver of G6PD^{S188F} as compared to WT rats (Fig. 1B). G6PD is the rate limiting enzyme in the PPP that reduces six carbon glucose to five carbon ribose sugars and produces NADPH, a major reducing potential that maintains a ratio of reduced glutathione (GSH)-to-oxidized glutathione (GSSG), in the cell (Fig. 1C). The PPP integrates with other pathways in the cell (Supplement figure S1) Metabolomics analyses revealed that ribose phosphate (and isobaric pentose phosphate metabolites of the PPP) are decreased, and G6P and GSSG-to-GSH ratio are increased, in arteries of G6PD^{S188F} as compared to WT rats (Fig. 1D, E). In addition, metabolites of the TCA cycle and ancillary metabolic pathways (polyamine pathway and one-carbon metabolism) that are involved in NADPH homeostasis were significantly lower in arteries of G6PD^{S188F} rat (Fig. 2).

High fat diet elicited less severe hypertension and arterial stiffness in G6PD^{S188F} mutants compared to WT rats:

Metabolic syndrome is on the rise worldwide.[28] It is caused by high fat/calorie diet and is a major risk factor for life-threating coronary artery disease and stroke.[15, 28] High fat diet (HFD) and hormonal changes increase expression and activity of G6PD.[29] Therefore, our goal was to determine whether the HFD-mediated increase in risk factors for vascular diseases, large artery stiffness and hypertension, are amplified or reduced in G6PD^{S188F} as compared to WT rats. We fed WT and G6PD^{S188F} rats with HFD (containing 20% protein, 60% fat, and 20% carbohydrate; 60% fat of total calories; D12492, Research Diets Inc., USA) for four months. Both WT and G6PD^{S188F} rats gained identical weight on HFD (Supplement figure S2). We performed echocardiography and catherization on these rats and found that carotid peak velocity and pulse wave velocity were elevated more (21.1% versus

14.3%) in WT than G6PD^{S188F} rats (Fig. 3A, B) as determined from the blood flow in carotid and transit time between Doppler flow signals in the carotid and iliac arteries of WT and G6PD^{S188F} rats on normal chow or HFD (Fig. 3C). Further, systolic and diastolic blood pressure (Fig. 3D–E) and arterial elastance (Fig. 3F) were increased in HFD fed WT but not in HFD fed G6PD^{S188F} rats. There was no major remodeling (medial hypertrophy or neo-intimal formation) of carotid artery and aorta isolated from WT and G6PD ^{S188F} rats fed with normal chow or HFD (Supplement figure S3).

Inhibition of nitric oxide synthase elicited less increase in hypertension and arterial elastance in G6PD^{S188F} than WT rats:

Nitric oxide (NO) is a potent vasodilator, and loss of endothelial-derived NO has been implicated in the pathogenesis of metabolic syndrome-associated vascular diseases. NO is synthesized by NO synthase, which requires NADPH co-factor as an electron donor. Therefore, since G6PD activity is reduced by G6PD^{S188F} mutation, we measured NO levels and determined whether inhibition of NO synthase with L-NAME exacerbates hypertension and arterial stiffness in G6PD^{S188F} versus WT rats. Nitrite levels were showed slight reducing trend (P=0.312) in aorta of G6PD^{S188F} (934±135 μM/mg protein) as compared to WT (1049±179 μM/mg protein) rats. L-NAME (1 mg/ml in drinking water) increased systolic and diastolic blood pressure (Fig. 4A–B) and elastance (Fig. 4C) more in WT than G6PD^{S188F} rats. Furthermore, isometric tension studies in carotid arterial rings revealed that KCl (Fig. 4D–F)- and phenylephrine (Supplement figure S4A–F)-induced contraction curves shifted to the left and maximum contraction was increased (Supplement figure S4B) in WT but not in G6PD^{S188F} carotids. Acetylcholine-induced relaxation was reduced in WT (Supplement figure S4E) but not in G6PD^{S188F} (Supplement figure S4F) rats. There was no major remodeling (medial hypertrophy or neo-intimal formation) of carotid artery and aorta from WT and G6PD ^{S188F} rats treated with L-NAME (Supplement figure S5).

Blood pressure elicited by L-type Ca^{2+} channel agonist but not by α_1-adrenergic agonist was decreased in G6PD^{S188F} as compared to WT rats:

Since KCl (Fig. 4)- and phenylephrine (Supplement figure S4)-induced contraction of carotid artery significantly increased in L-NAME-treated WT but not G6PD^{S188F} rats, we sought to determine whether L-type Ca^{2+} channel- and α_1-adrenergic receptor-mediated pathways of vasoconstriction were suppressed in G6PD^{S188F} rats. To do so, we intravenously injected methyl 2.6-dimethyl-5-nitro-4-[2-(trfluoromethyl)phenyl]-1,4-dihydropyridine-3-carboxylate (Bay K8644; 1 μM), a selective L-type Ca^{2+} channel agonist, [30] and phenylephrine (0.01, 0.100 and 1.0 μM), a specific α_1-agonist that constricts smooth muscle in L-type Ca^{2+} channel-independent but predominantly by Rho kinase-dependent manner,[31] in WT and G6PD^{S188F} rats and determined blood pressure over a period of 30 minutes. Interestingly, systolic and diastolic blood pressure elicited by Bay K8644 (Fig. 5A, B) but not by phenylephrine (1 μM; Supplement figure S6) was significantly reduced in G6PD^{S188F} as compared to WT rats. A lower concentration of phenylephrine did not increase blood pressure.

Ca^{2+} and K^+ channel genes and proteins are increased in arteries of $G6PD^{S188F}$ rats:

Bay K8644 binds to the α-subunit of L-type Ca^{2+} channel[32] and increases the open channel probability,[33] which augments Ca^{2+} influx into SMCs to evoke contraction.[34] This way Bay K8644 constricts arteries and raises blood pressure. Therefore, since Bay K8644 elicited less increase of blood pressure in $G6PD^{S188F}$ than WT rats , we considered whether L-type Ca^{2+} channel proteins are reduced in vascular smooth muscle of $G6PD^{S188F}$ rats. On the contrary, we found the expression of genes that encode *Cacna1c*, α-subunit of L-type Ca^{2+} channel, *Cacnb3*, β-subunit of L-type Ca^{2+} channel, *Cacna2d1*, δ-subunit of L-type Ca^{2+} channel, and *Kcnmb1*, β-subunit of big conductance K^+ channel, were significantly increased in aorta of $G6PD^{S188F}$ as compared to WT rats (Fig. 5C). Furthermore, we found that CACNA2D1 and KCNMB1 protein expression were higher in aorta of $G6PD^{S188F}$ as compared to WT rats (Fig. 5D). Additionally, we determined the expression of these genes in aorta of HFD-fed WT and $G6PD^{S188F}$ rats (Supplement table S1). Intriguingly, HFD feeding, as compared to normal chow feeding, increased *Cacna1c* by 20-fold, *Cacnb1* by 10-fold, *Cacnb3* by 27-fold, and *Cacna2d1* by 2.3-fold in WT rats, but not in $G6PD^{S188F}$ rats. Instead, HFD feeding significantly increased (3.1-fold) *Kcnma1*, α-subunit of big conductance K^+ channel, in $G6PD^{S188F}$ but not in WT rats.

Membrane potential is less and Ca^{2+}-influx-induced contraction is attenuated in arteries of $G6PD^{S188F}$ than in WT rats:

Since we found that *Cacna1c* was paradoxically increased in arteries of $G6PD^{S188F}$ rats, in which Bay K8644-elicited less increase of blood pressure than WT rats, we postulated that Bay K8644 binding to the open channels, which ensues with the depolarization of the membrane potential, would be reduced, perhaps due to less depolarized membrane potential in the smooth muscle cells of $G6PD^{S188F}$ versus WT rats. Therefore, we determined membrane potential by a slow-response membrane potential indicator, $DiBAC_4(3)$, in aorta of $G6PD^{S188F}$ and WT rats at resting condition and in response to KCl. Fluorescence of $DiBAC_4(3)$ increases with depolarization of the membrane potential.[20] Our results showed that fluorescence of $DiBAC_4(3)$ was higher in the resting and KCl (20 and 40 mM)-treated arteries of WT versus $G6PD^{S188F}$ rats (Fig. 6A, B). Additionally, since nitrendipine, a structural analog of Bay K8644, decreases Ca^{2+} channel activity in SMCs with more depolarized membrane potentials,[35] we determined whether nitrendipine elicited less relaxation of arteries isolated from $G6PD^{S188F}$ and WT rats pre-contracted with KCl (30 mM). As expected, nitrendipine-elicited less relaxation of aorta from $G6PD^{S188F}$ than WT rats (Fig. 6C). Consequences of lower depolarized membrane potential are: 1) reduced opening of the L-type Ca^{2+} channels; 2) less Ca^{2+} influx into the SMCs; and 3) attenuated contraction of arteries. Therefore, we measured intracellular Ca^{2+} ($[Ca^{2+}]i$) levels in resting and KCl (30 mM)-treated SMCs by fluorescence imaging. These results showed that $[Ca^{2+}]i$ is higher in resting and KCl-treated SMCs from WT as compared to $G6PD^{S188F}$ rats (Fig. 6D). Next, we determined the Ca^{2+} influx-elicited isometric force generation (contraction) of aorta and carotid artery isolated from $G6PD^{S188F}$ and WT rats pre-treated with KCl (30 M) or Bay K8644 (1 μM). We found that KCl (Fig. 6E, F)- and Bay K8644 (Supplement figure S7)-elicited less contraction of aorta and carotid artery from $G6PD^{S188F}$ rats as compared to WT rats. Taken together, these findings suggest that $G6PD^{S188F}$-induced

hyperpolarization of membrane potential reduced Ca^{2+} influx and Ca^{2+}-mediated SMC contraction.

Discussion

The results presented in this study provide the first experimental evidence that a mutation resulting in the replacement of a single amino acid (S188F) in G6PD, the rate-limiting enzyme in the PPP, protected the rat from developing large artery stiffness (compromised Windkessel function) and hypertension (HTN) that is associated with the metabolic syndrome and loss of nitric oxide. Furthermore, our results have illuminated new insight into the mechanisms through which the loss-of-function mutation in G6PD protects the rats from HTN – a major risk factor for coronary artery disease and stroke – and stiffness of large arteries – a critical risk for systolic HTN and heart failure in hypertensive patients.[36, 37]

$G6PD^{S188F}$ rat is an authentic experimental model of *G6PD* Mediterranean variant with genotype-phenotype similar to human, such as; X-linked c.563C>T (S188F) switch, severe loss of activity, increased GSSG-to-GSH ratio, decreased nucleotide levels, and little change in protein expression.[4, 38] In contrast, although different inbred mouse strains exhibit variability in G6PD activity in RBCs, there are no differences in GSH levels reported in them.[38, 39] Moreover, X-linked G6PD deficient mice, which was recovered from the offspring of male mice treated with 1-ethyl-1-nitrosourea, have normal protein synthesized in decreased quantity due to a mutation in the 5' untranslated region.[40] Thus, the latter mouse model is not a model of the human *G6PD* Mediterranean polymorphism. Because *G6PD* is coded by a gene on the X chromosome, X-linked G6PD deficiency is more common in males than in females. Generally, hemizygous males and females carrying two deficient variants (homozygous females) with defective *G6PD* alleles tend to have functionally faulty G6PD. Therefore, in this study hemizygous male and homozygous female rats were used as they are translationally relevant to humans.

HFD feeding, which produces metabolic syndrome-like symptoms in rats,[41] increased pulse wave velocity and arterial elastance (markers of large artery stiffness), common carotid peak velocity (a marker of carotid artery stenosis), and systolic and diastolic BP, more in WT rats than $G6PD^{S188F}$ rats. High fat and HFD-induced hormonal changes, viz-a-viz increased insulin, augment the expression and activity of G6PD.[29, 42, 43] Furthermore, G6PD is increased in various tissues of obese-diabetic individuals[44–47] and the elevated G6PD has been associated with increased inflammation – a potential cause of HTN and large artery stiffness – in obese-diabetic mice.[48] Our results support a mechanism(s) of preserved vasodilatory function and reduced arterial stiffness conferred by the $G6PD^{S188F}$ variant in rats fed a HFD.

Metabolic syndrome, a modifiable risk factor of cardiovascular diseases, is pandemic and a health concern in the US and worldwide.[28] In metabolic syndrome patients, neurohumoral imbalance, endothelial dysfunction, loss of nitric oxide, and inflammation of blood vessels elicit constriction and contribute to the remodeling of small resistance arteries that increases BP.[49] In rats, HFD feeding impairs endothelial function and contributes to increased vasoconstriction,[50] thus raising BP. Decreased endothelial function and endothelial-derived

nitric oxide bioavailability elicit vasoconstriction and remodeling of the arteries,[51] and contributes to the pathogenesis of HTN.[52, 53] Also, loss of nitric oxide causes and enhances arterial stiffness.[54, 55] Consistently, L-NAME treatment reduced acetylcholine-elicited relaxation – an indicator of endothelial dysfunction – of carotid artery isolated from WT, but not G6PD^{S188F}, rats. Furthermore, L-NAME increased BP as well as arterial elastance more in WT than G6PD^{S188F} rats. These results suggest that arteries of WT rats are stiffer than G6PD^{S188F} rats. This finding is indeed surprising and unexpected. Because, on the contrary, we expected that reduced levels of G6PD-derived NADPH, a critical co-factor for endothelial nitric oxide synthase, in arteries of G6PD^{S188F} rats would augment, not reduce, L-NAME-induced vasoconstriction and BP. This paradox could have occurred if there was unmasking of a dominant compensatory hyperpolarization of the membrane potential, subsequent to inhibition of nitric oxide synthase, in arteries of G6PD^{S188F} but not WT rats. Alternatively, since G6PD-derived NADPH fuels generation of superoxide anion instead of nitric oxide from inactivated nitric oxide synthase, which increases the Ca^{2+} sensitivity to the myofilament, we suggest that a loss-of-function mutation in G6PD might have decreased the Ca^{2+} sensitivity to the contractile apparatus and relaxed the vascular smooth muscle.

Phenylephrine, a selective α_1-agonist that increases vasoconstriction through augmenting Rho kinase-mediated Ca^{2+} sensitivity to the myofilament,[31] and KCl, which elicits vasoconstriction through depolarizing the membrane potential of SMCs, increased contraction of carotid arteries isolated from WT, but not G6PD^{S188F}, rats treated with L-NAME. We interpret these results to signify reductions inCa^{2+} influx through the voltage-gated Ca^{2+} channels and Ca^{2+} sensitivity to the myofilament in arteries of G6PD^{S188F} rats. However, BayK8644, but not phenylephrine, elicited less increase of BP in G6PD^{S188F} than WT rats. These results suggest that Ca^{2+} channel activity and not the Ca^{2+} sensitivity to the contractile apparatus was reduced in arteries of G6PD^{S188F} rats. Potentially, Bay K8644-induced increase of Ca^{2+} channel activity could be decreased if; 1) the α-subunit of L-type Ca^{2+} channel expression is decreased and 2) the membrane potential is more hyperpolarized. Because in both scenarios BayK8644, which binds to channels and increases the open channel probability in a membrane potential-dependent manner,[32] cannot activate L-type Ca^{2+} channel. However, we rule out the first scenario because expression of *Cacna1c*, *Cacnb3*, and *Cacna2d1* was higher (>5-fold) in arteries of G6PD^{S188F} than WT rats. Therefore, the second possibility of more hyperpolarization of the membrane potential likely explains why actions of Bay K8644 were blunted in G6PD^{S188F} rats. We suggest that: 1) augmented expression of *Kcnmb1*, which increases voltage- and Ca^{2+}-sensitivity of BK_{Ca} channel, 2) decreased ancillary polyamine pathway (spermine and spermidine; Fig. 2B), which inhibits the activity of inward rectifier K^+ channels,[56, 57] and 3) elevated polyunsaturated fatty acids (EPA and DHA; Fig. 2C), which increases K^+ channel activity;[58] altogether significantly contributed to a more hyperpolarized membrane potential in arteries isolated from G6PD^{S188F} versus WT rats. This notion is supported by our results indicating that the differences in the membrane potential observed between the arteries of WT and G6PD^{S188F} rats was abolished by high extracellular KCl (80 mM), which blocks all outward K^+ currents. Lower membrane potential decreases Ca^{2+} influx and contraction of the SMC. Consistently, in arteries of G6PD^{S188F} rats as compared to WT rats, we found decreased: 1) Ca^{2+} influx-induced contraction of KCl- and Bay K8644-treated arteries; 2) intracellular

Ca^{2+} in resting and KCl-treated SMCs; and 3) relaxation of pre-contracted arteries by nitrendipine, a Ca^{2+} channel blocker that inhibits channel activity by binding to a more depolarized SMC. These results suggest that lower resting membrane potential of SMCs moderated over excitement of the cell that elicits influx and overload of Ca^{2+}, a second messenger that not only elicits contraction of SMCs but also mediates gene expression and stiffening of blood vessels.[59, 60] This in turn would guard the $G6PD^{S188F}$ rats from abnormal vascular function and baneful vascular diseases often caused by HFD and loss of nitric oxide. Additionally, we propose that the lack of HFD-induced increase of *Cacna1c*, *Cacnb1*, and *Cacnb3* expression in arteries of $G6PD^{S188F}$ mutants fortified these rats from developing large artery stiffness and HTN .

The incidence of vascular diseases is less in the Mediterranean region as compared to Western countries. Some epidemiological studies suggest that individuals with the *G6PD* Mediterranean variant modeled here are even less susceptible to cardiovascular diseases.[12–14] Others suggest that the same loss-of-function SNP in *G6PD* is detrimental and contribute to the worsening of cardiovascular pathology/disease.[61, 62] Our findings support the epidemiological studies that suggest *G6PD* Mediterranean variant protects individuals from cardiovascular diseases,[12–14] and provides insight into the molecular mechanisms that shield the organism from vascular diseases. Many polymorphic variants of *G6PD* exist worldwide. The polymorphic *G6PD* African variant has less severe G6PD deficiency as compared to *G6PD* Mediterranean variant.[4] Therefore, it will be interesting to develop an experimental rat model of *G6PD* African variant and determine whether they are or are not protected from cardiovascular diseases. Nonetheless, based on our current findings, we propose that a modest inhibition of G6PD expression and/or activity may be beneficial, and not harmful, in reducing severity of vascular diseases associated with metabolic syndrome as compared to persons without the *G6PD* Mediterranean mutation.

Perspectives:

G6PD, an enzyme in a major metabolic pathway, unexpectedly contribute to the expression of genes, which encode ion channels that are critically involved in the regulation of membrane potential and Ca^{2+} homeostasis in SMCs, and to vascular function. A loss-of-function Mediterranean mutation in G6PD protected from arterial diseases like, large artery stiffness and HTN, elicited by HFD and loss of nitric oxide. Therefore, these findings give us a new opportunity to exploit this enzyme as a novel pharmacotherapeutic target, in the future, to reduce the load of all forms of vascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance:

What is new:

Epidemiological studies suggest that individuals in the Mediterranean region with a loss-of-function, non-synonymous single nucleotide polymorphism (SNP; S188F), in glucose-6-phosphate dehydrogenase (*G6pd*) are less susceptible to vascular diseases. Therefore, we developed the first rat model with the Mediterranean SNP in G6PD using CRISPR-Cas9 genome editing.

What is relevant:

In this rat model, we determined whether Mediterranean G6PD variant is or is not protective to cardiovascular system.

Summary:

The results presented in this study provide the intriguing experimental evidence that a mutation resulting in the replacement of a single amino acid (S188F) in G6PD, the rate-limiting enzyme in the PPP, ascribed properties to the vascular smooth muscle that shields the organism from risk factors associated with vascular diseases.

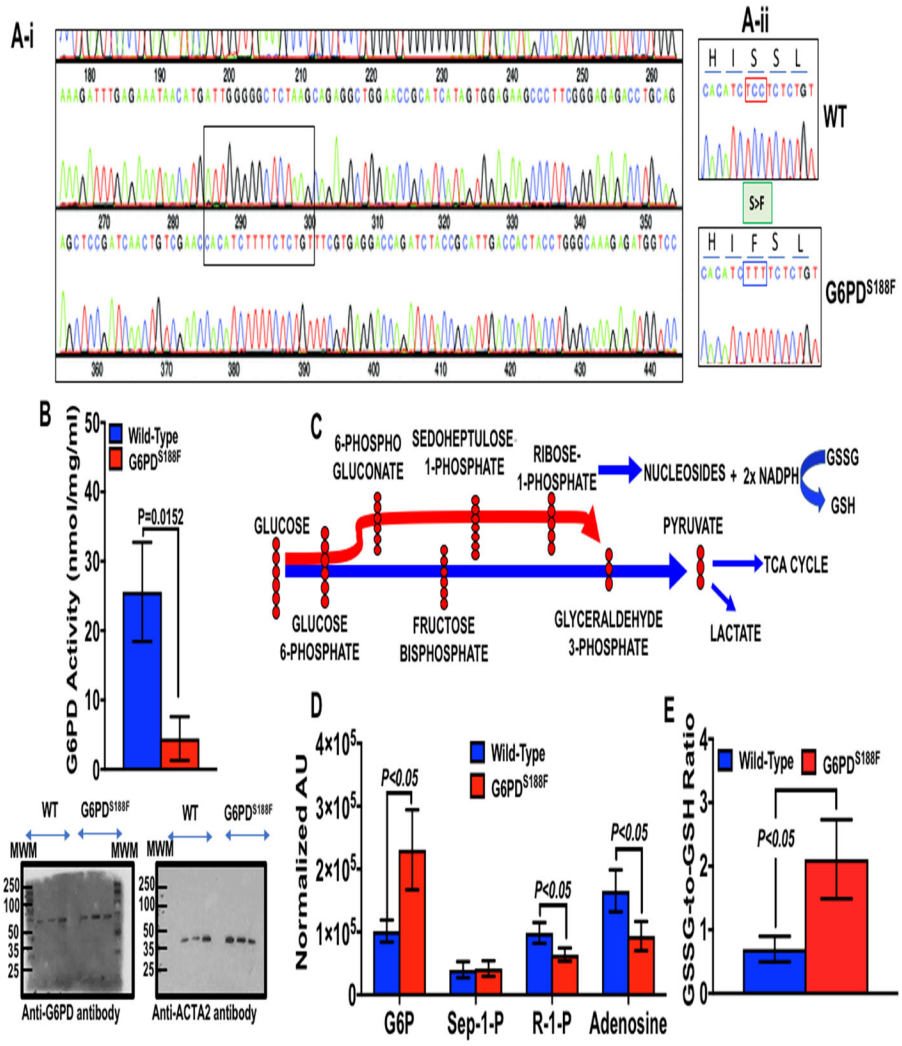

Figure 1:

Genotyping, G6PD activity and expression, and glucose metabolism through the pentose phosphate pathway. In (A-i) Sanger sequence rats was performed to determine mutation in G6PD between 285 and 300 bp (TCT; shown in square box) by CRISPR-cas9 editing. (A-ii) Sanger sequence demonstrating C>T in *G6pd* resulting S188F switch in G6PD of the mutant (G6PD^{S188F}) compared to wild-type (WT) rats. Red box is PAM and green box is S>F substitution. (B) G6PD activity (Top) and expression (Bottom) in liver of WT and G6PD^{S188F} rats. (C) A schematic illustrating metabolism six-carbon (glucose) to five-carbon (ribose sugar), nucleoside, and synthesis of NADPH in the PPP. (D) metabolomic analysis showing increased glucose 6-phosphate and reduction in the PPP metabolite ribose 1-phosphate (R-1-P) and adenosine in arteries of G6PD^{S188F} as compared WT rats. (E) The PPP-derived NADPH redox regulates oxidized (GSSG)-to-reduced (GSH) glutathione ratio in most cells and GSSG-to-GSH ratio is increased in arteries G6PD^{S188F} than WT rats. N=8 to 9 rats in WT and G6PD^{S188F} group.

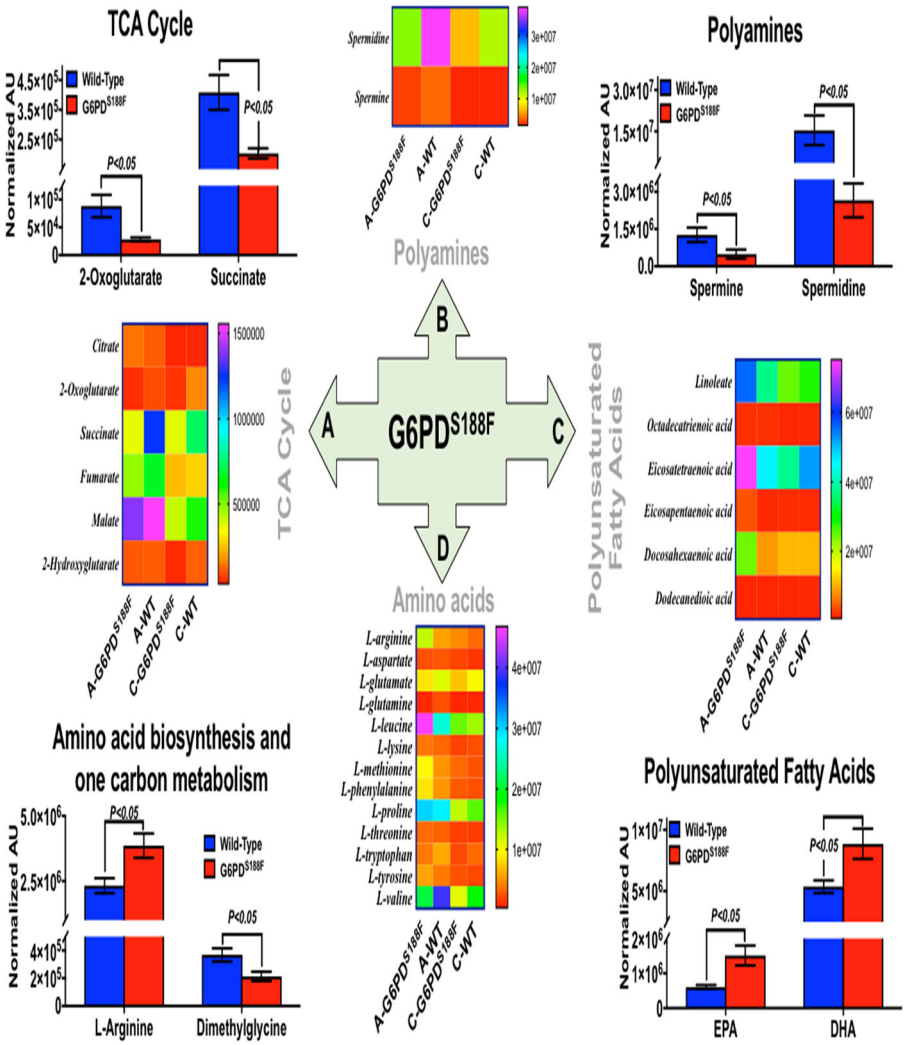

Figure 2:

Metabolism in arteries of WT and G6PD^{S188F} rats. Metabolic variations in arteries of WT and G6PD^{S188F} rats were quantitatively determined by metabolomic analysis. Selected metabolites of: Tricarboxylic acid (TCA) cycle; Polyamines; Polyunsaturated Fatty Acids; and Amino acids and one carbon metabolism, in aorta and carotid artery combined, are significantly altered in G6PD^{S188F} versus WT rats. Heat map illustrating levels of individual metabolites of four metabolic pathways quantitated in aorta (A-G6PD^{S188F} and A-WT) and in carotid artery (C-G6PD^{S188F} and C-WT). N=8 to 9 rats in WT and G6PD^{S188F} group.

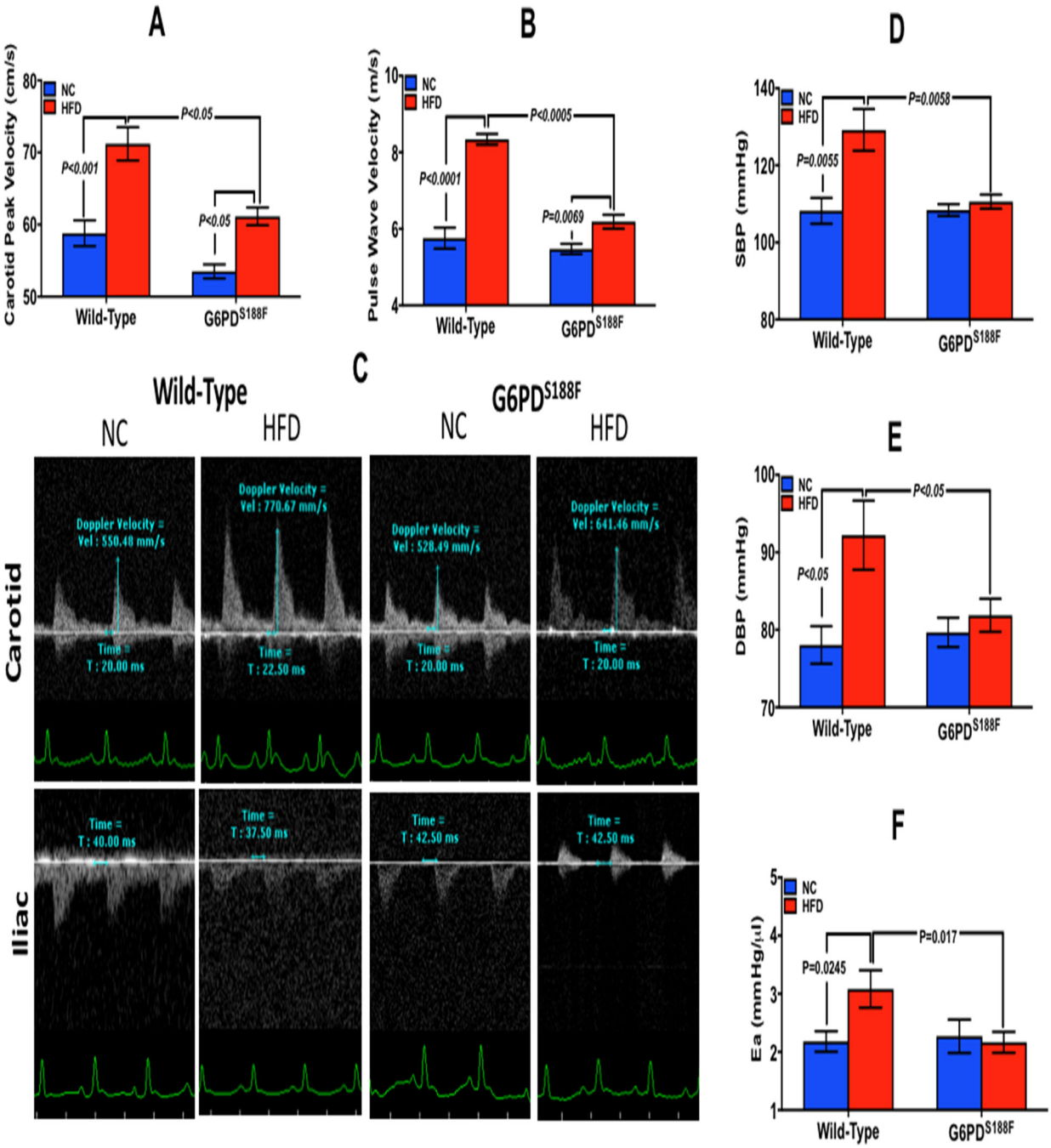

Figure 3:

Effect of HFD on blood pressure, large artery stiffness, and common carotid artery stenosis in WT and G6PD^{S188F} rats. (A) Common carotid artery peak velocity in G6PD^{S188F} and WT rats on normal chow (NC) and high fat diet (HFD). (B) Pulse wave velocity (PWV), determined by the distance between carotid and iliac artery divided by time, increased less in G6PD^{S188F} than in WT rats. (C) A representative echocardiography experiment showing the blood flow in carotid and iliac artery in WT and G6PD^{S188F} rats on NC and HFD. (D-F)

HFD-elicited systolic and diastolic blood pressure and arterial elastance (Ea) increased more in WT than G6PD^{S188F} rats. N=5 rats in WT and G6PD^{S188F} group.

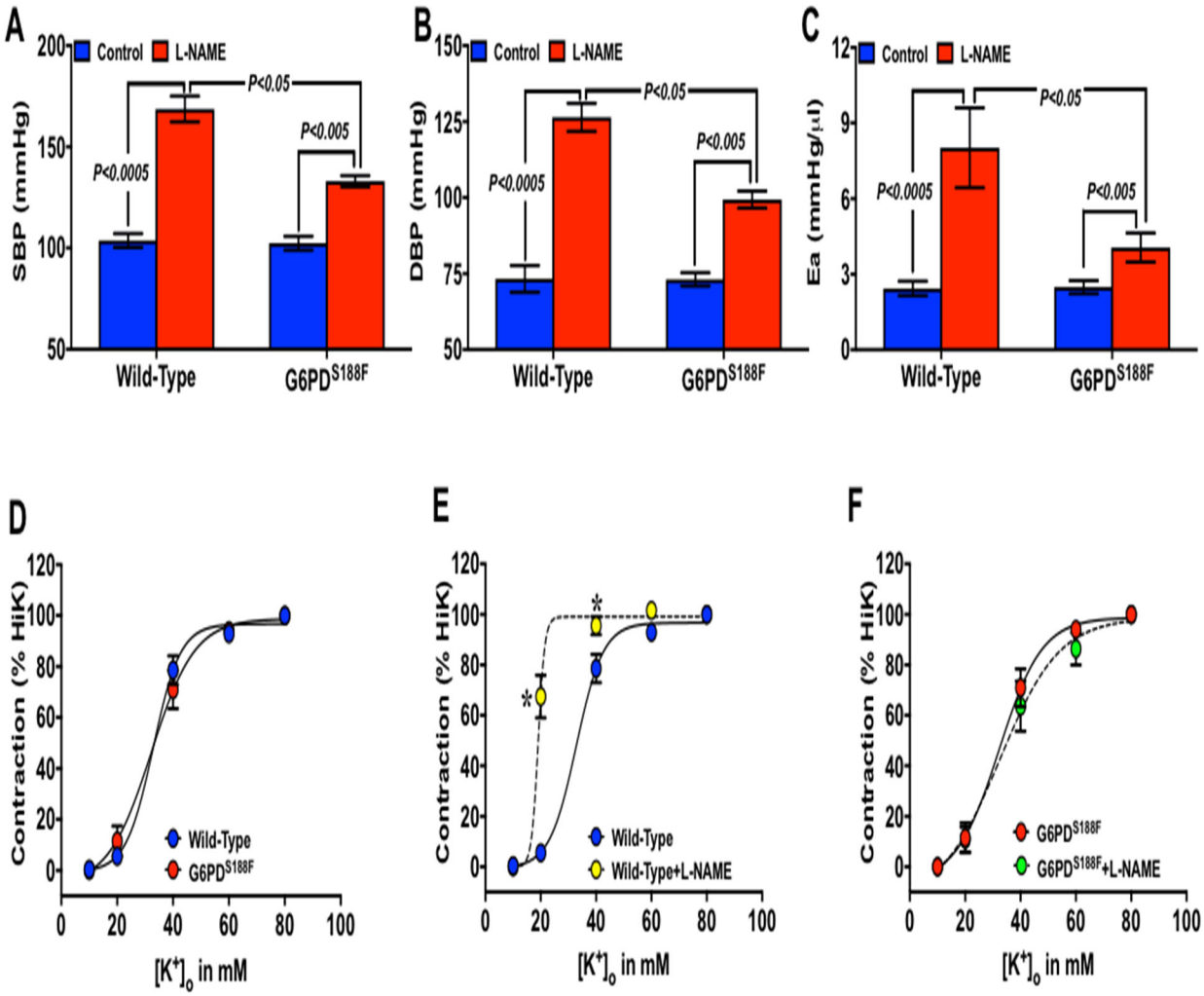

Figure 4:

Effect of L-NAME on blood pressure, arterial elastance, and carotid artery function in WT and G6PD^{S188F} rats. (A) Systolic and (B) diastolic pressure, and (C) arterial elastance in WT and G6PD^{S188F} rats treated with vehicle control and L-NAME (1 mg/ml in drinking water) for 5 days is shown. (D-F) KCl-induced contraction of common carotid arteries isolated from WT and G6PD^{S188F} rats treated with vehicle control and L-NAME. N=8 to 11 rats in WT and G6PD^{S188F} group.

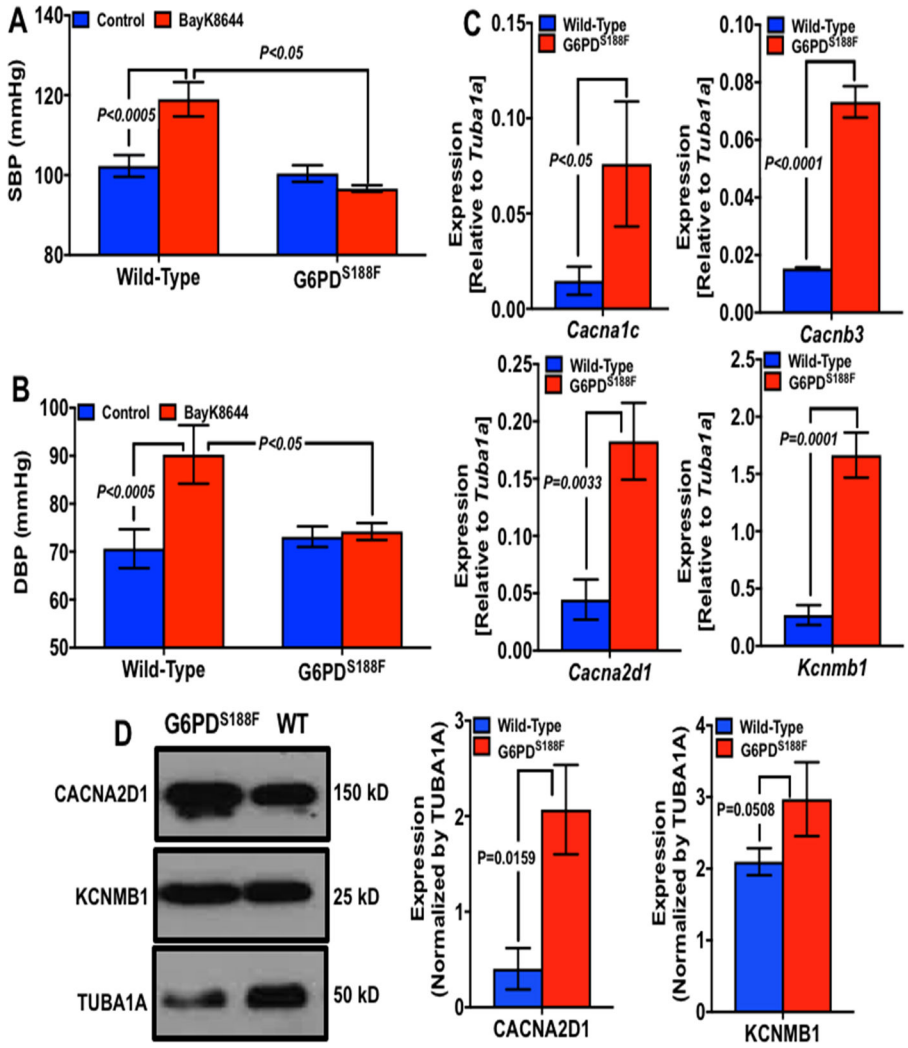

Figure 5:

Effect of BayK8644 and phenylephrine on blood pressure and ion channel gene expression in aorta of WT and G6PD^{S188F} rats. Systolic and diastolic blood pressure was recorded before (Control) and 10 minutes after intravenously injecting (A, B) BayK8644 (1 μM) to WT (N=7) and G6PD^{S188F} (N=5) rats. Expression levels of: (C) alpha-, beta- and delta-subunits of L-type Ca^{2+} channels, and beta-subunit of BK_{Ca} channel gene in aorta of WT and G6PD^{S188F} rats is shown. (D) Expression of delta-subunit of L-type Ca^{2+} (left and middle panel) and BK_{Ca} (middle and right panel) channel protein in aorta of WT and G6PD^{S188F} rats is shown. N=5 in each group.

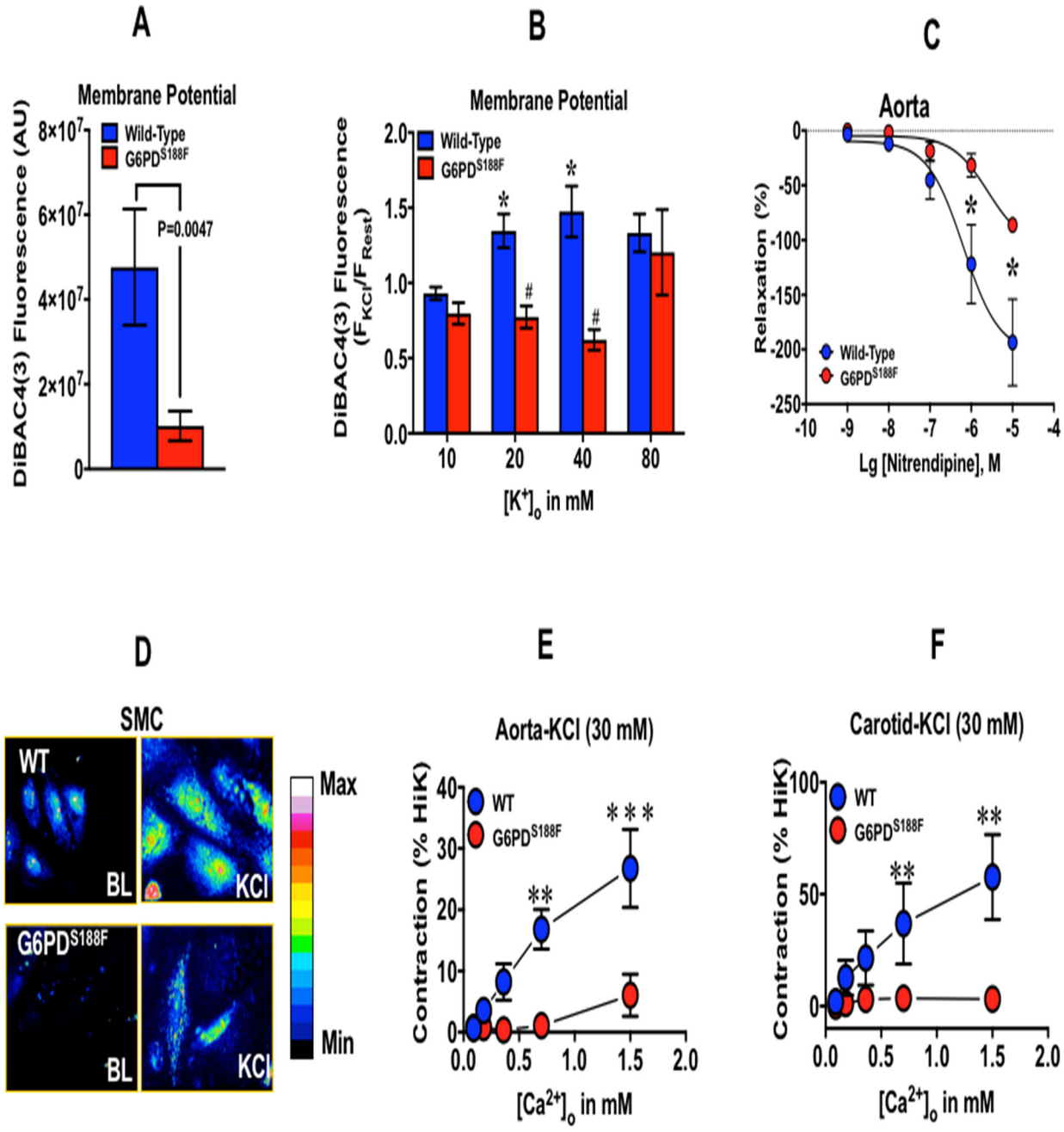

Figure 6:

Membrane potential, nitrendipine-mediated relaxation, intracellular Ca^{2+}, and Ca^{2+}-mediated contraction of arteries isolated from WT and $G6PD^{S188F}$ rats. Membrane potential of (A) resting and (B) KCl-treated aorta was determined by the fluorescence ($f_{480/520}$) of $DiBAC_4(3)$. N= 5 WT and $G6PD^{S188F}$ rats. (C) The effect of nitrendipine, a L-type Ca^{2+} channel blocker that inhibits channel activity at higher membrane potential, on aorta pre-contracted with KCl (30 mM). N= 5 WT and $G6PD^{S188F}$ rats. (D) A representative confocal image of four experiments demonstrating intracellular Ca^{2+} determined by Fluro-4 ($f_{480/520}$) in aortic SMCs. Extracellular Ca^{2+}-elicited contraction of aorta (E) and carotid artery (F)

pre-contracted with KCl (30 mM) is shown. Aorta (N= 5 WT and 10 G6PD^{S188F} rats) and carotid artery (N= 5 WT and 9 G6PD^{S188F} rats).