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The Role of S-Nitrosylation in Valosin-Containing Protein-Mediated Cardioprotection

by

Xiaomeng Shi

Under the Direction of Dr. Chunying Li, M.D., Ph.D.

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the Institute for Biomedical Sciences

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2023

ABSTRACT

Aims: Valosin-containing protein (VCP) has recently been identified as a novel mediator of mitochondrial respiration and cell survival in the heart, in which increased inducible nitric oxide synthase (iNOS) expression and activity is considered an essential mechanistic link in the cardioprotection conferred by VCP. iNOS is one of the three isoforms of nitric oxide synthase (NOS) that generates nitric oxide (NO) from L-arginine, which can then react with cysteine residues in proteins to form protein S-nitrosothiols (SNOs). The study aimed to investigate whether VCP directly mediates protein S-nitrosylation in the heart through the iNOS/NO/SNO pathway. We hypothesized that VCP plays a crucial role in mediating mitochondrial protein S-nitrosylation through an iNOS-dependent mechanism in the heart. To test this hypothesis, we utilized four distinct transgenic (TG) mouse models: cardiac-specific VCP TG mice, bigenic iNOS knockout (KO) with VCP overexpression mice (VCP TG/iNOS $KO^{-/-}$), cardiac-specific dominant-negative (DN) VCP TG mice, and cardiac-specific VCP KO mice.

Methods and results: To investigate the potential impact of VCP on both overall and specific protein S-nitrosylation in mouse heart tissues, we utilized a biotin switch assay combined with streptavidin purification. Our results showed that VCP overexpression increased S-nitrosylation of both VCP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the heart, which was diminished by genetic iNOS deletion. Conversely, function inhibition of VCP resulted in a decrease in the S-nitrosylation levels of VCP and the mitochondrial respiration complex I, but did not affect the S-nitrosylation level of GAPDH in the heart.

Conclusion: Taken collectively, these data provide compelling evidence that VCP could serve as a novel mediator of cardiac protein S-nitrosylation through an iNOS-dependent mechanism.

INDEX WORDS: valosin-containing protein, S-nitrosylation, inducible nitric oxide synthase

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The Role of S-Nitrosylation in Valosin-Containing Protein-Mediated Cardioprotection

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May 3, 2023

DEDICATION

To my loving family and close friends,

My main driving force in finishing my Ph.D. program has been your unwavering support and encouragement. Your constant belief in me and dedication to supporting me along my journey have been truly amazing. I am tremendously appreciative of all the love, support, encouragement, and compassion you have shown me; it has been what has motivated me to reach my goal.

There are no words to adequately express my gratitude to my mom and dad, for all your help, understanding, direction, and inspiration throughout my life as I have pursued knowledge. I'm extremely grateful for everything you've done, and I'm so fortunate to be your child. You are one of the most important and priceless people in my life who has had a profound effect on me and will continue to have an impact on who I become in the future.

To my dear sisters, you two have been by my side good or bad, and have always protected me and fought for me. You two are the love of my life and I cherish every moment we have shared. Thank you for always watching out for me, being there to encourage me, and guiding me through so many aspects of adulting. Thank you for being my spiritual beacon and leading me to independence and self-love.

In our life, people come and go, but you are the few that chose to stick around, support me, and never passed judgment on me. Because of you, I learned what true friendship and unselfish love are like. I appreciate you always being available to me when I needed to talk. You have brightened my life and encouraged me so much throughout this trip, although we live in different countries and are unable to frequently visit one another.

I sincerely dedicate this dissertation to each one of you. I appreciate all your support and love as we have traveled together. Without your help, I would not have been able to accomplish this. You have given me the best support I could ever hope for.

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I would also like to thank my committee members, Dr. Minghui Zou, and Dr. Chunying Li, for their insightful feedback and constructive criticism of my research work. Their support and encouragement have motivated me to advance my experiments and I feel more assured in my abilities to carry out better tests as a result of their support and guidance. Their advice has greatly aided me in improving my research methods and skills as well as gaining a deeper understanding of my topic of study.

Also, I would like to express my gratitude to Dr. Xiaoxu Zheng, Dr. Shaligram Sharma, and Dr. Jing Mu for their guidance, inspiration, and support throughout my Ph.D. journey. Their advice has significantly aided me in acquiring the knowledge and skills necessary for success in academia and beyond.

Finally, I would like to express my gratitude for the transforming influence that my mentors and peers have had on both my academic development and personal growth. They have made immeasurable contributions that have been crucial in assisting me in achieving my objectives and improving as a researcher.

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LIST OF ABBREVIATIONS

αMHC	α-myosin heavy chain
AAA	ATPases-Associated with diverse cellular Activities
CypD	Cyclophilin D
CX43	Connexin 43
DN	Dominant-negative
ETC	Electron transport chain
GSNO	S-nitrosoglutathione
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hsp 22	Heat shock protein 22
iNOS	Inducible nitric oxide synthase
IBMPFD	Inclusion body homeopathy with Paget's disease of bone and frontotemporal dementia
IPC	Ischemia precondition
IFM	Interfibrillar mitochondria
IR	Ischemia reperfusion
KO	Knockout
MtCx43	Mitochondrial Cx43
MPTP	Mitochondrial permeability transition pores
MEFs	Mouse embryonic fibroblasts
ND3	NADH dehydrogenase 3
NO	Nitric oxide

NOS	Nitric oxide synthase
PTM	Post-translational modification
SNO-MPG	S-nitroso-2-mercapto propionyl glycine
SSM	Subsarcolemmal mitochondria
TG	Transgenic
TCA	Tricarboxylic acid
VCP	Valosin-containing protein
WT	Wild type

PREFACE

This Ph.D. dissertation is a significant step in both my academic and professional development. The study that is being provided here is the result of many hours of reading, writing, and reflection, and I am incredibly appreciative of the chance to communicate my research findings to the academic community.

The research work presented in this dissertation primarily focuses on investigating the molecular mechanisms underlying VCP-mediated cardioprotection in physiological conditions and is aimed at addressing the knowledge gap in current literature regarding the largely unrecognized cardioprotective role of VCP through S-nitrosylation. Through utilizing a combination of qualitative and quantitative approaches, I strived to provide more insights into the potential mechanisms regarding the regulatory signaling pathways involved in VCP-mediated cardiac protective effects in the heart under physiological conditions.

Throughout my research project, I received numerous guidance and support from my primary investigator, dissertation advisor, committee members, colleagues, family, and friends. Their constructive feedback and encouragement played an indispensable role in the success of this project, and I am immensely grateful for their contributions.

I hope that the results presented in this dissertation will make a valuable addition to cardiovascular research. Specifically, I believe that this research can help to elucidate the potential therapeutic values of targeting the VCP in the heart, and I look forward to seeing how the presented findings be received by the academic community.

Thank you for investing your time to read this dissertation, and it is my sincere hope that it offers a valuable and meaningful contribution to the ongoing conversation in heart disease research.

1. INTRODUCTION

1.1. VCP is a highly conserved ATPase that plays a crucial role in many cellular processes

VCP is encoded by the VCP gene, which belongs to the type II ATPases-Associated with diverse cellular Activities (AAA) protein family. It has a molecular weight of 97 kDa (also called p97 in mammals and transitional endoplasmic reticulum ATPase) and consists of 806 amino acids. This protein is ubiquitously expressed in all tissues and is highly conserved in all eukaryotes. There are four domains in the protein: the N domain, the two ATPase domains (D1 and D2), and the C-terminal domain **(Figure.1)**. The ATPase domains catalyze ATP hydrolysis. The N-domain is the substrate-binding site and engages in extensive interactions with diverse biological partners and cofactors, which can subsequently mediate substantial post-translational modifications (PTMs) on the substrates, fulfilling the functional diversity of VCP. Some of the known substrates of the N-domain of VCP include ubiquitinated proteins, endoplasmic reticulum (ER)-associated degradation (ERAD) substrates, DNA damage repair proteins, and mitochondrial proteins, such as the transcription factor NF-κB, hydroxymethylglutaryl reductase degradation protein 1, UV excision repair protein RAD23[1-3]. The two best-characterized cofactors so far are the heterodimeric UFD1-NPL4 complex and homotrimeric p47 protein[4]. The UFD1-NPL4 complex designates the involvement of VCP in ERAD and various other pathways, while the p47 protein recruits VCP to initiate homotypic membrane fusion associated with autophagy.

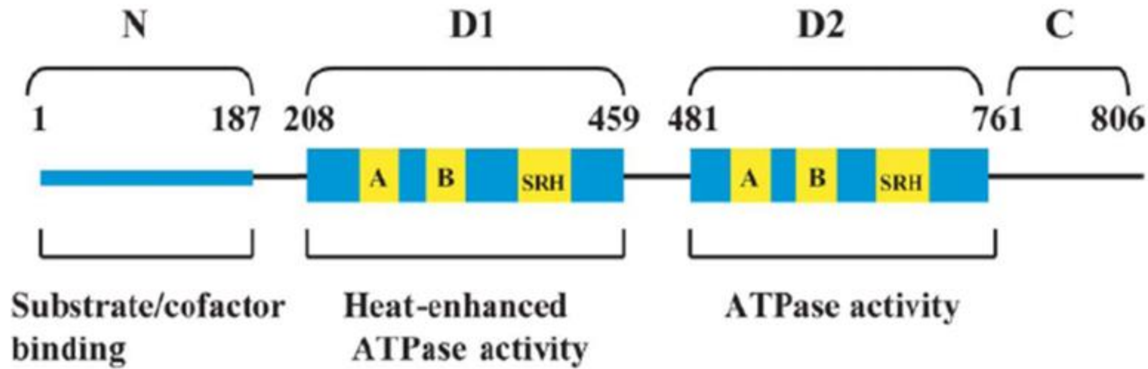

Figure 1. Domains of valosin-containing protein (VCP). *The protein is composed of multiple domains, including N-terminal binding domains, ATPase domains, and a C-terminal region. Source: adapted from www.geb.uni-giessen.de*

To date, the multifunctional properties of VPC have been documented for regulating various cellular processes such as organelle biogenesis, apoptosis, ubiquitin-mediated protein degradation, and autophagy[5]. VCP mutations are widely acknowledged in clinical settings as a causative factor for the development of inclusion body myopathy with Paget's disease of bone (PDB) and frontotemporal dementia (IBMPFD)[6]. This rare autosomal dominant multisystem degenerative disorder is characterized by proximal, progressive muscle weakness that typically arises during adulthood, in addition to early-onset IBMPFD. Apart from these symptoms, IBMPFD has also been observed to present with various cardiac and central nervous system manifestations, such as dilated cardiomyopathy, amyotrophic lateral sclerosis, and Parkinson's disease.

Despite being the most extensively studied member of the AAA protein family, much of the research on VCP has centered around its involvement in the ERAD pathway[3]. VCP is abundantly present in the cytosol and can be recruited to the outer, cytoplasmic surface of the ER with the aid of VCP recruitment proteins like gp78, an ERAD ubiquitin ligase. This recruitment enables VCP to assist in the removal of ubiquitin-tagged defective proteins from

the ER membranes. IBMPFD-associated VCP mutations have been found to specifically disrupt the ERAD pathway, thereby contributing to the development of IBMPFD[7]. Despite cardiac complications being frequently associated with IBMPFD, the specific roles of VCP in the cardiovascular system and associated heart conditions are yet to be fully understood.

1.2. VCP exerts a protective effect on the heart through an iNOS-dependent mechanism both in vivo and in vitro

The potential role of VCP in the heart was first characterized by Lizano et al. in 2013 as a downstream effector of heat shock protein 22 (Hsp22), which formed an intracellular complex with Akt in promoting cardiac cell survival in vitro[1]. Lizano et al. demonstrated co-localization and co-precipitation of both Hsp22 and Akt with VCP in neonatal rat cardiac myocytes. Furthermore, VCP overexpression resulted in a significant increase of NF-kB nuclear translocation and transcriptional activity while overexpression of VCP dominant-negative mutant did not exhibit the same effect. It was also observed that the overexpression of VCP in cardiac myocytes resulted in a dose-dependent upregulation of NOS, a gene that is known to be regulated by NF-kB. This upregulation of iNOS was found to be abolished when the activity of NF-kB was blocked, suggesting that VCP could mediate iNOS expression via NF-kB. iNOS, an isoform of NOS, is expressed solely in response to stimuli, including cytokines, bacterial lipopolysaccharides, and other inflammatory mediators[8]. The binding of NF-kB to specific DNA sequences in the iNOS promoter region leads to transcriptional activation of iNOS, which in turn leads to NO production. In contrast, the overexpression of VCP DN did not lead to an increase in iNOS expression and instead resulted in a dose-dependent decrease in the endogenous levels of iNOS in cardiac myocytes. Prior research has demonstrated that elevated expression of iNOS

in the heart plays a critical role in the cardioprotective mechanisms observed during the delayed phase of ischemic preconditioning[9]. In this study, the researchers found that chelerythrine-induced apoptosis in cardiac myocytes was significantly attenuated by VCP overexpression while over-expression of the VCP DN mutant did not achieve the same effect. Furthermore, the anti-apoptotic effects of VCP overexpression were effectively eliminated by both the NF-kB inhibitor and the pan-NOS inhibitor, indicating that VCP could protect cardiomyocytes against apoptosis through the NF-kB/iNOS pathway in vitro.

In 2017, Following these in vitro findings, Lizano et al progressed to in vivo studies and offered the first description of the expression, function, and mechanism of action of VCP in the mammalian heart by using a novel transgenic mouse model that specifically overexpressed VCP by around 3.5-fold in the heart[10]. In vitro, it has been established that VCP could increase iNOS expression in cardiomyocytes in a dose-dependent manner. Consistently, cardiac-specific overexpression of VCP significantly increased iNOS expression and activity in VCP TG mice compared to wild-type (WT). Additionally, there was an increase in the distribution of both VCP and iNOS in mitochondria within the hearts of VCP TG mice. Given that a significant proportion of VCP is localized within mitochondria, the researchers proceeded to explore the potential impact of VCP on mitochondrial respiration in the heart. It was discovered that complex I-driven state 3 respiration, which is the ADP-stimulated respiration rate was greatly enhanced in VCP TG mouse heart. However, the increase in mitochondrial respiration state 3 observed in VCP TG mouse hearts was abolished upon genetic deletion of iNOS in a bigenic VCP TG/iNOS KO$^{-/-}$ mouse model. This double transgenic mouse model was produced by breeding VCP TG mice with homozygous iNOS KO mice. There was also a significant decrease in maximum respiration

capacity and respiratory control ratio in VCP TG/iNOS KO$^{-/-}$ mouse hearts compared to VCP TG mice. Additional in vitro study revealed that iNOS inhibition with a selective iNOS inhibitor 1400W abolished VCP-induced increase in state 3 mitochondrial respiration in isolated cardiomyocytes. Hence, the results obtained from cultured cardiomyocytes provide additional evidence to support the findings from genetically modified mice, indicating that the beneficial effects of VCP on mitochondrial respiration are dependent upon increased iNOS activity. Furthermore, VCP overexpression was also observed to prevent the opening of mitochondrial permeability transition pores (MPTP), which was abolished by the deletion of iNOS in VCP TG/iNOS KO$^{-/-}$ mice. mPTP opening was also determined in isolated cardiomyocytes with or without the iNOS inhibitor 1400W and the preventive effect of VCP on mPTP opening was abolished by the addition of the iNOS inhibitor 1400W. Taken together, these results demonstrated that VCP overexpression could induce an increase in mitochondrial respiratory capacity and inhibition of MPTP opening, both of which are dependent on iNOS expression and activity. In addition, the investigators examined if VCP overexpression could provide cardioprotection against pathological stimuli in an in vivo setting. 3 to 4-month-old WT and VCP TG mice were subjected to 45 minutes of ischemia followed by 24 hours of reperfusion. There was a 50% reduction of infarction size in the heart of VCP TG mice compared with WT, providing substantial cardioprotection against ischemia-reperfusion injury that is comparable to that observed in the second window of preconditioning. Collectively, these findings offer compelling evidence for a new cardioprotective mechanism of VCP, which involves the upregulation of iNOS expression and activity in cardiomyocytes. This increase in iNOS

expression and activity represents a crucial mechanistic connection between VCP-mediated mitochondrial function preservation and cardioprotection **(Figure.2)**.

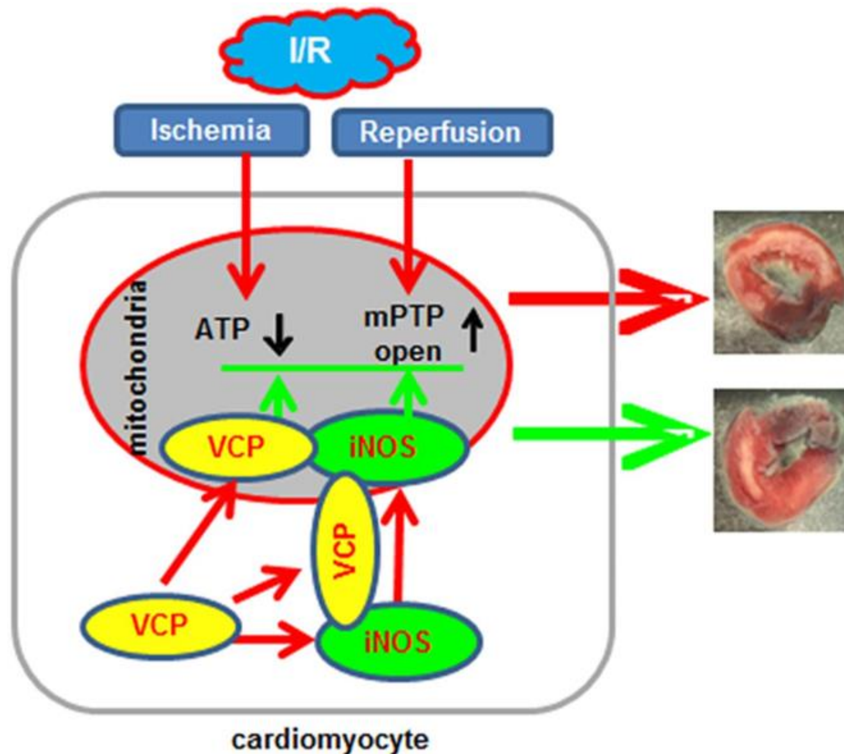

Figure 2. Valosin-containing protein (VCP)-mediated cardioprotection against ischemia-reperfusion injury via iNOS-dependent mechanism. *Illustrates the cardioprotective effects of VCP overexpression against myocardial ischemia-reperfusion injury, which is attributed to the preservation of mitochondrial respiration capacity and prevention of mitochondrial permeability transition pore (mPTP) opening. The mechanistic link between VCP-mediated cardioprotection and preservation of mitochondrial function under cardiac stress is shown to be through increased inducible nitric oxide synthase (iNOS) expression and activity. Source: Lizano et al. Scientific Reports. 2017. 10.1038/srep46324.*

1.3. S-nitrosylation has emerged as a potential new paradigm in redox signaling

NO is a free radical with a single, unpaired electron, which makes it highly labile (a half-life of only a few seconds or less) and chemically reactive[11]. Thus, the bioactivity of NO can be partially compromised because NO rapidly reacts with numerous inactivating species present in the bloodstream and cellular milieu[12]. Reduced protein thiol groups were reported decades ago to serve as rich NO carriers that could not only stabilize NO and extend its half-life but also protect its biological activity from oxidative inactivation[13].

The term S-nitrosylation was first coined by Jonathan S. Stamler in 1992 to describe the reversible formation of the SNOs of reduced thiols from protein sulfhydryl groups that were exposed to NO[13]. Most proteins in the human body possess such thiol groups and can function as substrates for S-nitrosylation, making it a ubiquitous PTM in biology[14]. Thus far, more than 4000 SNO sites involving over 3000 proteins have been experimentally identified and curated in terms of the structural characteristics, functionality, disease relevance, and regulatory networks of S-nitrosylated proteins in dbSNO, the first database of cysteine S-nitrosylation[15]. Since NO functions as an integrative element in electron transfer reactions, S-nitrosylation is now considered a key mechanism that mediates extensive redox-based cellular signal transduction.

Like other PTMs of proteins, such as phosphorylation, S-nitrosylation has emerged as a key regulatory mechanism in various cellular functions and has been studied in many physiological and disease conditions. It has been reported that S-nitrosylation plays an important role in cellular functions in aspects of gene regulation, immune modulation, vascular homeostasis, and respiratory and neuronal signaling. Conversely, its impairment has been implicated in many diseases such as neurodegenerative diseases, airway diseases, diabetes, and endotoxic/septic shock[3]. In recent years, the significance of protein S-nitrosylation has been revealed in the heart, leading to a great number of discoveries in the mechanisms underlying the pathogenesis of heart diseases. Emerging evidence indicates that S-nitrosylation is a potential target of cardiac protection that would provide new avenues for developing new therapeutic strategies.

The heart is one of the most affected organs by SNOs, and a large number of SNOs have been reported in cardiomyocytes[16, 17]. SNOs in the heart exert many functions, including vasodilation, anti-inflammatory effects, anti-thrombotic effects, oxygen homeostasis, the regulation of angiogenesis, apoptotic/necrotic cell death, and intracellular Ca^{2+} homeostasis. Aberrant or dysregulated SNO-dependent NO signaling has been linked to heart diseases and related conditions such as myocardial ischemia, heart failure, and atrial fibrillation[18]. However, the molecular mechanisms underlying these discoveries are still far from being fully elucidated.

1.4. S-nitrosylation plays an essential role in regulating mitochondrial function and homeostasis in the heart under stress

There is growing evidence that S-nitrosylation plays a critical role in regulating mitochondrial function and homeostasis in the heart. Mitochondria are essential organelles that are responsible for ATP production, calcium signaling, and apoptosis, and their dysfunction is implicated in a variety of cardiovascular diseases. Nadtochiy et al. investigated the protective efficacy of the mitochondrial S-nitrosating agent S-nitroso-2-mercapto propionyl glycine (SNO-MPG) in vivo using murine models of myocardial ischemia with permanent left anterior descending artery ligation[19]. Their results showed that in vivo SNO-MPG exhibited a similar extent of cardioprotective effects against ischemia-reperfusion (IR) injury as ischemia precondition (IPC), a golden standard strategy of cardiac protection. They detected that S-nitrosylated proteins in IPC mitochondria widely overlapped with those in SNO-MPG-treated mitochondria. Moreover, the cardioprotection conferred by either SNO-MPG or IPC requires functionally intact mitochondrial complex I. Chouchani et al. used a mitochondrial selective S-

nitrosating agent, MitoSNO, to explore the potential mechanism underlying the cardioprotective effects of mitochondrial S-nitrosylation in vivo during the reperfusion phase of acute murine myocardial infarction[20]. They identified the S-nitrosylation of Cys39 on the NADH dehydrogenase 3 (ND3) subunit of mitochondrial complex I to be responsible for MitoSNO's cardioprotective effects on ischemic myocardium during reperfusion. They suggested that the S-nitrosylation of ND3 Cys39 could mediate the reversible inhibition of complex I activity by disrupting its interaction with ubiquinone and decreasing ROS production. Their discovery of the unique local environment of the ND3 subunit within complex I provided the structural basis for exposing the occluded Cys39 to SNO modification during ischemia. SNO-Cys39 then switches complex I activity to a low state at reperfusion. Based on these findings, Methner et al. further explored the long-term cardioprotective effects of Mito-SNO against post-infarct heart failure using an in vivo mouse model of MI achieved by LAD ligation[21]. Their results showed that persistent infusion of Mito-SNO infusion greatly reduced the infarct size and troponin level in Mito-SNO-treated hearts at 24 h post-reperfusion. Moreover, cardiac functions in Mito-SNO-treated hearts at 28 days post-MI were also significantly improved, indicating that acute Mito-SNO administration could have both short-term and long-term protective effects for the heart. Additionally, the administration of Mito-SNO did not influence hemodynamic parameters, which made it potential for the clinical application of Mito-SNO.

Studies have also been conducted to explore the mechanism of SNO involved in cardiac protection. It has been demonstrated that aside from entering the cell nucleus as a nuclear transnitrosylase in the form of SNO-GADPH, GADPH can also be imported into the mitochondria and modulate mitophagy and apoptosis after myocardial IR injury[22]. Kohr et al. investigated

the interaction between SNO-GADPH and mitochondrial proteins in mice hearts with/without preceding myocardial IPC-IR injury[23]. Their results showed a significant increase of GADPH in the mitochondrial fraction of IPC hearts without increasing the total GADPH level. Purified GAPDH and SNO-GAPDH after trypsin digestion were detected in the mitochondrial matrix, thus indicating the ability of GAPDH to enter the mitochondrial matrix. Importantly, SNO-GAPDH could rapidly increase mitochondrial protein S-nitrosylation, which was found to be in correlation with mitochondrial SNO levels. The findings indicate that SNO-GADPH acts as a mitochondrial transnitrosylase mediating the transnitrosylation of heat shock protein 60 and acetyl-CoA Acetyltransferase 1.

Sun et al. explored the association of caveolar structures in the context of the cardioprotective signaling in Langendorff-perfused mouse hearts with/without IPC[24]. Two distinct mitochondrial subpopulations have been identified in the myocardium, namely, subsarcolemmal mitochondria (SSM), situated directly beneath the sarcolemmal membrane, and interfibrillar mitochondria (IFM), which are distributed between myofibrils. Their results showed that SSM exhibited a higher SNO level than IFM at baseline, and an increase of SNO content was only observed in SSM other than IFM in IPC hearts. A co-immunoprecipitation analysis revealed that only eNOS and caveolin-3 were associated with SSM, and both protein levels were elevated in IPC hearts. The SSM of caveolin-3−/− mouse hearts subjected to IPC treatment exhibited a loss of cardioprotection and the rise of SNO induced by IPC. Thus, SSM could be considered a favored target of the caveolae/eNOS/NO/SNO signaling in IPC-induced cardioprotection. Notably, the gap junction protein connexin 43 (Cx43) expressed in myocardial mitochondria exists exclusively at the inner membrane of SSM and serves as a marker for

SSM[25]. Mitochondrial Cx43 (mtCx43) has been considered an essential component of cardiac preconditioning. It has long been linked to IPC-induced cardioprotection and attenuated reperfusion-induced reactive oxygen species production, as well as the opening of mitochondrial KATP channels[26]. Soetkamp et al. investigated the regulatory effects of nitrite-induced SNO of mtCx43 during IR injury via pharmacological preconditioning with sodium nitrite in the Langendorff-perfused mouse heart model[27]. Their results showed that the NO-mediated increase in ROS production was driven by a proton gradient across the inner membrane. These increases in SSM could all be blocked by a Cx43 hemichannel blocker, carbenoxolone. These results together suggest that the SNO of mtCx43 plays an important role in mediating mitochondrial permeability, K^+ influxes, and ROS formation, thus participating in the cardioprotective signal transduction cascade.

Sun et al. showed that the effects of IPC on protein S-nitrosylation and cardioprotection against myocardial IR injury were mimicked by S-nitrosoglutathione (GSNO)[28]. They reported that GSNO not only greatly decreased calcium transients during IR but also altered the activities of sarcoplasmic reticulum Ca^{2+} ATPase and proteins involved in mitochondrial energetics, such as alpha-ketoglutarate dehydrogenase and F1-ATPase. The pathological opening of mPTP has been intensely linked to myocardial IR injury and apoptotic cell death. Mitochondrial cyclophilin D (CypD) is a key regulator of the mPTP and contains a redox-sensitive residue Cys-203 that can be S-nitrosylated in the heart after treatment with GSNO[17]. Similar results were also observed by Nguyen et al. in Langendorff-perfused hearts[29]. Their results showed that GSNO treatment significantly reduced H_2O_2-induced mPTP opening in WT mouse embryonic fibroblasts (MEFs). The demonstrated inhibition of mPTP opening with GSNO treatment

confirmed the cytoprotective role of S-nitrosylated CypD. Additionally, CypD$^{-/-}$ MEFs transfected with either WT CypD or C203S-CypD generated by the site-directed mutation of Cys 203 of CypD to a serine showed a similar level of inhibition on mPTP opening, suggesting that the inhibitory effects on the mPTP opening by SNO of Cys-203 were similar to that of C203S-CypD or CypD deletion. Amanakis et al. recently identified Cys-202 of CypD as a redox-sensitive site for multiple PTMs, including S-nitrosylation[30]. The collective findings from these studies have provided valuable insights into the crucial role played by S-nitrosylation in regulating mitochondrial function and homeostasis in the heart during IR injury. This knowledge highlights the importance of continued investigation into the molecular mechanisms of S-nitrosylation in mediating mitochondrial protein function in the heart.

1.5. Preliminary data indicate VCP plays a potential role in mediating mitochondrial protein S-nitrosylation in the heart

Having established the background of the study, initial investigations were carried out to determine whether VCP could affect protein S-nitrosylation in the heart. Specifically, Table. 1 illustrates the preliminary data obtained from examining whether cardiac-specific overexpression of VCP could mediate protein S-nitrosylation in the heart of VCP TG mice. To accomplish this, heart tissue samples were treated with the biotin switch assay, which allowed for the enrichment of biotinylated proteins. These proteins were then separated using liquid chromatography and analyzed with mass spectrometry to identify and quantify their protein S-nitrosylation levels. This table summarized a series of cardiac proteins that were significantly more S-nitrosylated in the hearts of mice with VCP overexpression compared to WT mice, with the highest fold change observed among all the identified proteins **(Table.1)**.

Among these most S-nitrosylated cardiac proteins, some participate in toxin metabolism and detoxification such as microsomal glutathione S-transferase 3 and epoxide hydrolase 2. Some play a crucial role in the tricarboxylic acid (TCA) cycle, such as NADP-dependent malic enzyme. Some extensively engage in fatty acid metabolism such as peroxisomal acyl-CoA, citrate lyase subunit beta-like protein, and 3-hydroxy acyl-CoA dehydrogenase type 2. More importantly, a majority of these proteins are heavily associated with the mitochondrial respiration apparatus, especially mitochondrial complex I located in the inner mitochondrial membrane. Complex I is the first and largest enzyme complex in the electron transport chain and is responsible for generating ATP. In the heart of VCPTG mice, a significant increase in S-nitrosylation was observed in several essential components of complex I, such as NADH dehydrogenase flavoprotein 2, NADH dehydrogenase iron-sulfur protein 6, NADH dehydrogenase 1 alpha subunit 10, and NADH dehydrogenase 1 subunit 9, as compared to the WT mice. Additionally, cytochrome C1 as an electron carrier and cytochrome c oxidase subunit 6C as a subunit of complex IV, and ATP synthase subunit O as a subunit of the mitochondrial ATP synthase complex in the electron transport chain (ETC) were also greatly S-nitrosylated in the heart of VCP TG mice. To sum up, these identified S-nitrosylated proteins are heavily involved in the four major aspects of cellular functions including mitochondrial respiration, fatty acid metabolism, TCA cycle, and detoxification. Moreover, a significant number of the S-nitrosylated proteins identified by the initial study are structural subunits of complex I and crucial components of the ETC chain, implying that VCP overexpression could substantially upregulate the S-nitrosylation of key mitochondrial proteins, thereby potentially modulating mitochondrial function in the heart. An especially captivating finding revealed in this table is

that the level of S-nitrosylation of VCP was significantly higher in the hearts of VCP TG mice than in the WT. This indicates that not only VCP could potentially mediate the S-nitrosylation of multiple cardiac proteins but also acts as an SNO protein itself that could be further S-nitrosylated upon VCP overexpression. Given the background information and the preliminary data presented, it is reasonable to hypothesize that VCP mediates mitochondrial protein S-nitrosylation through the iNOS-dependent NO signaling in the heart. The first aim is to determine whether VCP directly mediates protein S-nitrosylation in the heart, using the VCP overexpression transgenic mouse model and the bigenic VCP TG/iNOS KO$^{-/-}$ mouse model. Conversely, our second aim is to confirm the role of VCP in mediating protein S-nitrosylation in the heart through two additional transgenic mouse models that were specifically engineered to inhibit VCP function or expression in the heart, which are cardiac-specific DN-VCP TG mice model and cardiac-specific VCP KO model.

VCPTG	WT	VCPTG/WT	Gene Name
4.7	0		Microsomal glutathione S-transferase 3
4.7	0		NADP-dependent malic enzyme
2.8	0		Peroxisomal acyl-coenzyme A oxidase 1
2.8	0		Sarcalumenin
1.9	0		Cytochrome c1, heme protein
1.9	0		Peptidyl-prolyl cis-trans isomerase A
1.9	0		NADH dehydrogenase flavoprotein 2
22.5	2.2	10.4	Transitional endoplasmic reticulum ATPase
6.6	1.1	6.1	Succinate-semialdehyde dehydrogenase
3.7	1.1	3.5	60 kDa heat shock protein
3.7	1.1	3.5	Complement C3
3.7	1.1	3.5	26S proteasomeregulatory subunit 2
2.8	1.1	2.6	NADH dehydrogenase iron-sulfur protein 6
2.8	1.1	2.6	14-3-3 protein epsilon
2.8	1.1	2.6	Estradiol 17-beta-dehydrogenase 8
5.6	2.2	2.6	NADH dehydrogenase 1 alpha subunit 10
5.6	2.2	2.6	Citrate lyase subunit beta-like protein
5.6	2.2	2.6	Tubulin alpha-4A chain
10.3	4.3	2.4	Epoxide hydrolase 2
16.8	7.5	2.2	NADH dehydrogenase 1 alpha subunit 9
4.7	2.2	2.2	Cytochrome c oxidase subunit 6C
13.1	6.4	2	3-hydroxyacyl-CoA dehydrogenase type-2
6.6	3.2	2	Moesin
16.8	10.7	1.6	ATP synthase subunit O

Table 1. Cardiac proteins that were most S-nitrosylated in the hearts of mice with cardiac-specific valosin-containing protein (VCP) overexpression. *Lists the top cardiac proteins that were found to be significantly more S-nitrosylated in the hearts of transgenic (TG) mice with cardiac-specific VCP overexpression compared to the wild-type. The S-nitrosylation of these proteins was analyzed using a biotin-switch assay followed by mass spectrometry. For each protein, the fold change in S-nitrosylation level and the corresponding ratio are shown.*

2. MATERIALS AND METHODS

2.1. Animal models

2.1.1. Generation of the VCP TG mouse

A genetic construct containing a 2.4 Kb coding sequence of VCP was created. The transgene expression was controlled by a cardiac-specific promoter of the α-myosin heavy chain (αMHC). The construct was then cut using the restriction enzyme BamHI and introduced

into the zygotes of FVB mice through pronuclear microinjection. Mice that tested positive for the transgene were bred with WT mice, and their offspring were analyzed and identified by performing the polymerase chain reaction on DNA extracted from their tails.

2.1.2. Generation of the VCP TG/iNOS KO$^{-/-}$ bigenic mouse

VCP TG mice of the FVB strain were bred with homozygous iNOS KO mice of the C57BL/6 strain (obtained from the Jackson Laboratory with stock number 002609). The resulting F1 generation (consisting of both VCP TG/iNOS KO$^{+/-}$ and WT/iNOS KO$^{+/-}$ mice of the FVB strain) were interbred for five generations to eliminate any background genetic variations. The resulting offspring of this interbreeding was VCP TG/iNOS KO$^{-/-}$ homozygote mice of the FVB strain. Litter-matched VCP TG (VCP TG/iNOS$^{+/+}$) (FVB) and WT (WT/iNOS$^{+/+}$) (FVB) mice were recruited in this study.

2.1.3. Generation of the DN-VCP TG mouse

A transgenic mouse carrying a cardiac-specific dominant-negative VCP mutation was generated with an FVB background. The construct containing the coding sequence of DN-VCP was designed such that it lacked the first 600 nucleotides of the human VCP coding sequence. The DN-VCP coding sequence was then inserted downstream of αMHC. To maintain the mouse colony, transgenic mice were bred with WT FVB mice.

2.1.4. Generation of the VCP KO mouse

A cardiac-specific heterozygous knockout mouse model was developed by crossbreeding VCP Flox$^{+/-}$ mice with a-MHC-Cre-mice, resulting in the generation of mice with a 45% reduction in VCP expression in the heart.

Litter-matched or age-matched WT mice were used as controls for the experimental groups. Both male and female mice were included in the study. All animal procedures were performed following the guidelines set by the National Institutes of Health on the Care and Use of Laboratory Animals, revised in 2011. The protocols used in the study were approved by the Institutional Animal Care and Use Committee of both Loma Linda University and Georgia State University.

2.2. Protein extraction and western blot

To extract the total protein from the left ventricular heart tissue of mice, the tissue was first washed with ice-cold phosphate-buffered saline and weighed. The tissue was then cut into small pieces and homogenized using a bead mill homogenizer (OMNI, bead mill homogenizer) in RIPA buffer with EDTA (Boston bioproducts, BP-115D), and protease inhibitor (Roche,11836153001). The homogenate was then centrifuged at 13,000 rpm for 15 minutes at 4°C to remove any insoluble debris. The total protein content of the supernatant was measured using the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA, 1859078), and the proteins were separated by electrophoresis on SDS-PAGE gels before being transferred to nitrocellulose membranes for detection. To determine protein levels, western blotting was performed using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Primary antibodies specific to the target proteins and corresponding secondary antibodies (LI-COR, Lincoln, NE, USA, IRDye800CW, IRDye680LT) were used for detection. Primary antibodies utilized for protein incubation include anti-VCP-N terminal (Abcam, Cambridge, UK. ab109240), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA. 2118), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA. 97166), and OxPhos Rodent WB

Antibody (Thermo Fisher Scientific, Waltham, MA, USA. 45-8099). The chemiluminescence signal was detected using the LI-COR Odyssey CLx and the resulting image was analyzed.

2.3. Modified biotin switch assay to measure protein S-nitrosylation

A modified biotin switch assay using a commercially available kit (ab236207, Abcam) was performed with minor alterations. To block S-nitrosylation, 1 vial of S-Nitrosylation Blocking Reagent was dissolved in 100 µL N-dimethylformamide and then mixed with 900 µL of Buffer A. The resulting solution, designated as Buffer A containing Blocking Reagent, was added to 9 mL of Buffer A and thoroughly mixed. Heart tissue samples were homogenized in S-Nitrosylation Buffer A containing Blocking Reagent, up to a total volume of 0.5 mL, and incubated on a rocker for 30 minutes at 4°C. The lysates were clarified by centrifugation and the supernatants were mixed with four volumes of ice-cold acetone, incubated at -20°C for 1 hour, and then centrifuged to produce protein pellets. After centrifugation, each protein pellet was resuspended in 0.5 mL of reconstituted Buffer B containing Reducing and Labeling Reagents, incubated for 1 hour at room temperature, and then subjected to acetone precipitation as described earlier. The protein pellets were resuspended in cold 1X S-Nitrosylation Wash Buffer and prepared for western blotting by adding Laemmli buffer and boiling for 5 minutes followed by cooling on ice. Membranes were blocked with 2% BSA in PBS buffers and probed with S-Nitrosylation Detection Reagent I, which was diluted to a ratio of 1:75 for optimal performance. The membranes were developed using enhanced chemiluminescence reagents.

2.4. Streptavidin purification to detect specific S-nitrosylated proteins

The purification of SNO-proteins was performed using Dynabeads™ M-270 Streptavidin (65305, Invitrogen). The beads were resuspended in the vial by rotating for 5 minutes. Beads were transferred to a tube and 1 mL of PBS was added and resuspended. The tube was then placed on a magnet for 1 minute, and the supernatant was discarded. This process was repeated for a total of 3 washes. After the beads were washed, the biotinylated molecule was added to the washed beads, and the tube was incubated for 30 minutes at room temperature with gentle rotation. The tube was then placed in a magnet for 3 minutes, and the supernatant was discarded. The coated beads were washed 3 times with PBS. The washed beads were then resuspended with elution buffer containing 0.1% SDS and heated at 95°C for 5 minutes. The heated tubes containing beads and the elution buffer were centrifuged for 30 seconds at 4000rpm 4°C. The tube was then placed on a magnet for 1 minute, and the supernatant containing eluted proteins was collected.

2.5. Griess assay

Heart tissue (10 mg) was rapidly homogenized with 100 µl of ice-cold Nitrite Assay Buffer. The mixture was then kept on ice for 10 minutes. After that, it was centrifuged at 10,000 x g for 5 minutes, and the supernatant was carefully transferred to a fresh tube. Next, standards, samples, and controls were prepared and added to a 96 microwell plate in duplicate. Griess Reagent I, Griess Reagent II, and the Nitrite Assay Buffer were added separately to each well, and the mixture was thoroughly mixed. In the background control sample wells, 100 µl of Background Reaction Mix was added, and the mixture was again thoroughly mixed. The plate

was then incubated for 10 minutes at room temperature. Finally, the absorbance of each well was measured at 540 nm in end-point mode at room temperature.

2.6. Statistical analysis

The data presented in this study were analyzed as fold change relative to the control group. Statistical analysis was performed using ordinary one-way ANOVA followed by Holm-Sidak's multiple comparisons analysis. A p-value of less than 0.05 was considered statistically significant ($P<0.05$).

RESULTS

3.1. Cardiac-specific VCP overexpression did not alter overall protein S-nitrosylation in the heart

We first investigated the potential role of cardiac-specific VCP overexpression on overall protein S-nitrosylation in heart tissues. To achieve this, we utilized two different methods to detect protein S-nitrosylation at both the expression and functional levels in transgenic mice with cardiac-specific VCP overexpression and their littermate controls. To detect S-nitrosylation at the protein expression level, we performed the biotin switch assay in combination with western blot analysis. Our results revealed no significant differences in protein S-nitrosylation expression levels between VCP TG and WT **(Figure 3. A and B)**, indicating that cardiac-specific VCP overexpression did not affect overall protein S-nitrosylation at the expression level in the heart. To further investigate protein S-nitrosylation at the functional level, we employed the Griess assay, which measures the reaction product of S-nitrosylation, nitrites. Our data showed no significant differences in the presence of S-nitrosylation-derived nitrites between VCP TG and WT **(Figure 3. C and D)**, suggesting that cardiac-specific VCP overexpression did not affect

overall protein S-nitrosylation levels in the heart at the functional level as well. Taken together, our findings suggest that cardiac-specific VCP overexpression does not alter the overall protein S-nitrosylation levels in heart tissues.

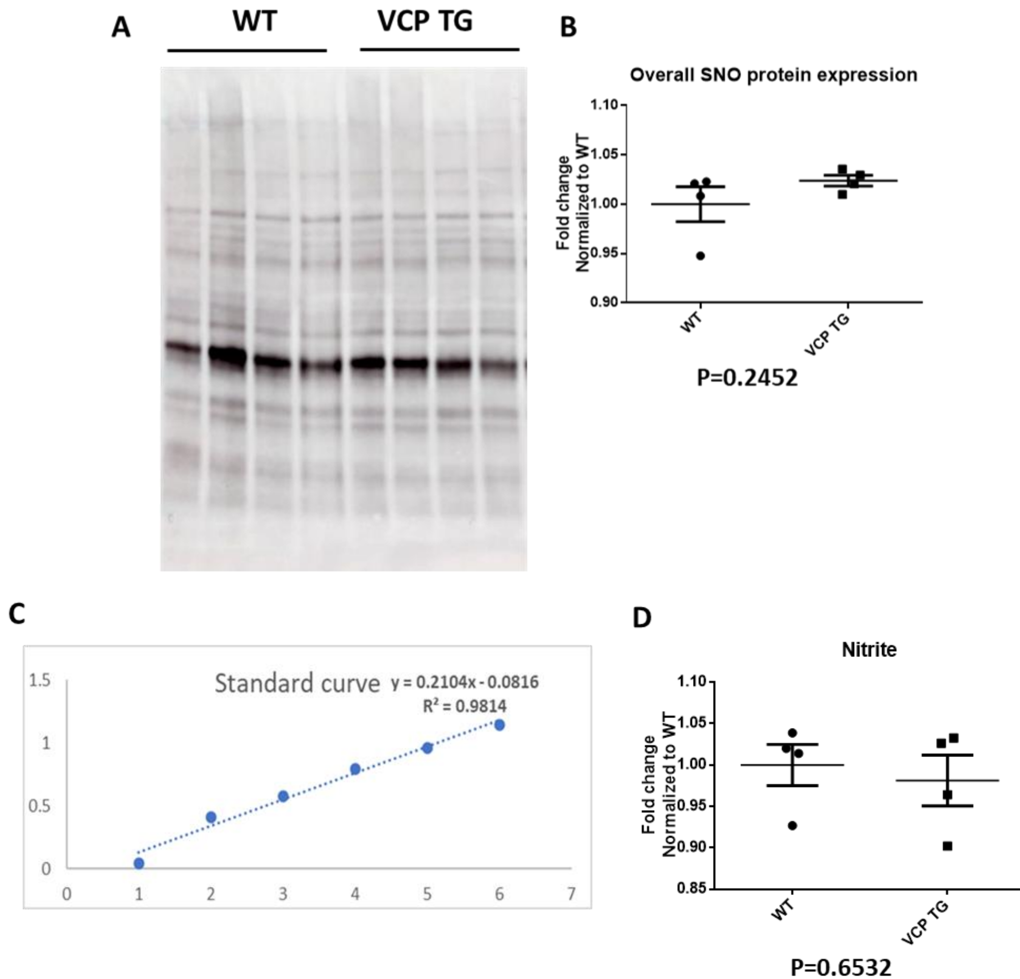

Figure 3. Overall protein S-nitrosylation remained unchanged in response to valosin-containing protein (VCP) overexpression. *A. Biotin switch assay analyzed by western blotting showing overall S-nitrosylated protein expression in the heart of wild type (WT) and VCP transgenic (TG) mice. B. Quantification of western blot showing overall protein S-nitrosylation in total protein extracts from WT and VCP TG mice. C. A standard curve in the Griess assay was used for the accurate quantification of nitrite of unknown concentration. D. Quantification of nitrite concentration in the heart of WT and VCP TG mice.*

3.2. VCP overexpression results in increased S-nitrosylation of VCP and GAPDH in the heart

Based on our preliminary data with mass spectrometry, we have identified a potentially significant difference in the S-nitrosylation of VCP between VCP TG mice and their WT

littermates in the heart. Specifically, we observed a significant increase in the S-nitrosylation of VCP in the hearts of VCP TG mice compared to WT littermates. This finding has led us to primarily focus on further investigating the level of S-nitrosylation of VCP in the hearts of these two groups of mice. To achieve this aim, we employed a combination of the biotin switch assay and streptavidin purification. The biotin switch assay is a widely used technique for detecting S-nitrosylation, while streptavidin purification provides a highly specific and efficient means of isolating biotinylated proteins for subsequent analysis. GAPDH is a frequently used loading control protein in western blot analysis. However, recent studies have shown that GAPDH could also be modified by S-nitrosylation, which would interfere with its role as a reliable normalization control[23, 31]. To address this potential issue, we adopted the overall expression of protein S-nitrosylation as a control panel. This approach allowed us to interpret our findings more accurately. In this study, GAPDH serves a dual purpose as a regular loading control at baseline and as a critical target for investigation.

We first sought to confirm the overexpression of VCP in the hearts of VCP TG mice compared to their WT counterparts. The baseline group shown in **Figure 4. A** demonstrated that VCP expression was significantly increased in the hearts of VCP TG mice compared to WT mice on western blot, thus verifying the successful overexpression of VCP in the VCP TG mouse model. Since our previous findings suggest that cardiac-specific VCP overexpression does not alter the overall protein S-nitrosylation levels in heart tissues, we next investigated the effect of VCP overexpression on S-nitrosylation levels of specific proteins in heart tissue. Therefore, we performed biotin switch assays combined with streptavidin purification to purify the S-nitrosylated proteins from heart tissue lysates of both VCP TG and WT mice.

Upon isolation, we compared the level of S-nitrosylation of two target proteins, VCP and GAPDH, in the hearts of VCP TG mice with their corresponding WT counterparts **(Figure. 4A)**. Interestingly, in the test group in which SNO-proteins were specifically labeled and purified, there were almost non-existing expressions of SNO-VCP and SNO-GAPDH observed in the WT group, while it was significantly higher in the VCP TG group **(Figure. 4 E)**. This is a substantial increase in expression and implies that the SNO modification of VCP may play a crucial role in regulating the function of this protein in VCP TG mice. In contrast, the baseline group showed only about a 2.5-fold increase in VCP expression between the VCP TG and WT mice **(Figure 4. B and D)**. These results together suggest that the overexpression of VCP could significantly increase the S-nitrosylation level of VCP and GAPDH in the heart. In comparison to the baseline panel, wherein GAPDH was employed as a trustworthy loading control, the test group exhibited a noteworthy disparity in SNO-GAPDH expression between the WT group and the VCP TG group **(Figure 4. A)**. SNO-GAPDH expression in the hearts of the WT group was essentially absent, except for a solitary sample band. While the observed difference in SNO-GAPDH expression between the hearts of VCP TG mice and the WT group was not deemed statistically significant, a closer examination of the mean value depicted in the graph reveals a notable increase in SNO-GAPDH expression **(Figure. 4 C and F)**. Specifically, there was a robust 2.5-fold increase in SNO-GAPDH expression in the hearts of VCP TG mice when compared to the WT group. Although the p-value did not meet the conventional threshold for statistical significance, this finding should not be overlooked as it indicates a potential association between VCP TG and heightened levels of SNO-GAPDH expression. These results together potentially suggest that cardiac VCP overexpression could increase the S-nitrosylation levels of both VCP and GAPDH in the heart.

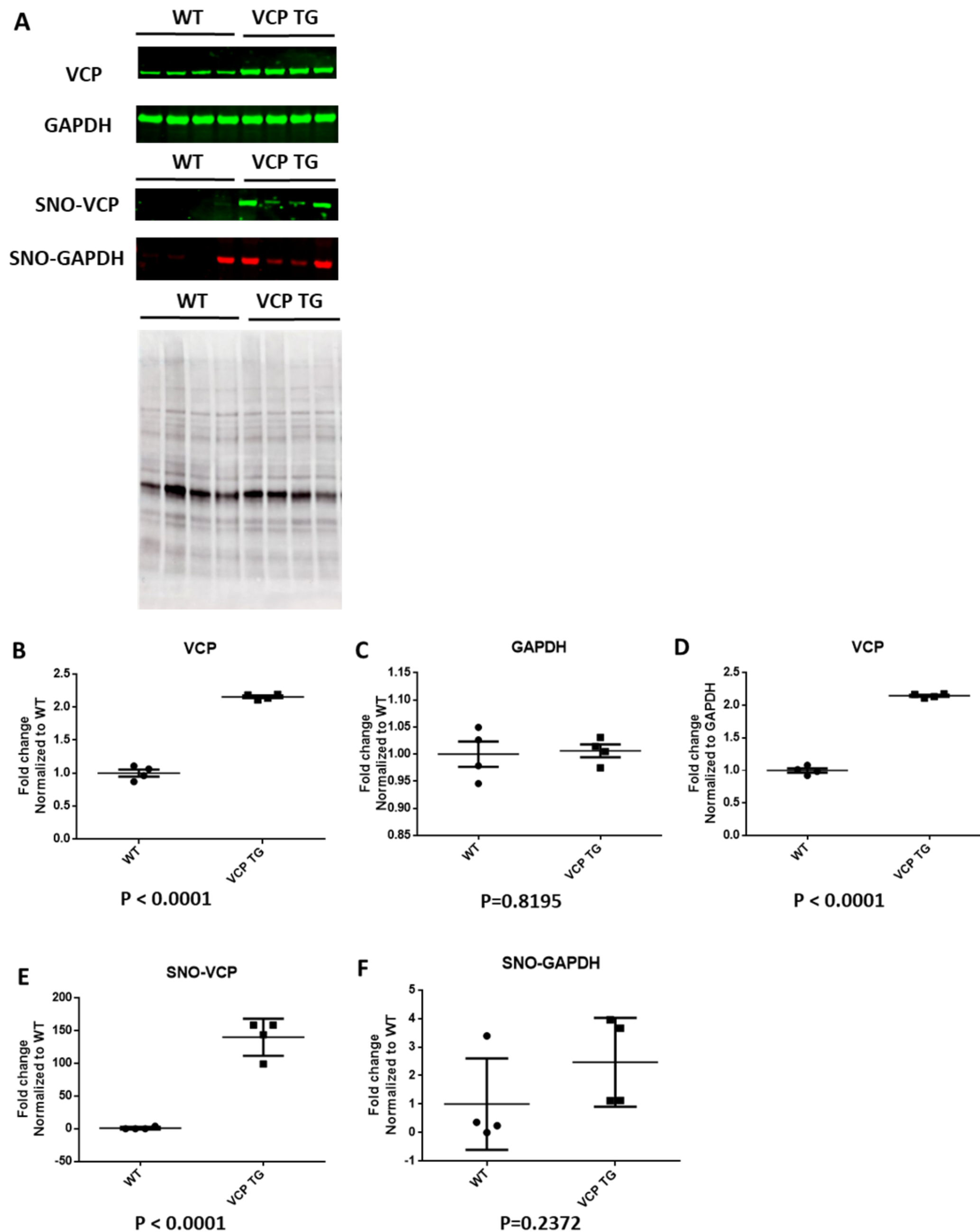

Figure 4. S-nitrosylation of valosin-containing protein (VCP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was increased in response to VCP overexpression. *A. Western blot analysis of untreated proteins at baseline showing the protein expression of VCP and GAPDH, as well as purified biotinylated proteins showing S-nitrosylated VCP and GAPDH expression, in the heart of VCP transgenic (TG) and wild-type (WT) mice. GAPDH used as a loading control in total protein extracts. Total protein S-nitrosylation expression obtained from biotin switch assay used as the loading control for purified biotinylated protein extracts. B. Quantification of western blot showing fold change of VCP expression normalized to wild-type. C. Quantification of western blot showing fold change of GAPDH expression normalized to wild-type. D. Quantification of western blot showing fold change of VCP expression normalized to GAPDH. E. Quantification of western blot showing expression of S-nitrosylated VCP (SNO-VCP) normalized to wild-type. F. Quantification of western blot showing expression of S-nitrosylated GAPDH (SNO-GAPDH) normalized to wild-type.*

3.3. iNOS deletion did not alter overall protein S-nitrosylation in the heart with VCP overexpression

Our investigation has revealed that VCP overexpression could lead to a significant increase in the S-nitrosylation level of both VCP and GAPDH in the heart. While this finding is intriguing, the underlying mechanism that results in VCP and GAPDH being S-nitrosylated to a higher degree when VCP is overexpressed remains unclear.

Previous studies have already established VCP as a novel mediator of iNOS-dependent cardioprotection in vitro and in vivo. Given the protective effects of VCP in the heart were found to be mediated through the iNOS signaling pathway, we hypothesized that the increase in VCP and GAPDH S-nitrosylation induced by VCP overexpression might be associated with iNOS-mediated S-nitrosylation formation. To recapitulate the relationship between iNOS and S-nitrosylation: iNOS is one of the three key NO synthases that produce NO, whose electrons later attack a cysteine thiol to form SNOs. To test our hypothesis, we recruited the cardiac-specific bigenic VCP TG/iNOS KO$^{-/-}$ mouse model described above. The overall SNO protein expression in the hearts of all four groups of mice was measured and the results of the biotin switch assay revealed no significant differences in SNO protein expression levels between the four experimental groups and the WT group **(Figure 5. A and B)**. Subsequently, the Griess assay was used to measure the overall S-nitrosylation of the heart tissues at the functional level of all four groups of mice. The results showed that there were no significant differences regarding the concentration of SNO products nitrite between the four groups **(Figure 5. C and D)**. These results suggest that iNOS deletion did not alter overall protein S-nitrosylation either at the protein expression level or the functional level upon VCP overexpression.

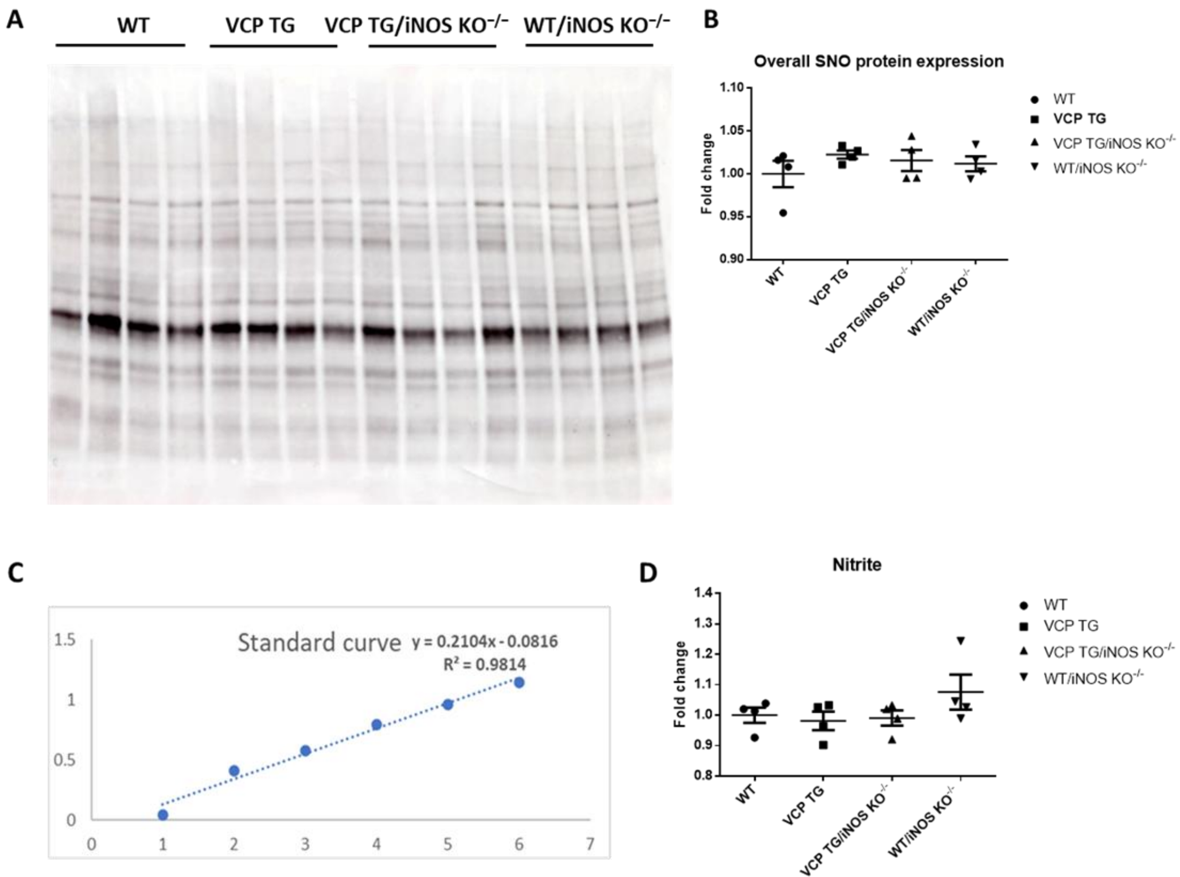

Figure 5. Overall protein S-nitrosylation in the heart remained unchanged upon genetic knockout (KO) of inducible nitric oxide synthase (iNOS). *A. Biotin switch assay analyzed by western blotting showing overall S-nitrosylated protein expression in the heart of four groups of mice: wild type (WT), VCP transgenic (TG), VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ mice. B. Quantification of western blot showing overall S-nitrosylated protein expression in total protein extracts. C. A standard curve in the Griess assay used for the accurate quantification of nitrite of unknown concentration. D. Quantification of nitrite concentration in total protein extracts.*

3.4. iNOS deletion attenuated VCP overexpression-induced increase in VCP and GAPDH S-nitrosylation in the heart

Based on the previous results, iNOS deletion did not significantly alter the overall S-nitrosylation level in the heart, indicating that iNOS may not play a major role in regulating overall protein S-nitrosylation in the heart. To further explore the effects of iNOS deletion on protein S-nitrosylation, the biotin switch assay combined with streptavidin purification was performed to determine whether iNOS deletion could impact individual protein S-nitrosylation,

with a particular emphasis on the two key protein targets in our study, VCP and GAPDH. VCP expression was significantly increased in the heart of both VCP TG and VCP TG/iNOS KO$^{-/-}$ mice when compared to their respective controls **(Figure 6. A-D)**. This result confirmed successful VCP overexpression in the bigenic VCP TG/iNOS KO$^{-/-}$ mouse model. Western blot results regarding the deletion of iNOS expression in VCP TG/iNOS KO$^{-/-}$ mice are not presented here as it has been previously verified. Additionally, there was no significant difference in cardiac VCP expression between VCP TG mice and VCP TG/iNOS KO$^{-/-}$ mice. Protein expression of both SNO-VCP and SNO-GAPDH were observed to be notably increased in VCP TG mice compared to WT as described above. While the statistical significance of the findings may be debatable, there appears to be a notable decreasing trend in SNO-VCP expression in the hearts of VCP TG/iNOS KO$^{-/-}$ mice as compared to VCP TG mice **(Figure. 6 A and E)**. This trend suggests that iNOS deletion could potentially attenuate SNO-VCP expression in the heart of VCP TG mice. In addition, the VCP TG/iNOS KO$^{-/-}$ group demonstrated almost undetectable levels of SNO-VCP expression, suggesting that deletion of iNOS could potentially abolish S-nitrosylation of VCP in cases where VCP is not overexpressed **(Figure. 6 A and E)**. Aside from greatly increased SNO-VCP expression in the VCP TG group, the expression of SNO-GAPDH was also notably increased compared to the WT group as described above **(Figure. 6 A and F)**. In contrast, there is a notable decreasing trend regarding the expression of SNO-GAPDH in the VCP TG/iNOS KO$^{-/-}$ group compared to the VCP TG group although this difference did not reach statistical significance. These findings suggest that iNOS deletion could potentially attenuate VCP overexpression-induced increase in S-nitrosylation of GAPDH in the heart of VCP TG/iNOS KO$^{-/-}$ mice. Drawing from these results, it is reasonable to assert that iNOS deletion could potentially

attenuate VCP overexpression-induced increase in S-nitrosylation of both VCP and GAPDH in the heart.

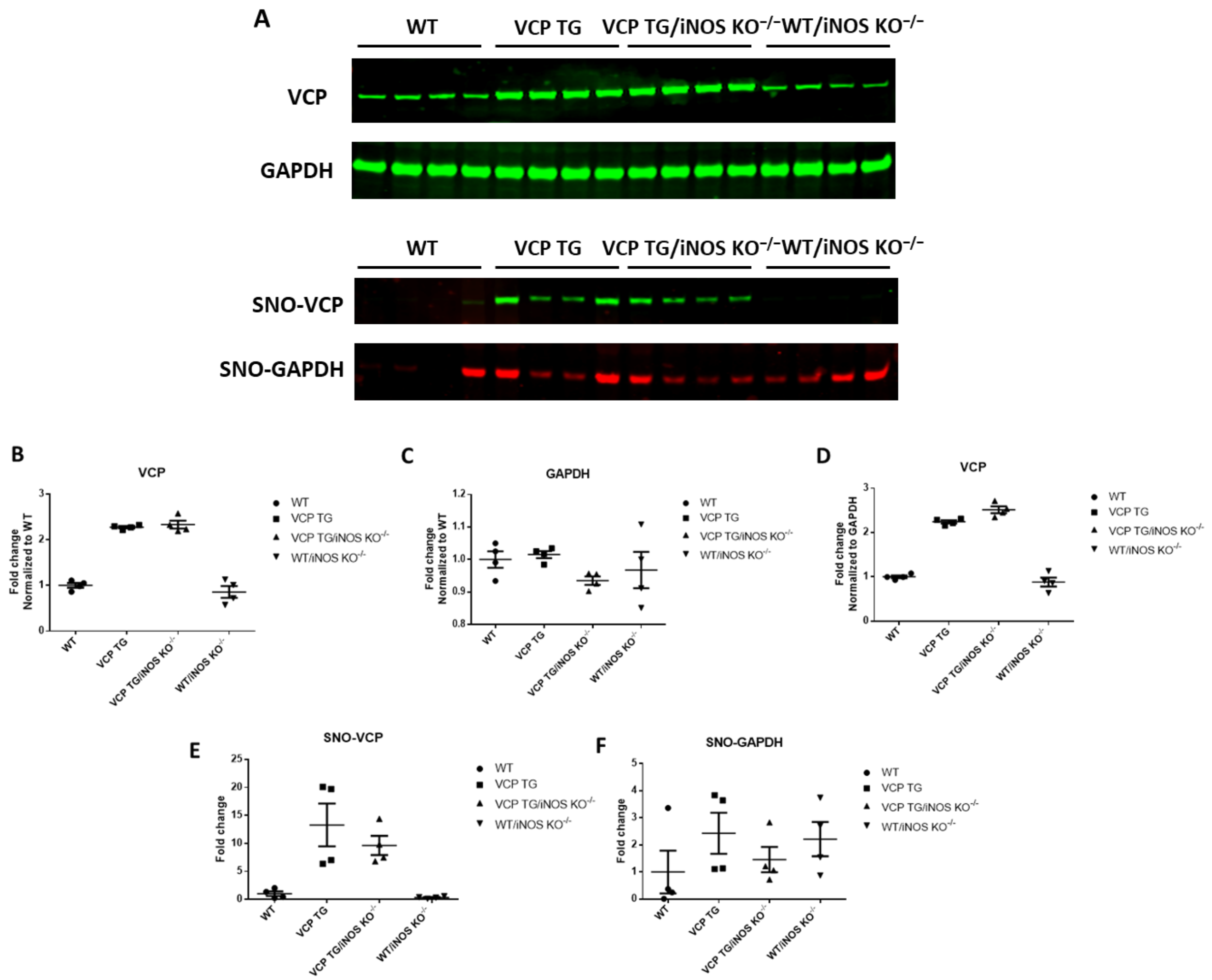

Figure 6. The increase in S-nitrosylation of valosin-containing protein (VCP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) induced by VCP overexpression was attenuated by inducible nitric oxide synthase (iNOS) knockout (KO). *A. Western blot analysis of untreated proteins at baseline showing the protein expression of VCP and GAPDH, as well as purified biotinylated proteins showing S-nitrosylated VCP and GAPDH expression, in the heart of VCP transgenic (TG), wild-type (WT), VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ mice. GAPDH used as a loading control in total protein extracts. B. Quantification of western blot showing fold change of VCP expression normalized to wild-type. C. Quantification of western blot showing fold change of GAPDH expression normalized to wild-type. D. Quantification of western blot showing fold change of VCP expression normalized to GAPDH. E. Quantification of western blot showing expression of S-nitrosylated VCP (SNO-VCP) normalized to wild-type. F. Quantification of western blot showing expression of S-nitrosylated GAPDH (SNO-GAPDH) normalized to wild-type.*

3.5. iNOS deletion decreased VCP overexpression-induced increase in S-nitrosylation of VCP and GAPDH in female mouse heart

To verify and strengthen the observed findings in the heart of male mice and to explore any potential gender-based differences in VCP overexpression-mediated protein S-nitrosylation, additional experiments were conducted, this time focusing on female subjects. Specifically, four groups of female mice including WT, VCP TG, VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ were recruited for the study. In the baseline panel, cardiac VCP expression was significantly increased in both VCP TG and VCP TG/iNOS KO$^{-/-}$ mice compared to WT and WT/iNOS KO$^{-/-}$ mice, respectively **(Figure 7. A-D)**. In line with the findings in male mice hearts, cardiac SNO-VCP expression in the WT group was significantly diminished to an almost undetectable level compared to SNO-VCP expression shown in the VCPTG group, suggesting that VCP overexpression could also induce a significant increase in the S-nitrosylation level of VCP in the heart of female VCP TG mice **(Figure 7. A)**. Despite the absence of data in the WT group that interfered with conducting statistical analysis, it remains reasonable to state that the extent of fold change in SNO-VCP expression between the VCP TG group and the WT group was considerably higher than the corresponding fold change in VCP expression observed between these groups at baseline **(Figure 7. E)**. In comparison to the VCP TG group, the VCP TG/iNOS KO$^{-/-}$ group demonstrated a notable decreasing trend in SNO-VCP expression, implying that iNOS deletion could potentially impair the increase in SNO-VCP expression induced by VCP overexpression **(Figure 7. A)**. Additionally, the WT/iNOS KO$^{-/-}$ group exhibited an almost negligible presence of SNO-VCP expression. These results are consistent with the observations made in the heart of male mice. Notably, the expression of SNO-GAPDH was nearly non-

existent in the male mouse heart of the WT group, whereas there was no significant difference in SNO-GAPDH expression between the VCP TG group and the WT group in the female mouse heart **(Figure 7. A and F)**. What's particularly intriguing is that both the VCP TG/iNOS KO$^{-/-}$ group and the WT/iNOS KO$^{-/-}$ group showed a significant decrease in SNO-GAPDH expression to near non-existent levels. These results, obtained from the hearts of female mice, provide further evidence that iNOS plays a critical role in mediating S-nitrosylation of VCP and GAPDH expression in the heart. Additionally, it appears that iNOS deletion may have a more pronounced effect on diminishing SNO-GAPDH expression in female hearts.

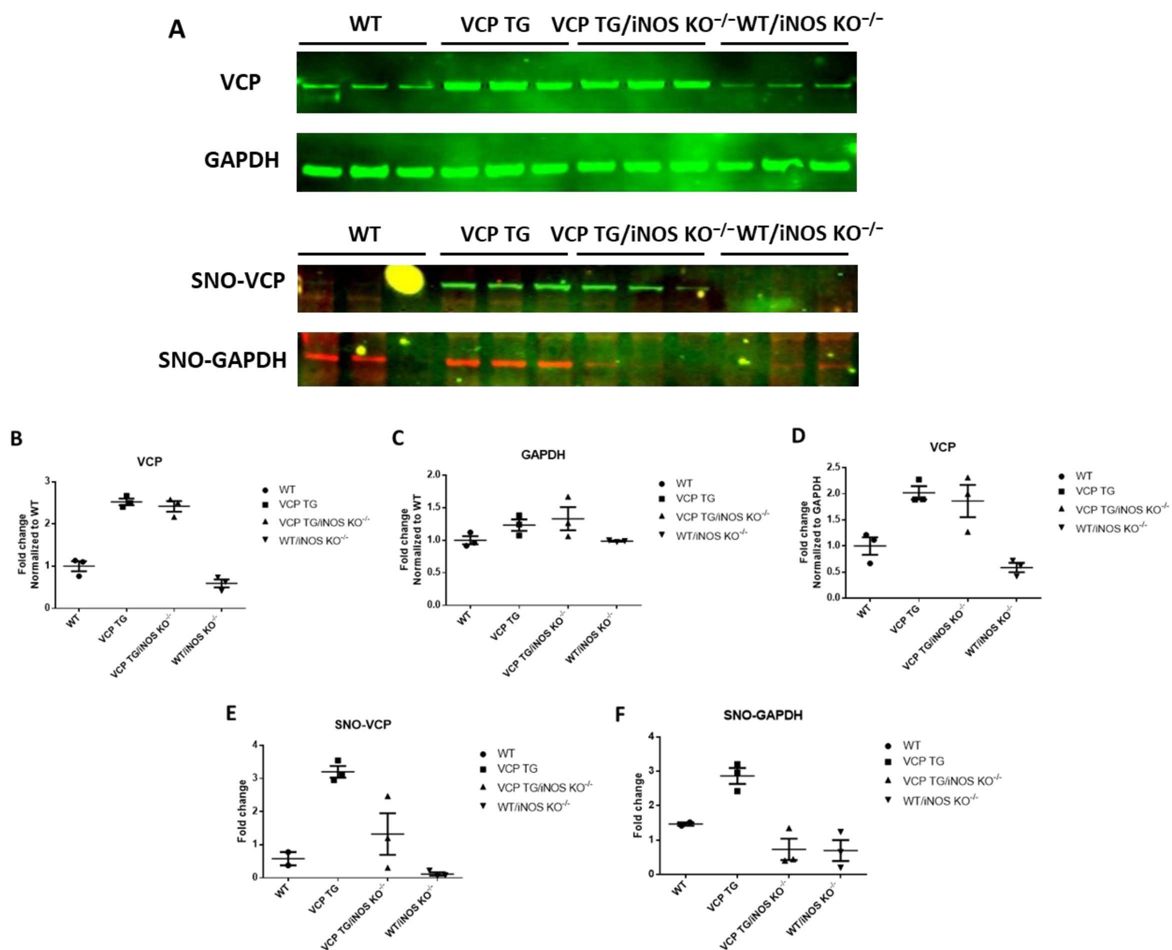

Figure 7. Inducible nitric oxide synthase (iNOS) knockout (KO) decreased valosin-containing protein (VCP) overexpression-induced increase in S-nitrosylation of VCP and GAPDH in female mouse hearts. *A. Additional western blot analysis of untreated proteins at baseline showing the protein expression of VCP and GAPDH, as well*

as purified biotinylated proteins showing S-nitrosylated VCP and GAPDH expression, in the heart of female VCP transgenic (TG), wild-type (WT), VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ mice. B. Quantification of western blot showing fold change of VCP expression normalized to wild-type. C. Quantification of western blot showing fold change of GAPDH expression normalized to wild-type. D. Quantification of western blot showing fold change of VCP expression normalized to GAPDH. E. Quantification of western blot showing expression of S-nitrosylated VCP (SNO-VCP) normalized to wild-type. F. Quantification of western blot showing expression of S-nitrosylated GAPDH (SNO-GAPDH) normalized to wild-type.

3.6. A single cysteine residue located at the N-terminal of VCP was identified to be S-nitrosylated as the S-Nitrosylated site in response to VCP overexpression

The findings revealed in the aforementioned results indicate a significant increase in the S-nitrosylation level of both VCP and GAPDH in VCP TG mice as a result of cardiac VCP overexpression. More importantly, the deletion of iNOS resulted in a marked reduction in SNO-VCP and even completely abolished SNO-GAPDH expression in the female group. Following the establishment of the significant impact of VCP overexpression on S-nitrosylation levels, it was evident that a more comprehensive understanding of the underlying mechanisms driving this effect was needed. Therefore, we aimed to identify and determine the specific cysteine residues within VCP that are responsible for VCP S-nitrosylation upon VCP overexpression. To achieve this objective, mass spectrometry analysis was conducted to determine the peptide sequences of a group of vital S-nitrosylated proteins in the heart. The resulting table revealed that there was only one cysteine residue in VCP found to be S-nitrosylated in response to VCP overexpression in the heart of VCP TG mice **(Table. 2)**. Upon further analysis of the peptide sequence, it was discovered that the cysteine residue was located at the N-terminal of VCP. Specifically, the amino acid sequence containing the identified cysteine residue at the N-terminal of VCP was LGDVISIQPCPDVK. This finding provides additional evidence supporting the essential role of the substrate-binding N-terminal of VCP in the S-nitrosylation induced by VCP overexpression. These results shed light on the regulatory mechanisms underlying VCP-

mediated S-nitrosylation in response to VCP overexpression and provide a structural foundation for further investigations into the functional role of VCP in S-nitrosylation modification of myocardial proteins.

	Protein name (Swissprot)**	MW	Number of S-Nitrosylated peptides*		
			WT	VCP	VCP/WT
Heat shock cognate 71 kDa protein OS=Bos taurus GN=HSPA8 PE=1 SV=2	HSP7C_BOVIN (+6)	71 kDa	0	1	#DIV/0!
2,4-dienoyl-CoA reductase, mitochondrial OS=Mus musculus GN=Decr1 PE=1 SV=1	DECR_MOUSE	36 kDa	0	1	#DIV/0!
Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial OS=Mus musculus GN=Etfdh PE=1 SV=1	ETFD_MOUSE (+1)	68 kDa	0	1	#DIV/0!
Cytochrome c1, heme protein, mitochondrial OS=Mus musculus GN=Cyc1 PE=1 SV=1	CY1_MOUSE	35 kDa	0	1	#DIV/0!
Phosphoglucomutase-1 OS=Macaca fascicularis GN=PGM1 PE=2 SV=3	PGM1_MACFA (+1)	61 kDa	0	1	#DIV/0!
60 kDa heat shock protein, mitochondrial OS=Mus musculus GN=Hspd1 PE=1 SV=1	CH60_MOUSE (+1)	61 kDa	0	1	#DIV/0!
Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial OS=Mus musculus GN=Sucla2 PE=1 SV=2	SUCB1_MOUSE	50 kDa	0	1	#DIV/0!
Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1 SV=4	TERA_HUMAN (+2)	89 kDa	0	1	#DIV/0!
Sarcalumenin OS=Mus musculus GN=Srl PE=1 SV=1	SRCA_MOUSE	99 kDa	0	1	#DIV/0!
2-oxoglutarate dehydrogenase, mitochondrial OS=Mus musculus GN=Ogdh PE=1 SV=3	ODO1_MOUSE	116 kDa	1	3	3
Isocitrate dehydrogenase [NADP], mitochondrial OS=Mus musculus GN=Idh2 PE=1 SV=3	IDHP_MOUSE (+1)	51 kDa	2	4	2
ADP/ATP translocase 1 OS=Mus musculus GN=Slc25a4 PE=1 SV=4	ADT1_MOUSE	33 kDa	1	2	2
ATP synthase subunit gamma, mitochondrial OS=Mus musculus GN=Atp5c1 PE=1 SV=1	ATPG_MOUSE (+1)	33 kDa	1	2	2
Beta-enolase OS=Mus musculus GN=Eno3 PE=1 SV=3	ENOB_MOUSE (+1)	47 kDa	1	2	2
Protein NipSnap homolog 2 OS=Mus musculus GN=Gbas PE=2 SV=1	NIPS2_MOUSE	33 kDa	1	2	2
14-3-3 protein zeta/delta OS=Bos taurus GN=YWHAZ PE=1 SV=1	1433Z_BOVIN (+5)	28 kDa	1	2	2
Ceruloplasmin OS=Mus musculus GN=Cp PE=1 SV=2	CERU_MOUSE	121 kDa	1	2	2
Aconitate hydratase, mitochondrial OS=Mus musculus GN=Aco2 PE=1 SV=1	ACON_MOUSE	85 kDa	3	5	1.666666667
Long-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Mus musculus GN=Acadl PE=2 SV=2	ACADL_MOUSE	48 kDa	2	3	1.5
Cytochrome c oxidase subunit 6B1 OS=Mus musculus GN=Cox6b1 PE=1 SV=2	CX6B1_MOUSE	10 kDa	2	3	1.5
Triosephosphate isomerase OS=Mus musculus GN=Tpi1 PE=1 SV=3	TPIS_MOUSE	27 kDa	3	4	1.333333333
Creatine kinase S-type, mitochondrial OS=Mus musculus GN=Ckmt2 PE=1 SV=1	KCRS_MOUSE	47 kDa	11	14	1.272727273
Fructose-bisphosphate aldolase A OS=Mus musculus GN=Aldoa PE=1 SV=2	ALDOA_MOUSE (+1)	39 kDa	5	6	1.2

Table 2. A cysteine residue located at the N-terminal of valosin-containing protein (VCP) was found to be the sole site of S-nitrosylation in response to VCP overexpression. *The number of S-nitrosylated peptides detected in each of the listed S-nitrosylated proteins in the hearts of VCP transgenic (TG) and wild-type (WT) mice is displayed.*

3.7. Functional inhibition of VCP activity decreased overall protein S-nitrosylation in the heart

Previous findings have indicated that the site of S-nitrosylation in response to VCP overexpression in the heart is a single cysteine residue located at the N-terminal of the VCP sequence. The discovery holds great importance as the N-terminal domain of VCP has been established as critical for its ATPase activity and plays an essential role in regulating protein function via S-nitrosylation [32]. Therefore, we sought to investigate the potential effects of impaired VCP activity on protein S-nitrosylation in the heart by utilizing a cardiac-specific DN-VCP TG mouse model designed to lack the initial 600 nucleotides in the N-terminal region of the

human VCP coding sequence. In the first step, overall SNO protein expression was measured in the heart of DN-VCP and WT mice. The biotin switch assay revealed that there was a significant decrease in overall protein S-nitrosylation expression level in the heart of DN-VCP TG mice **(Figure 8. A and B)**. At a functional level, the comparison between the nitrite concentration in the heart of DN-VCP TG mice and WT mice did not yield a statistically significant difference **(Figure 8. C and D)**. However, it is worth noting that there was a discernible decreasing trend in the nitrite concentration in the hearts of DN-VCP TG mice compared to their WT counterparts. The decrease in overall protein S-nitrosylation observed in response to functional inhibition of VCP-mediated cardiac ATPase activity implies that the N-terminal region of VCP may play a crucial role in maintaining overall protein S-nitrosylation homeostasis in the heart.

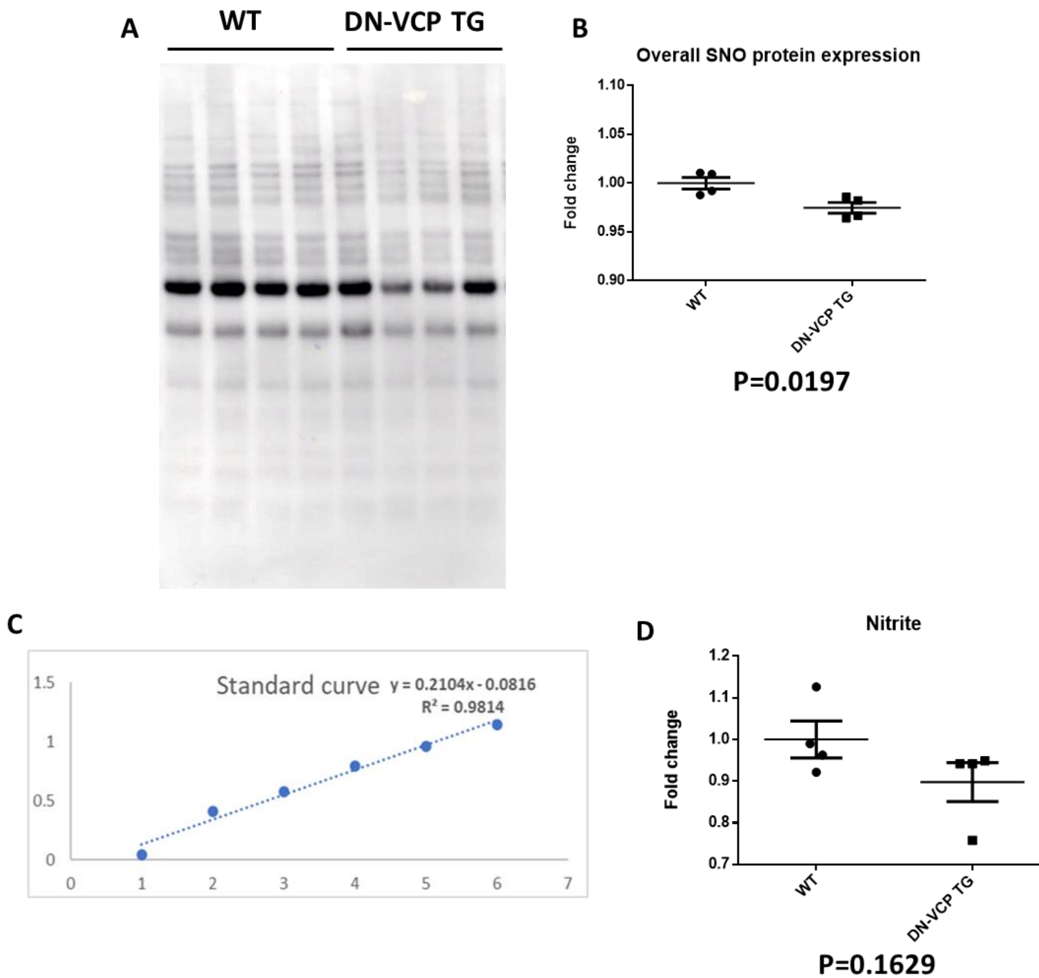

Figure 8. Functional inactivation of valosin-containing protein (VCP) resulted in reduced overall protein S-nitrosylation in the heart. *A. Biotin switch assay analyzed by western blot showing the overall S-nitrosylated protein expression in the heart of wild-type (WT) and dominant-negative (DN) VCP transgenic (TG) mice. B. Quantification of western blot showing overall S-nitrosylated protein in total protein extracts from WT and DN-VCP TG mice. C. A standard curve in the Griess assay was used for the accurate quantification of nitrite of unknown concentration. D. Quantification of nitrite concentration in the heart of WT and DN-VCP TG mice.*

3.8. Impaired VCP activity attenuated S-nitrosylation of VCP in the heart

Having characterized the overall protein S-nitrosylation in response to VCP function inhibition, specific protein S-nitrosylation in the heart of DN-VCP TG mice and their WT littermates was then analyzed on western blot following streptavidin purification and biotin switch assay. The results of the baseline group showed no significant difference in endogenous VCP expression in the heart between the DN-VCP TG group and the WT group **(Figure 9. A-D)**.

The exogenous VCP expression was observed as a distinct band located directly beneath the endogenous VCP bands, confirming the validity of the DN-VCP TG mouse model. In the test group, SNO-VCP expression was found to be significantly decreased in the heart of DN-VCP TG mice compared to the WT group **(Figure 9. A and E)**. This result suggests that functional inhibition of VCP activity could attenuate the S-nitrosylation level of VCP in the heart of DVCPTG mice. Interestingly, our data also revealed that there was no significant change regarding SNO-GAPDH expression between the DN-VCP TG group and the WT group **(Figure 9. A and F)**. One plausible explanation as to why SNO-GAPDH expression was not altered is that the endogenous expression of VCP was not compromised in this model, which may have prevented any significant impact on the iNOS pathway and the subsequent reduction in SNO-GAPDH expression. These results provide compelling evidence that compromised VCP ATPase activity exhibited in this unique functional-inactivation model could potentially impair overall protein S-nitrosylation and SNO-VCP expression in the heart of DN-VCP TG mice when compared to WT, thus suggesting a key role of the N-terminal region in mediating cardiac protein S-nitrosylation. Additionally, the unaltered expression levels of SNO-GAPDH in the heart of DN-VCP TG mice revealed intriguing insights regarding the critical role of the iNOS pathway as a direct mediator in regulating the S-nitrosylation level of GAPDH in the heart, thus emphasizing the intricate nature of the regulatory mechanisms governing protein S-nitrosylation.

Although functional inhibition of VCP activity by truncating the N-terminal region resulted in decreased overall S-nitrosylation and VCP S-nitrosylation, this model falls short of providing conclusive evidence to establish whether the specific cysteine residue identified in the N-terminal region was mechanistically responsible for achieving VCP-mediated S-

nitrosylation in the heart. To address this limitation, further investigations are warranted to examine the function of this specific cysteine residue. One possible approach would be to mutate the cysteine residue at this site to alanine using site-directed mutagenesis, and compare overall and specific protein S-nitrosylation in the heart of the resulting cysteine-to-alanine mutant with the control group[33]. This approach could better help determine whether the identified cysteine residue plays a direct role in VCP-mediated S-nitrosylation in the heart.

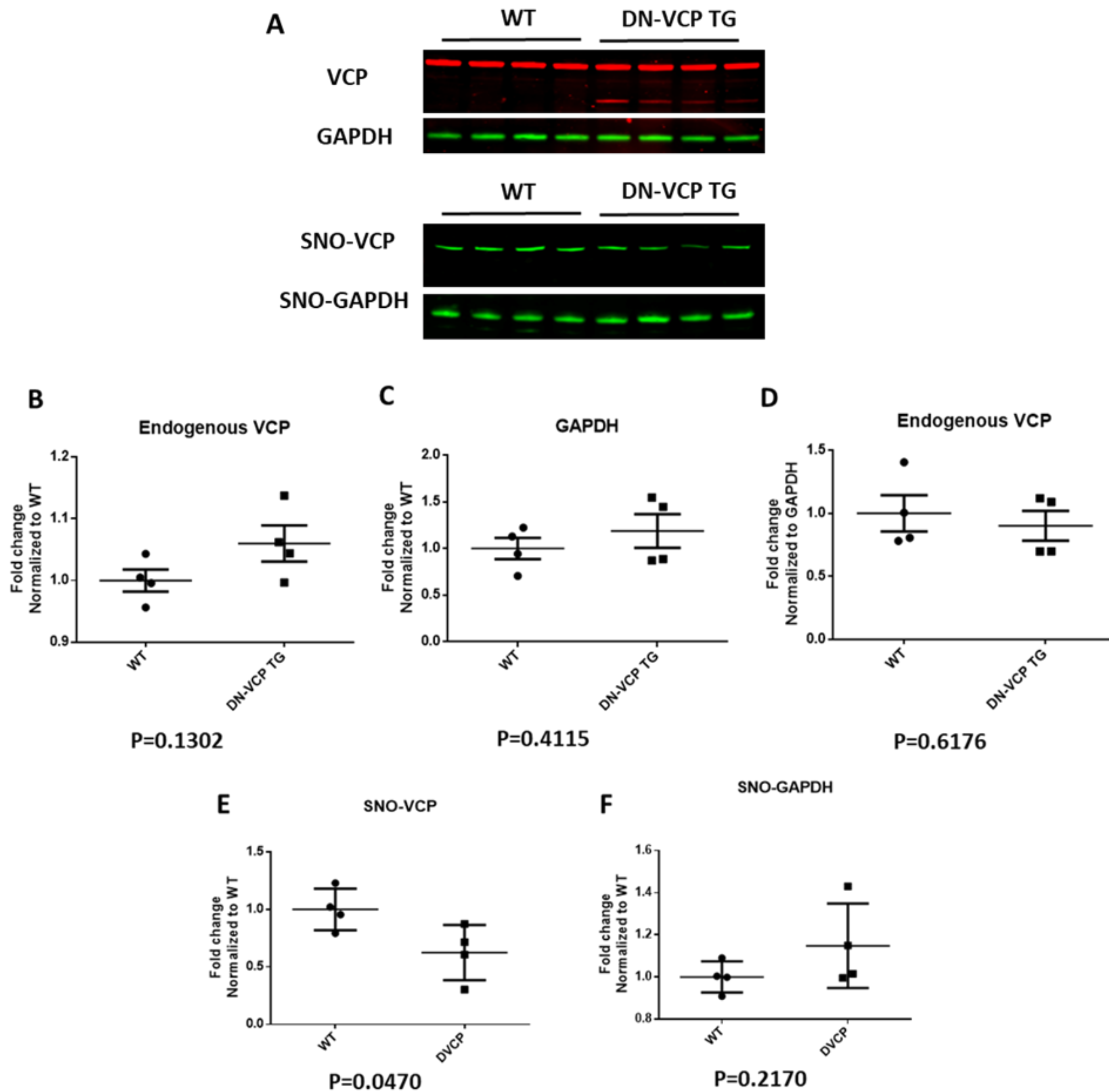

Figure 9. Functional inhibition of valosin-containing protein (VCP) ATPase activity reduced VCP S-nitrosylation in the heart. *A. Western blot analysis of untreated proteins at baseline showing the protein expression of endogenous and exogenous VCP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as of purified biotinylated proteins showing S-nitrosylated VCP and GAPDH expression, in the heart of cardiac-specific dominant-negative (DN) VCP transgenic (TG) and wild-type (WT) mice. B. Quantification of western blot showing fold change of endogenous VCP expression normalized to WT. C. Quantification of western blot showing fold change of GAPDH expression normalized to WT. D. Quantification of western blot showing fold change of endogenous VCP expression normalized to GAPDH. E. Quantification of western blot showing fold change of S-nitrosylated VCP (SNO-VCP) normalized to wild-type. F. Quantification of western blot showing fold change of S-nitrosylated GAPDH (SNO-GAPDH) expression normalized to WT.*

3.9. VCP deficiency resulted in increased overall protein S-nitrosylation in the heart

After examining the effects of cardiac-specific VCP overexpression and function inhibition on protein S-nitrosylation in the heart, a novel cardiac-specific VCP knockout mouse model was recruited to explore the potential impact of VCP deficiency on protein S-nitrosylation in the heart. First, overall SNO protein expression was measured in the heart of VCP KO and WT mice. The biotin switch assay showed that there was a significant increase in overall protein S-nitrosylation expression level in the heart of VCP KO mice **(Figure. 10 A and B)**. At a functional level, the comparison between the nitrite concentration in the heart of VCP KO mice and WT mice did not yield a statistically significant difference **(Figure. 10 C and D)**. However, there was an increasing trend in the nitrite concentration in the hearts of VCP KO mice compared to their WT counterparts. Therefore, it seems that VCP deficiency could potentially result in increased overall protein S-nitrosylation levels in the heart. However, the current body of evidence is insufficient to provide a definitive interpretation of the observed rise in general S-nitrosylation levels in the hearts of VCP knockout mice.

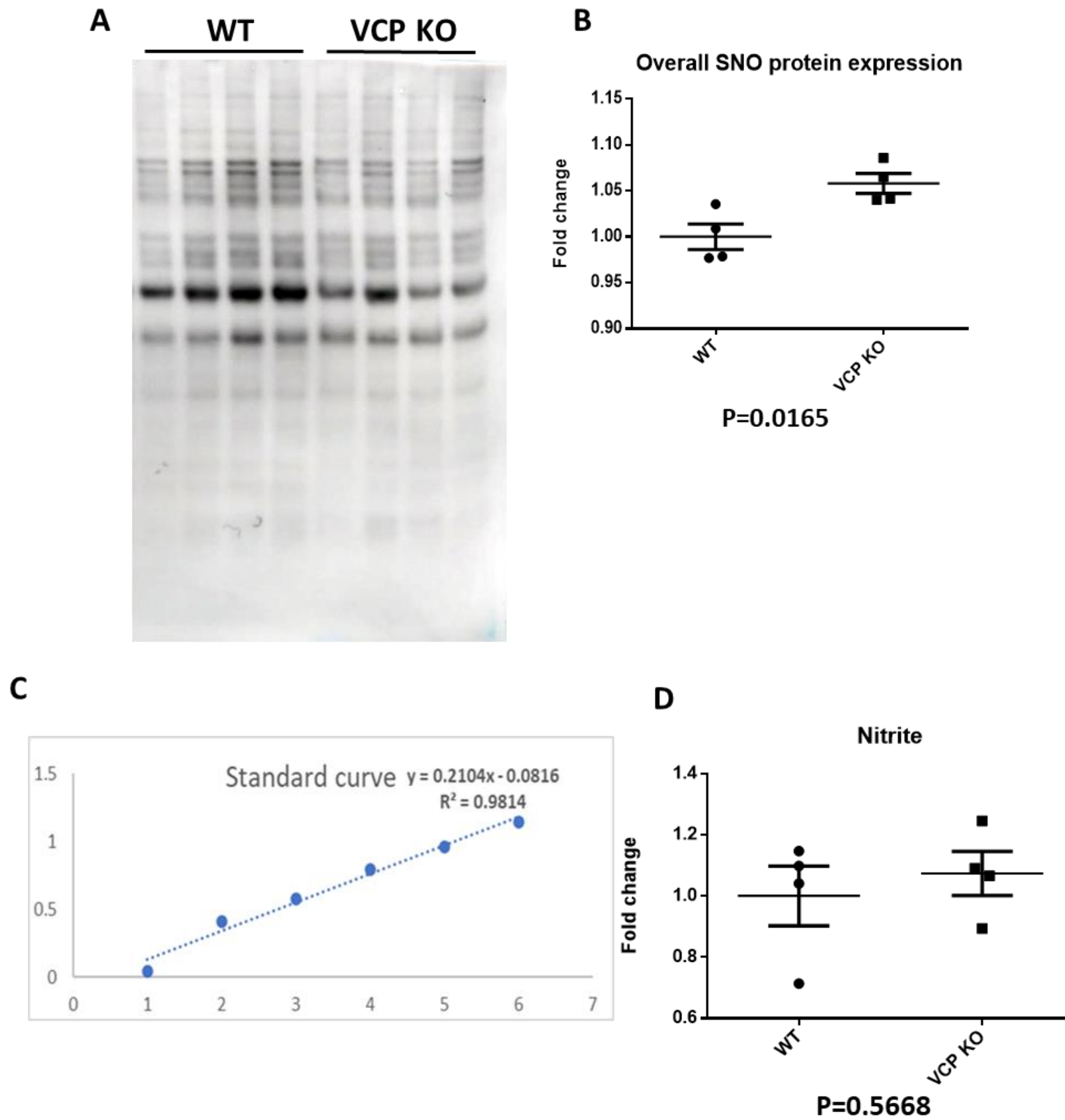

Figure 10. Valosin-containing protein (VCP) deficiency by genetic knockout (KO) led to increased overall protein S-nitrosylation in the heart. *A. Biotin switch assay analyzed by western blot showing overall S-nitrosylated protein expression in the heart of wild type (WT) and VCP KO mice. B. Quantification of western blot showing overall S-nitrosylated protein in total protein extracts from WT and VCP KO mice. C. A standard curve in the Griess assay was used for the accurate quantification of nitrite of unknown concentration. D. Quantification of nitrite concentration in the heart of WT and VCP KO mice.*

3.10. Deficiency in VCP did not result in a significant alteration in S-nitrosylation of VCP and GAPDH in the heart

After studying the overall S-nitrosylation, specific protein S-nitrosylation in the heart of VCP KO mice and their WT littermates was analyzed on western blot following streptavidin

purification and biotin switch assay. The results of the baseline group showed significantly decreased VCP expression in the heart of VCP KO mice compared to WT, confirming the validity of the VCP KO mouse model **(Figure. 11 A-D)**. In the test group, SNO-VCP expression was found to be significantly decreased in the heart of VCP KO mice compared to the WT group **(Figure 11. A and E)**. However, the fold change observed in VCP expression between the baseline group and the test group is not substantial enough to support the conclusion that VCP deficiency might lead to impaired expression of SNO-VCP. Additionally, there was no significant change in SNO-GAPDH expression between the VCP KO group and the WT group **(Figure 11. A and F).** To obtain a more thorough understanding of these results, further investigations and analyses are required in the future. This would allow for a more robust interpretation of the current data, potentially revealing underlying patterns or relationships that were not apparent in these initial findings.

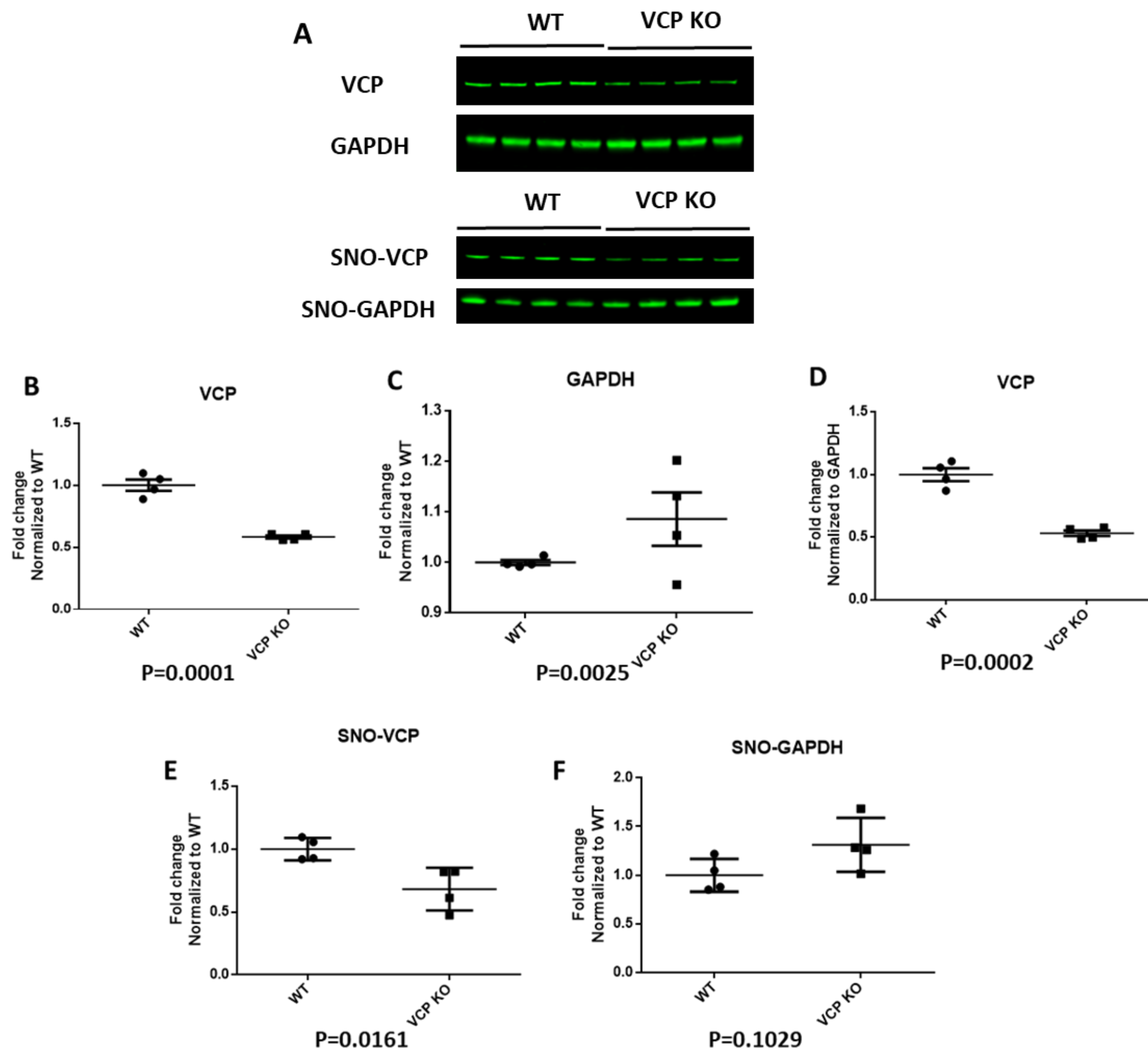

Figure 11. Valosin-containing protein (VCP) knockout (KO) did not lead to any significant changes in the S-nitrosylation of VCP or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. A. Western blot analysis of untreated proteins at baseline showing the protein expression of VCP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as of purified biotinylated proteins showing S-nitrosylated VCP and GAPDH expression, in the heart of cardiac-specific VCP KO and wild-type (WT) mice. B. Quantification of western blot showing fold change of VCP expression normalized to WT. C. Quantification of western blot showing fold change of GAPDH expression normalized to WT. D. Quantification of western blot showing fold change of GAPDH expression normalized to GAPDH. E. Quantification of western blot showing fold change of S-nitrosylated VCP (SNO-VCP) normalized to wild-type. F. Quantification of western blot showing fold change of S-nitrosylated GAPDH (SNO-GAPDH) expression normalized to WT.*

3.11. VCP overexpression did not result in a significant increase in S-nitrosylation of target proteins in mitochondrial respiratory complexes

Previous research has established the cardioprotective role of VCP through mediating mitochondrial respiration capacity and preventing mitochondrial mPTP opening. Additionally, our preliminary data demonstrated that among the most S-nitrosylated cardiac proteins identified, there were a great number of mitochondrial proteins, particularly those involved in mitochondrial respiratory complexes. Therefore, we first sought to investigate whether VCP could mediate protein S-nitrosylation of the oxidative phosphorylation system (OXPHOS) located at the mitochondrial inner membrane, which is composed of five enzymes known as mitochondrial respiratory complexes I through V. A cocktail of multiple OXPHOS antibodies was utilized for western blotting analysis of the relative levels of the 5 OXPHOS complexes in mitochondria from mice. Specifically, NDUF88 was used to probe for complex I (CI-NDUF88), SDHB for complex II (CII-SDHB), UQCRC2 for complex III (CIII-UQCRC2), MTCO1 for complex IV (CIV-MTCO1), and ATP5A for complex V (CV-ATP5A). In the baseline group, there were no significant differences in the expressions of CI-NDUF88, CIII-UQCRC2, CIV-MTCO1, and CV-ATP5A in the hearts of VCP TG and WT mice, regardless of whether they were normalized to WT or GAPDH **(Figure 12. A, B, D-G)**. The data for the former is not shown here. However, there was a significant decrease in CII-SDHB expression in the heart of VCP TG mice compared to WT mice **(Figure 12. C)**. In the biotin-treated group, there were no significant differences in the expression of S-nitrosylated CI-NDUF88 (SNO-CI-NDUF88), CII-SDHB (SNO-CII-SDHB), CIII-UQCRC2 (SNO-CIII-UQCRC2), CIV-MTCO1 (SNO-CIV-MTCO1), and CV-ATP5A (SNO-CV-ATP5A) in the heart between VCP TG and WT mice **(Figure. 12 H-L)**. Additionally, there was no significant

change in SNO-GAPDH expression in the heart between WT and VCP TG mice **(Figure. 12 M)**. Taken together, these results indicate that overexpression of VCP did not lead to a significant upregulation of S-nitrosylation in the probed proteins of mitochondrial respiratory complexes.

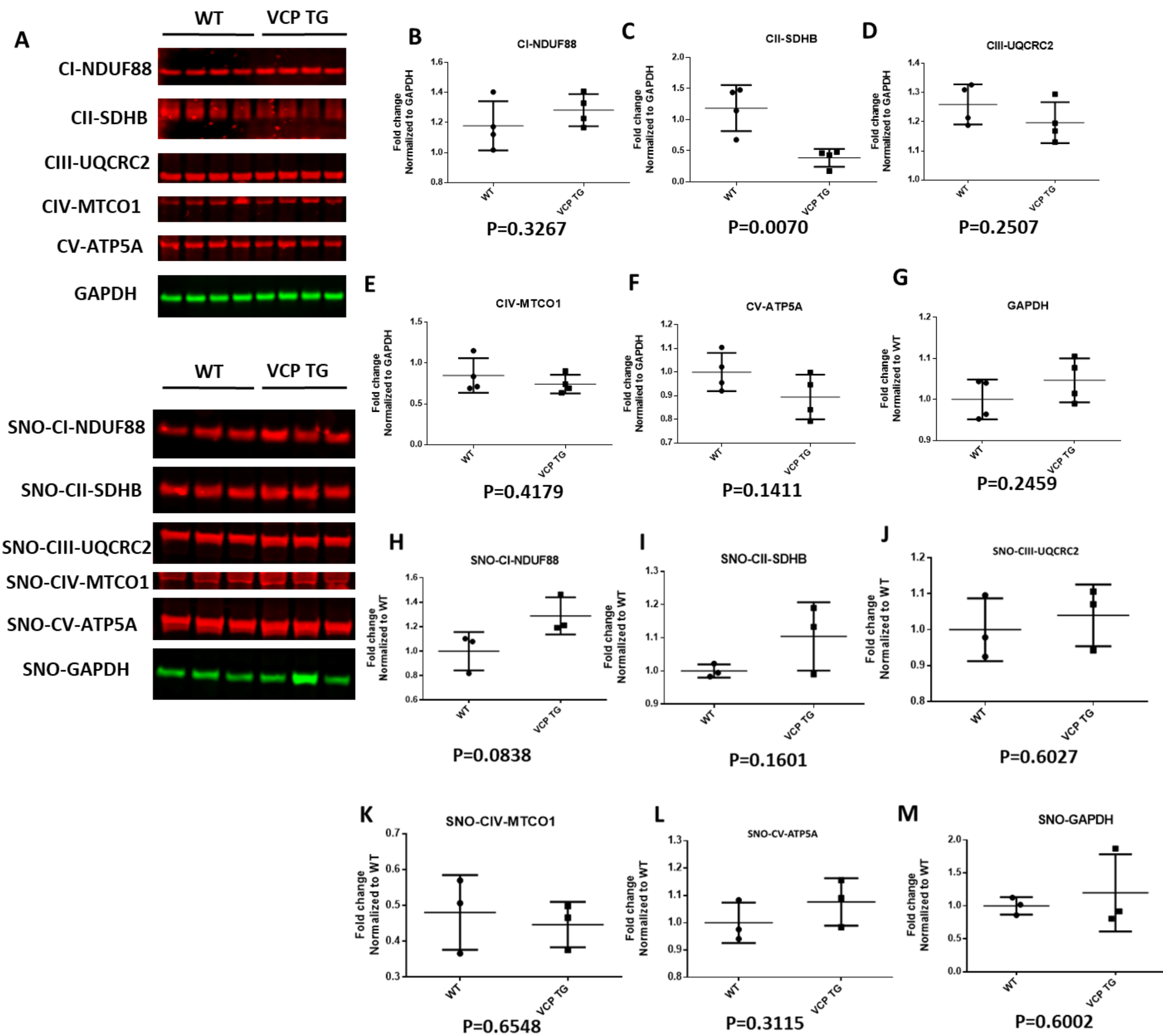

Figure 12. Overexpression of valosin-containing protein (VCP) did not lead to a significant increase in S-nitrosylation of proteins probed for mitochondrial respiration complexes. *A. Western blot analysis of untreated proteins at baseline showing the protein expression of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as of purified biotinylated proteins showing protein expression of S-nitrosylated CI-NDUF88 (SNO-CI-NDUF88), CII-SDHB (SNO-CII-SDHB), CIII-UQCRC2 (SNO-CIII-UQCRC2), CIV-MTCO1 (SNO-CIV-MTCO1), and CV-ATP5A (SNO-CV-ATP5A), and GAPDH (SNO-GAPDH) in the heart of VCP transgenic (TG) and wild-type (WT) mice. B-F. Quantification of western blot showing fold change of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A normalized to GAPDH. G. Quantification of western blot showing fold change of GAPDH normalized to WT. H-L. Quantification of western blot showing the fold change in the expression of SNO-CI-NDUF88, SNO-CII-SDHB, SNO-CIII-UQCRC2, SNO-CIV-MTCO1, and SNO-CV-ATP5A*

normalized to WT. M. Quantification of western blot showing the fold change in the expression of SNO-GAPDH normalized to WT.

3.12. iNOS deletion dramatically attenuated S-nitrosylation of target proteins in mitochondrial respiratory complexes

Based on the aforementioned findings and observations, it has been demonstrated that genetic iNOS deletion can effectively attenuate VCP overexpression-induced increase in S-nitrosylation of both VCP and GAPDH in the hearts of VCP TG mice. This crucial insight has led to an important question regarding the potential implications of iNOS deletion on the S-nitrosylation of mitochondrial proteins that have been probed for respiratory complexes. Given the central role that these proteins play in facilitating cellular respiration and energy production, it is essential to explore whether iNOS deletion may compromise the S-nitrosylation of these proteins and consequentially affect mitochondrial function in the heart. The purified biotinylated proteins extracted from the heart of WT, VCP TG, VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ mice were then subjected to western blot analysis and incubated with the OXPHOS antibody cocktails described above. There were no significant changes in baseline protein expression of CIII-UQCRC2, CIV-MTCO1, and CV-ATP5A in the heart across the four groups of mice **(Figure. 13 A and D-F)**. However, a significant increase in CI-NDUF88 expression was observed in the heart of WT/iNOS KO$^{-/-}$ mice when compared to WT, VCP TG, and VCP TG/iNOS KO$^{-/-}$ mice, respectively **(Figure. 13 A and B)**. Additionally, there was a significant decrease in CII-SDHB expression in the heart of VCP TG mice compared to WT, while a marked increase in CII-SDHB expression was observed in the heart of both VCP TG/iNOS KO$^{-/-}$ and WT/iNOS KO$^{-/-}$ when compared to VCP TG mice, respectively **(Figure. 13 A and C)**. The increased expression levels of both CI-NDUF88 and CII-SDHB in two of the iNOS KO groups of

mice warrant further investigation for a precise interpretation. In the biotin-treated groups, the expression of SNO-CI-NDUF88, SNO-CII-SDHB, SNO-CIII-UQCRC2, and SNO-CV-ATP5A in the hearts of both iNOS KO groups demonstrated a drastic decrease when compared to either VCP TG or WT mice **(Figure. 13 A, G, H, I, K)**. Particularly, SNO-CI-NDUF88 expression was almost nonexistent in the heart of WT/iNOS KO$^{-/-}$ mice and was also significantly decreased compared to VCP TG/iNOS KO$^{-/-}$ mice. SNO-CIV-MTCO1 expression was markedly reduced in the heart of WT/iNOS KO$^{-/-}$ when compared to WT, VCP TG, and VCP TG/iNOS KO$^{-/-}$ mice, respectively **(Figure. 13 A and J)**. Additionally, SNO-GAPDH expression was significantly decreased in the heart of both iNOS KO groups compared to WT **(Figure. 13 A and L)**. There was also a marked reduction in SNO-GAPDH expression in the heart of WT/iNOS KO$^{-/-}$ compared to VCP TG/iNOS KO$^{-/-}$ mice. These findings illustrated a progressive and substantial reduction in the S-nitrosylation levels of the five targeted mitochondrial proteins as well as SNO-GAPDH in the hearts of VCP TG, VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ mice. Taken together, these results provide strong and consistent evidence that genetic deletion of iNOS can significantly diminish VCP-mediated S-nitrosylation of proteins probed for the mitochondrial respiratory complexes.

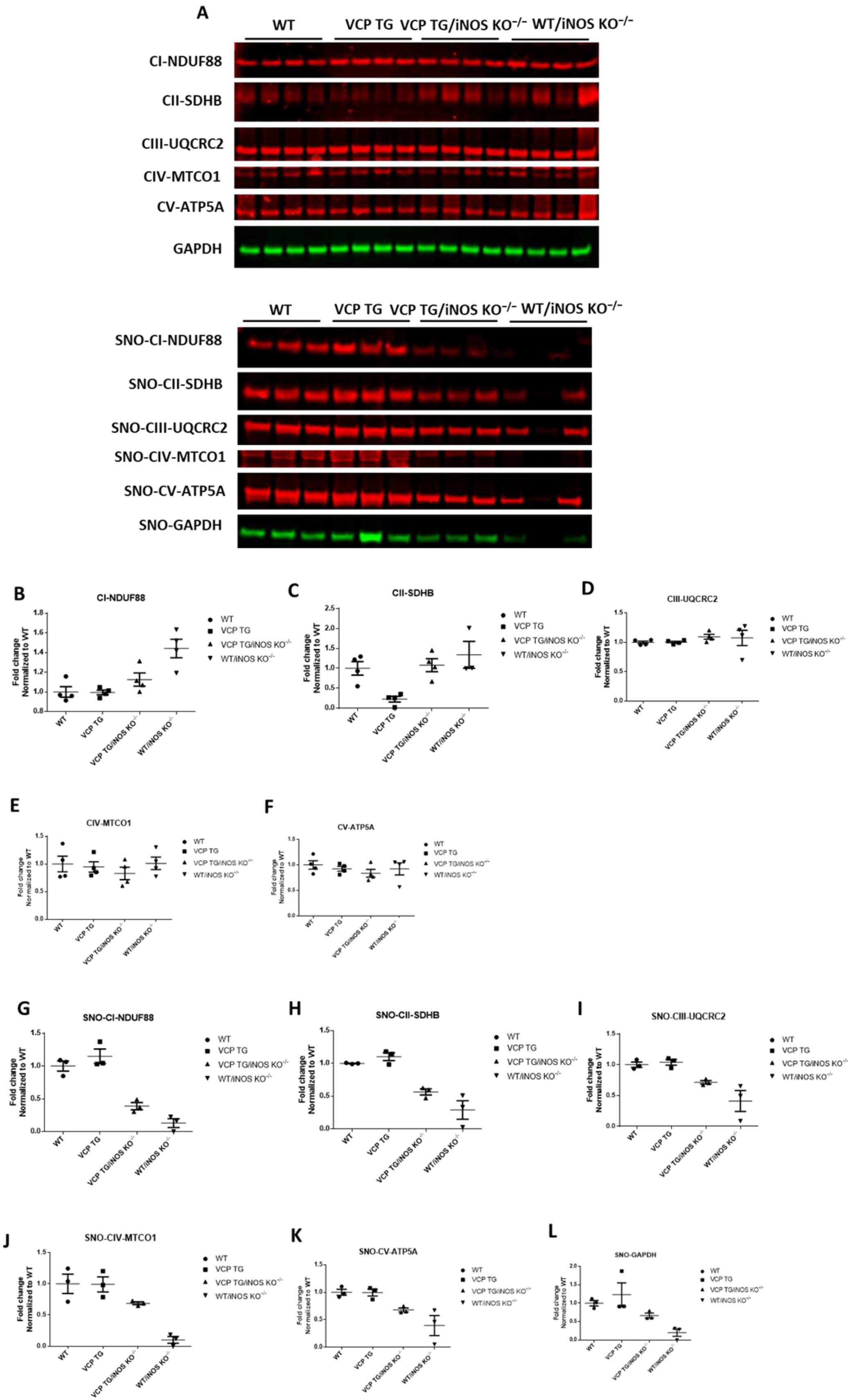
A
WT
VCP TG
VCP TG/iNOS KO-/-
WT/iNOS KO-/-
CI-NDUF88
CII-SDHB
CIII-UQCRC2
CIV-MTCO1
CV-ATP5A
GAPDH
SNO-CI-NDUF88
SNO-CII-SDHB
SNO-CIII-UQCRC2
SNO-CIV-MTCO1
SNO-CV-ATP5A
SNO-GAPDH
B
C
D
E
F
G
H
I
J
K
L
Fold change Normalized to WT

Figure 13. Genetic knockout (KO) of inducible nitric oxide synthase (iNOS) resulted in a substantial reduction in the S-nitrosylation of proteins probed for mitochondrial respiratory complexes. *A. Western blot analysis of untreated proteins at baseline showing the protein expression of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as of purified biotinylated proteins showing protein expression of S-nitrosylated CI-NDUF88 (SNO-CI-NDUF88), CII-SDHB (SNO-CII-SDHB), CIII-UQCRC2 (SNO-CIII-UQCRC2), CIV-MTCO1 (SNO-CIV-MTCO1), CV-ATP5A (SNO-CV-ATP5A), and GAPDH (SNO-GAPDH) in the heart of wild-type (WT), VCP transgenic (TG), VCP TG/iNOS KO$^{-/-}$, and WT/iNOS KO$^{-/-}$ mice. GAPDH used as a loading control in total protein extracts. B-F. Quantification of western blot showing fold change of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A normalized to WT. G-k. Quantification of western blot showing the fold change in the expression of SNO-CI-NDUF88, SNO-CII-SDHB, SNO-CIII-UQCRC2, SNO-CIV-MTCO1, and SNO-CV-ATP5A normalized to WT. L. Quantification of western blot showing expression of S-nitrosylated GAPDH (SNO-GAPDH) normalized to wild-type.*

3.13. Functional inhibition of VCP decreased S-nitrosylation of the target protein in mitochondrial respiration complex 1

Having conducted an in-depth analysis of the effects of VCP overexpression on the S-nitrosylation of target proteins in mitochondrial respiratory complexes in the heart, we endeavored to further advance our understanding by examining whether the functional inhibition of VCP activity could potentially alter the S-nitrosylation of the same target proteins within the mitochondrial respiratory complexes. The motivation behind this investigation was to comprehensively explore the multifaceted roles of VCP and its potential contributions to the regulation of protein S-nitrosylation in the mitochondria. In the baseline group, there were no significant differences in the expressions of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATP5A in the hearts of WT and DN-VCP TG mice **(Figure 14. A-F)**. In the biotin-treated group, SNO-CII-SDHB expression was significantly decreased in the heart of DN-VCP TG mice compared to WT. However, there were no significant differences in the expression of SNO-CII-SDHB, SNO-CIII-UQCRC2, SNO-CIV-MTCO1, and SNO-CV-ATP5A in the heart between WT and DN-VCP TG mice **(Figure. 14 I-L)**. These results suggest that functional inhibition of VCP could potentially attenuate protein S-nitrosylation of mitochondrial complex I.

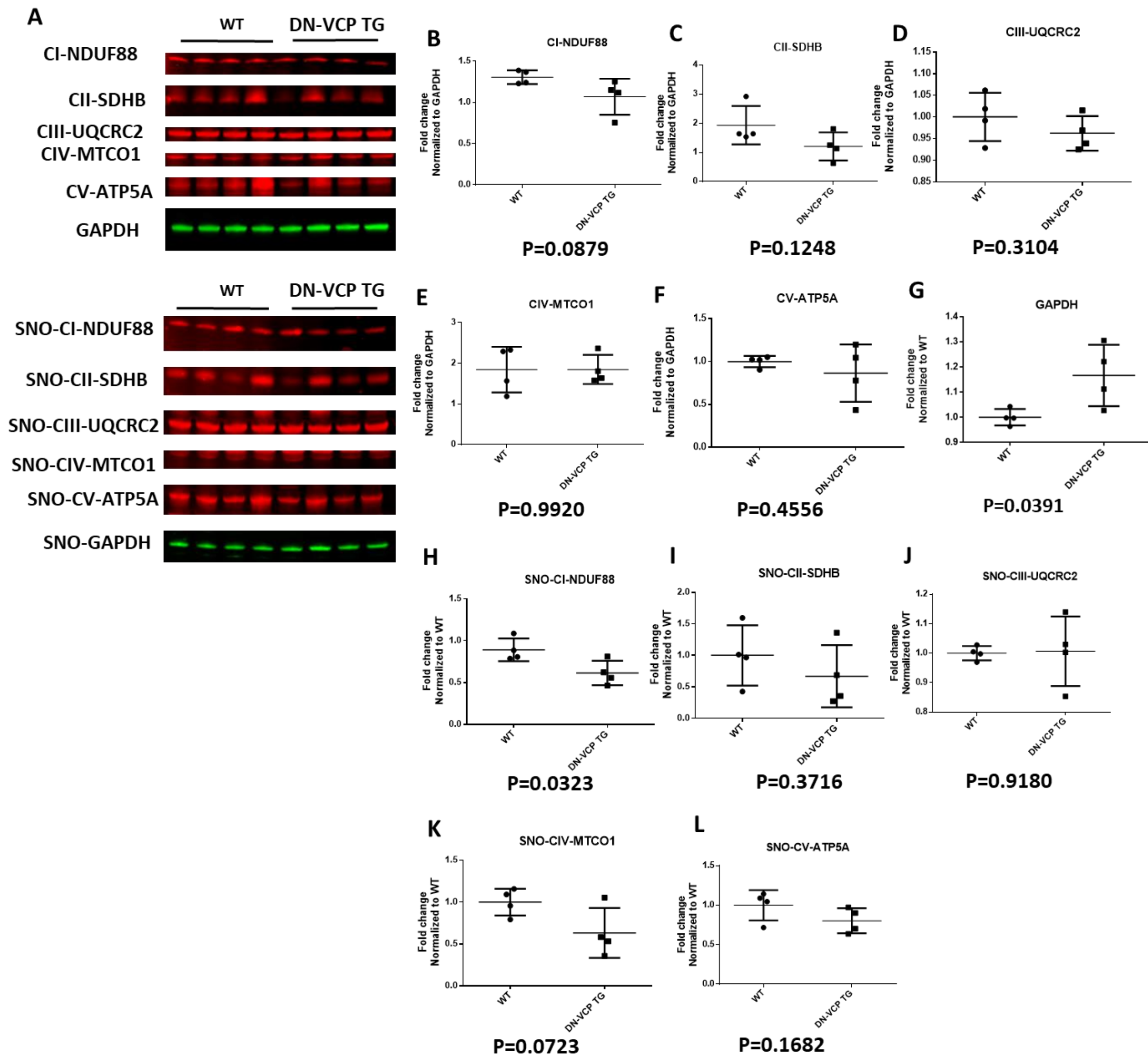

***Figure 14. Functional inhibition of valosin-containing protein (VCP) resulted in decreased S-nitrosylation of the target protein in mitochondrial respiratory complex 1**. A. Western blot analysis of untreated proteins at baseline showing the protein expression of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as of purified biotinylated proteins showing protein expression of S-nitrosylated CI-NDUF88 (SNO-CI-NDUF88), CII-SDHB (SNO-CII-SDHB), CIII-UQCRC2 (SNO-CIII-UQCRC2), CIV-MTCO1 (SNO-CIV-MTCO1), CV-ATP5A (SNO-CV-ATP5A), and GAPDH (SNO-GAPDH) in the heart of wild-type (WT), dominant-negative (DN) VCP transgenic (TG) mice. GAPDH used as a loading control in total protein extracts. B-F. Quantification of western blot showing fold change of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A normalized to GAPDH. G. Quantification of western blot showing fold change of GAPDH normalized to WT. H-L. Quantification of western blot showing the fold change in the expression of SNO-CI-NDUF88, SNO-CII-SDHB, SNO-CIII-UQCRC2, SNO-CIV-MTCO1, and SNO-CV-ATP5A normalized to WT.*

3.14. VCP deficiency did not induce a significant change in the S-nitrosylation of target proteins in mitochondrial respiratory complexes

Having observed that VCP overexpression did not lead to a significant increase in the S-nitrosylation of target proteins in mitochondrial respiratory complexes, we sought to further investigate the role of VCP in protein S-nitrosylation by examining the potential impact of VCP deficiency on the S-nitrosylation of these same target proteins within the mitochondrial respiratory complexes. This investigation will enable us to gain a deeper understanding of the complex interplay between VCP and protein S-nitrosylation within the mitochondria. In the baseline group, there were no significant differences in the expressions of CI-NDUF88 and CII-SDHB in the hearts of WT and VCP KO mice **(Figure 15. A-C)**. However, CIII-UQCRC2, CIV-MTCO1, and CV-ATP5A expression levels were significantly decreased in the heart of VCP KO mice compared to WT. In the biotin-treated group, there were no significant differences in the expression of SNO-CI-NDUF88 SNO-CII-SDHB, SNO-CIII-UQCRC2, SNO-CIV-MTCO1, and SNO-CV-ATP5A in the heart between WT and DN-VCP TG mice **(Figure. 15 H-L)**. Despite the collective results obtained from the study, a definitive conclusion regarding the impact of VCP deficiency on S-nitrosylation of target proteins in the mitochondrial complexes could not be drawn due to the lack of conclusive evidence. Consequently, further investigations are required to obtain a more comprehensive understanding of the underlying mechanisms and to accurately interpret the results obtained.

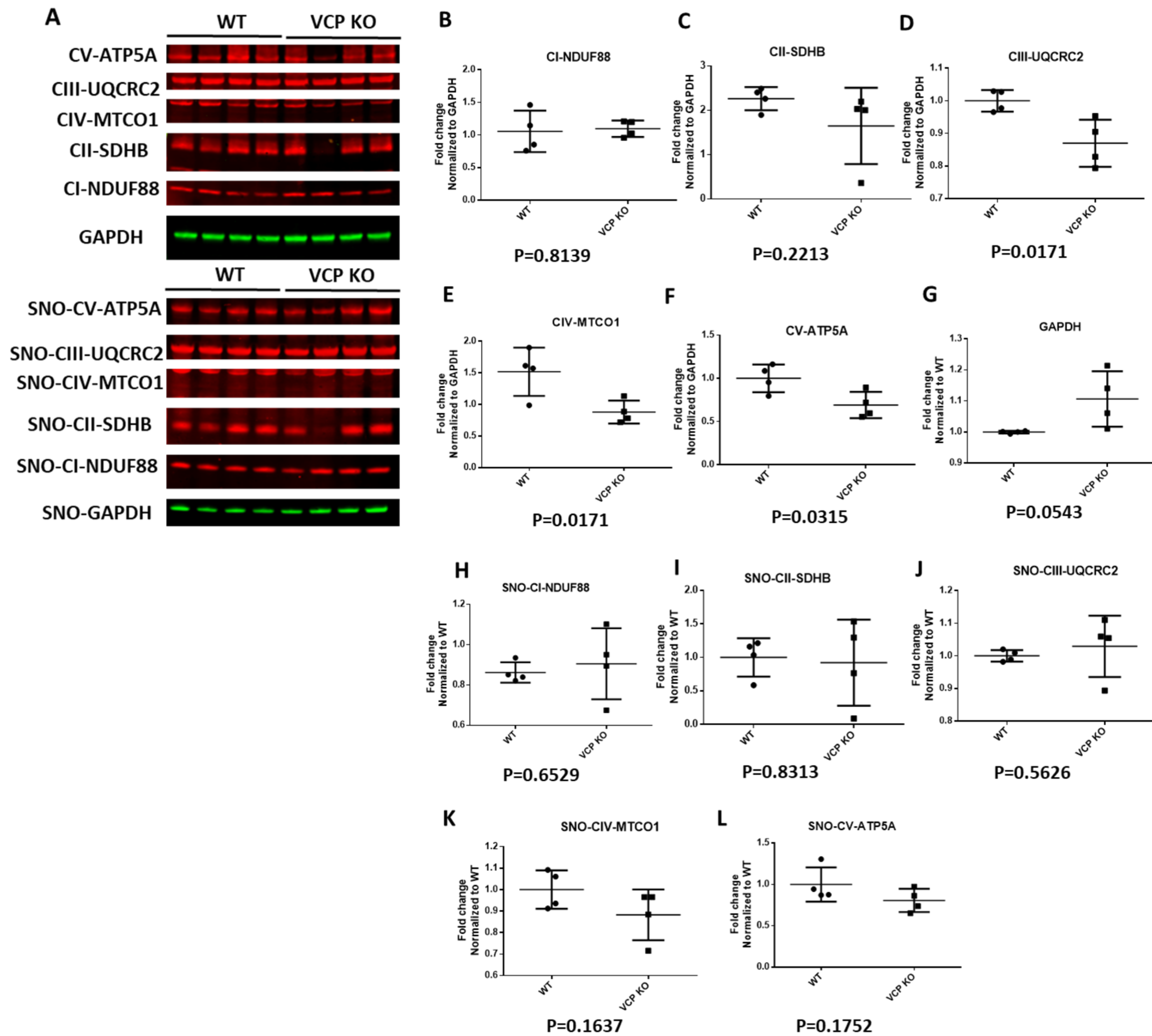

Figure 15. Genetic deletion of valosin-containing protein (VCP) did not result in a significant alteration in the S-nitrosylation of the targeted proteins in mitochondrial respiratory complexes. *A. Western blot analysis of untreated proteins at baseline showing the protein expression of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as of purified biotinylated proteins showing protein expression of S-nitrosylated CI-NDUF88 (SNO-CI-NDUF88), CII-SDHB (SNO-CII-SDHB), CIII-UQCRC2 (SNO-CIII-UQCRC2), CIV-MTCO1 (SNO-CIV-MTCO1), CV-ATP5A (SNO-CV-ATP5A), and GAPDH (SNO-GAPDH) in the heart of wild-type (WT),* and VCP knockout (VCP KO) *mice. GAPDH used as a loading control in total protein extracts. B-F. Quantification of western blot showing fold change of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, CV-ATP5A normalized to GAPDH. G. Quantification of western blot showing fold change of GAPDH normalized to WT. H-L. Quantification of western blot showing the fold change in the expression of SNO-CI-NDUF88, SNO-CII-SDHB, SNO-CIII-UQCRC2, SNO-CIV-MTCO1, and SNO-CV-ATP5A normalized to WT.*

CONCLUSION

This study employed four distinct transgenic mouse models to comprehensively investigate VCP-mediated S-nitrosylation in the heart, with a focus on examining overall protein S-nitrosylation and specific S-nitrosylation of VCP, GAPDH, and target proteins in the mitochondrial respiratory complexes. The findings revealed that VCP overexpression did not lead to a significant change in overall protein S-nitrosylation in the heart but resulted in increased S-nitrosylation of VCP and GAPDH in the heart. Additionally, iNOS deletion did not alter overall protein S-nitrosylation in the heart but attenuated VCP overexpression-induced increase in S-nitrosylation of VCP and GAPDH. Furthermore, iNOS deletion could substantially attenuate the S-nitrosylation level of target proteins probed for mitochondrial respiratory complexes. Functional inhibition of VCP activity led to decreased overall S-nitrosylation as well as reduced expression of SNO-VCP, and SNO-CI-NDUF88 in the heart while SNO-GAPDH expression remain unchanged. VCP deficiency resulted in increased overall protein S-nitrosylation in the heart but did not induce significant changes in SNO-VCP and SNO-GAPDH. Our investigation represents an important step towards characterizing the role of VCP in protein S-nitrosylation in the heart and has highlighted the crucial involvement of iNOS in VCP-mediated protein S-nitrosylation in this context. However, further investigations are necessary to advance our current understanding of the complex molecular mechanisms underlying this process. For instance, future studies could aim to confirm the role of VCP in mediating protein S-nitrosylation in cultured cardiomyocytes in vivo. Additionally, directed mutagenesis of specific cysteine residues within VCP could be employed to determine the precise sites of S-nitrosylation within the protein, providing valuable insights into the molecular mechanisms

involved in this process. Furthermore, investigating the potential effects of pathological stimuli, such as ischemia-reperfusion injury, on VCP-mediated protein S-nitrosylation in the heart could help to shed light on the functional consequences of these modifications in stressed heart, thus providing potential therapeutic targets for the treatment of heart diseases.

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VITAE

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EDUCATION

Georgia State University (Georgia, US) 2019 – 2023/05

Ph.D. Candidate in the Institute for Biomedical Sciences (IBMS)

Activities and achievements: Acquired a 3.92 GPA for my Ph.D. curriculum and have published four articles so far, including three first-author review papers and one second-author data paper. My research focuses on investigating the cardioprotective effects of valosin-containing protein (VCP) in the myocardium through S-nitrosylation. VCP belongs to the type II AAA protein family. VCP has only recently been associated with cardioprotective effects against the development of heart diseases such as myocardial infarction and heart failure, primarily through improving mitochondrial structural and functional integrity essential for mitochondrial respiration and cellular apoptosis. S-nitrosylation (SNO) is the covalent attachment of endogenous nitric oxide to the thiol side chain of cysteine to form an SNO. SNO proteins have been linked to cardioprotection against pathological stimuli in vivo and in vitro. My current project aims to investigate SNO-targeted mitochondrial proteins for their potential roles in VCP overexpression-induced cardioprotection.

Dalian Medical University (Dalian, China) 2017- 2019

Medical Doctor-General Surgery

Activities and achievements: Finished a two-year residency program in the Department of Gastrointestinal Surgery at the Second Affiliated Hospital of Dalian Medical University.

Received advanced training with laparoscopic abdominal procedures for colorectal and stomach cancer resection. Conducted preoperative and postoperative chemotherapy treatment for cancer patients with colorectal and stomach cancer. Passed the National Medical Licensing Examination and gained my doctor's license to practice medicine in China. Conducted and published clinical research regarding radiomics development in tumor diagnosis and histopathological features of colorectal cancer patients. Participated in basic research regarding the molecular mechanisms involved in the pathogenesis of colorectal cancer.

Dalian Medical University (Dalian, China) 2011-2017

Bachelor's Degree-Clinical Medicine

Activities and achievements: Completed all the required courses of the 5-year undergraduate program with satisfactory results and qualified for graduation with a major in Clinical Medicine.

Ohio State University (Ohio, US) 2015 -2016

International exchange student

Activities and achievements: Studied at the Dorothy M. Davis Heart and Lung Research Institute of the Ohio State University for 10 months through the Excellent Undergraduate International Exchange Program of the China Scholarship Council in 2015. Received a great amount of knowledge regarding conducting literature searches, improving literature reading and writing ability, preparing conference presentations, and assisting with basic experiment procedures.

SKILLS

- Extensive experience designing and conducting research studies, including developing research questions, selecting appropriate methods, collecting, and analyzing data, and interpreting findings.
- Strong writing and communication skills, with a track record of publishing research articles, presenting at conferences, and communicating research findings to diverse audiences.
- Excellent problem-solving and critical thinking skills, with a demonstrated ability to identify and address complex research questions and analyze data using advanced statistical methods.
- Proven project management skills, including the ability to prioritize tasks, manage timelines and work independently while meeting deadlines.
- Effective collaboration and teamwork skills, with experience working collaboratively on research projects with colleagues and peers from diverse academic backgrounds.
- Demonstrated ability to teach and mentor graduate and undergraduate students, including designing and delivering lectures, providing constructive feedback on assignments and papers, and mentoring students in research projects.
- Proficiency in a range of statistical analysis tools and software related to biomedical research.

CLINICAL & RESEARCH HIGHLIGHTS

Clinical work:

- A strong understanding of clinical terminology and procedures to properly interpret medical records and communicate with medical professionals.
- A strong understanding of colorectal cancer including its causes, risk factors, diagnosis, and treatment options.
- A comprehensive knowledge of chemotherapy drugs used in treating colorectal cancer, including their mechanisms of action, side effects, and potential drug interactions.
- Experience in surgical techniques for treating colorectal cancer, such as laparoscopic and open surgeries.
- Effective communication with patients and their families, as well as other healthcare professionals involved in the patient's care.
- Multidisciplinary teamwork that includes surgeons, medical oncologists, radiation oncologists, and other healthcare professionals.

Laboratory transgenic mice work:

- Breeding, weaning, maintenance, and genotyping.
- Echocardiogram under anesthesia
- Vessel dissection and organ retrieval
- Catheterization for arterial blood pressure monitoring
- Microscopic surgical procedures for coronary artery ligation and myocardial infarction

Laboratory assay development:

- Protein modification: protein S-nitrosylation detection with classic biotin-switch technique (BST) and modified BST with iodoacetyl tandem mass tag labeling
- S-nitrosylation purification with streptavidin pulldown assay
- Co-immunoprecipitation

PUBLICATIONS

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AWARDS AND HONORS

The Third Prize of Academic Scholarship of Dalian Medical University in 2019

The First Prize of the Academic Scholarship of Dalian Medical University in 2018

National Scholarship for overseas studies issued by the China Scholarship Council (CSC) in 2015

PROFESSIONAL MEMBERSHIP

American Heart Association (AHA)