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Nitrative Modification of Caveolin-3: A Novel Mechanism of Cardiac Insulin Resistance and a Potential Therapeutic Target against Ischemic Heart Failure in Prediabetic Animals

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Abstract

Background: Myocardial insulin resistance is a hallmark of diabetic cardiac injury. However, the underlying molecular mechanisms remain unclear. Recent studies demonstrate that the diabetic heart is resistant to other cardioprotective interventions, including adiponectin and preconditioning. The "universal" resistance to multiple therapeutic interventions suggests impairment of the requisite molecule(s) involved in broad pro-survival signaling cascades. Caveolin (Cav) is a scaffolding protein coordinating transmembrane signaling transduction. However, the role of Cav3 in diabetic impairment of cardiac protective signaling and diabetic ischemic heart failure (HF) is unknown.

Methods: WT and gene manipulated mice were fed a normal diet (ND) or high-fat diet (HFD) for 2–12 weeks and subjected to myocardial ischemia and reperfusion. Insulin cardioprotection was determined.

Results: Compared with the ND group, the cardioprotective effect of insulin was significantly blunted as early as four weeks of HFD feeding (pre-diabetes), a time point where expression levels

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Supplemental Materials
Expanded Methods
Figures S1 – 12
References 51–70

of insulin signaling molecules remained unchanged. However, Cav3/IRβ complex formation was significantly reduced. Among multiple post-translational modifications altering protein/protein interaction, Cav3 (not IRβ) tyrosine nitration is prominent in the prediabetic heart. Treatment of cardiomyocytes with SIN-1 reduced the signalsome complex and blocked insulin transmembrane signaling. Mass spectrometry identified Tyr^{73} as the Cav3 nitration site. Phenylalanine substitution of Tyr^{73} ($Cav3^{Y73F}$) abolished SIN-1 induced Cav3 nitration, restored Cav3/IRβ complex, and rescued insulin transmembrane signaling. Most importantly, AAV9-mediated cardiomyocyte-specific $Cav3^{Y73F}$ re-expression blocked HFD-induced Cav3 nitration, preserved Cav3 signalsome integrity, restored transmembrane signaling, and rescued insulin protective action against ischemic HF. Finally, diabetic nitrative modification of Cav3 at Tyr^{73} also reduced Cav3/AdipoR1 complex formation and blocked adiponectin cardioprotective signaling.

Conclusion: Nitration of Cav3 at Tyr^{73} and resultant signal complex dissociation results in cardiac insulin/adiponectin resistance in the prediabetic heart, contributing to ischemic HF progression. Early interventions preserving Cav3-centered signalsome integrity is an effective novel strategy against diabetic exacerbation of ischemic HF.

Keywords

Caveolin-3; Diabetes Mellitus; Ischemia-Reperfusion Injury; Post-Translational Modification

INTRODUCTION

Type 2 diabetes affects 23.6 million people in the United States, with annual prevalence rising alarmingly. Cardiovascular complications, particularly ischemic heart disease, comprise the primary cause of death in diabetic patients[1]. Diabetes causes coronary vascular injury and myocardial ischemia (MI). Additionally, diabetes renders cardiomyocytes more susceptible to ischemia/reperfusion injury, resulting in increased ischemic heart failure (HF) after a comparable initial ischemic insult[2, 3]. Tightened glycemic control does not prevent increased MI mortality in diabetic patients[4]. Novel strategies capable of protecting the diabetic heart against excessive ischemic HF are urgently needed.

As terminally differentiated cells, adult cardiomyocytes host an array of pro-survival signaling pathways that prevent cell death. The ultimate fate of cardiomyocytes after MI is thus determined by the balance between endogenous pro-survival signals and stress-activated pro-death signals. Many cardioprotective signaling pathways are pathologically suppressed, and pro-survival interventions are attenuated or lost in the diabetic heart[5–11]. Strong and consistent clinical observations and experimental studies demonstrate that the heart becomes insulin resistant in obesity and type 2 diabetes[5, 6]. Cardiac insulin resistance impairs cardiometabolism, increases oxidative stress, blocks cell survival signaling, and reduces cardiac function[12]. However, molecular mechanisms underlying diabetic cardiac insulin resistance remains incompletely understood, and effective therapeutic intervention is currently unavailable. Moreover, we recently demonstrated that the cardioprotective action of adiponectin (APN), an adipokine with strong anti-diabetic and cardioprotective actions, is blunted in the diabetic heart[7]. Strikingly, ischemic preconditioning (the most potent cardioprotective intervention in the nondiabetic heart that activates multiple cell survival pathways) response is also significantly attenuated in type 2 diabetic heart[8–11]. The

"universal" resistance to multiple cardioprotective interventions in the diabetic heart strongly suggests impairment of the requisite molecule(s) involved in broad pro-survival signaling cascades.

Caveolae are small flask-like invaginations of the plasma membrane enriched in select lipids and structural proteins, caveolins (Cav). Cav interacts with multiple intracellular signaling molecules[13–15]. Cav-centered signaling complexes (signalsomes) are critical in facilitating rapid, precise, and coordinated signal transduction involved in cell protection and survival[14–17]. Cav3 (the primary caveolin subtype in cardiomyocytes) is an essential molecule in multiple cardioprotective signaling, including insulin and preconditioning[14, 18, 19]. Finally, we recently demonstrated that Cav3 directly interacts with adiponectin receptor 1 (AdipoR1, the adiponectin receptor subtype in cardiomyocytes) and forms a signaling complex, mediating APN transmembrane signaling[20]. However, whether and how diabetes may impair the integrity of the Cav3-centered signalsome, causing "universal" impairment of cardioprotective signaling, has not been previously investigated.

Therefore, the aims of the present study are to 1) determine whether obesity/diabetes may impair Cav3-centered signalosome integrity, contributing to cardiac insulin resistance; 2) clarify molecular mechanisms impairing Cav3 signalsome-mediated transmembrane protective signaling; and 3) investigate whether preserving Cav3 signalsome integrity may restore cardioprotective signaling, protecting against diabetic exacerbation of ischemia HF.

METHODS

Detailed methods for cell isolation and culture, plasmids constructions and transfections, adeno-associated virus 9 vector production and infection, cell viability and apoptosis, Western and co-immunoprecipitation, echocardiography and strain analysis, immunofluorescent cellular and Masson's trichrome staining, and quantitative PCR methods are provided in online supplementation. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animal study protocol

All animal experiments were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care. Wild-type (WT) and Caveolin-3 knockout (Cav3KO) mice were utilized in the study.

To generate a cardiomyocyte-specific Cav3 mutation mouse line, a previously reported neonatal mouse AAV administration method[21] was utilized. In brief, 1×10^{11} viral genome particles/mouse of AAV9-cTNT-eGFP, AAV9-cTNT-Cav3^{WT,} or AAV9-cTNT-Cav3^{Y73F} were injected subcutaneously in the nape of 5–7 day-old Cav3KO neonatal mice. Cav3KO mice injected with AAV9-cTNT-eGFP exhibited cardiac-specific eGFP expression for at least 20 weeks.

Adult (8 weeks old) male WT C57BL/6J mice, Cav3KO mice re-expressing Cav3^{WT} (Cav3KO^{Cav3WT}) or Cav3KO mice re-expressing Cav3^{Y73F} (Cav3KO^{Cav3Y73F}) mice were

fed a high-fat diet (HFD, 60% kcal fat, 20% kcal protein, 20% kcal carbohydrate, Cat #D12492; Research Diets) for 4 weeks (obesity) or 12 weeks (diabetes), as previously reported[22]. The age-matched C57BL/6J nondiabetic mice were fed a standard normal diet (ND) and served as control.

To induce MI/R injury, mice were anesthetized with 2% isoflurane. The heart was temporarily exteriorized via a left thoracic incision. A 6–0 silk suture slipknot was tied around the left anterior descending (LAD) coronary artery as previously described[23]. 50 minutes after MI, mice were randomized to receive either PBS (vehicle) or insulin (0.5 U/kg) (intraperitoneal injection). 60 minutes after MI, the slipknot was released, allowing myocardial reperfusion for 3 hours (for apoptosis assays) or 24 hours (for infarct size assays). To evaluate sustained cardioprotective action, another group of animals was subjected to 60 minutes MI followed by 4 weeks of reperfusion. 10 minutes before reperfusion, an insulin bolus was administered. One day after reperfusion, an ALZET osmotic pump was subcutaneously implanted. PBS (vehicle) or insulin was continuously administered (0.5 U/kg/day) during the 4-week reperfusion period.

Mass spectrometry

Cardiac tissue from the left ventricular free wall of normal mice or prediabetic mice (4 weeks post-HFD) was removed and homogenized. Samples were immunoprecipitated with antibodies against Cav3 and subjected to electrophoresis separation. Mass spectrometry was performed as previously reported[24].

Statistical analysis

All numerical data are presented as mean ± SEM. The Shapiro-Wilk test was used to determine the normality of data. Comparisons between two groups were performed by Student’s t test. Comparison of three or more groups was completed by one-way or two-way ANOVA followed by Tukey’s multiple comparison test. All statistical analyses were performed by GraphPad Prism 9.4.1. P values <0.05 were considered significant.

RESULTS

Blunted cardioprotective response preceded HFD-induced diabetes via mechanisms other than signaling molecule downregulation

To investigate the full spectrum of obesity/diabetes impairment of cardioprotective signaling and the underlying mechanisms, time course experiments were performed. Adult mice were fed either ND or HFD diet for 2–12 weeks and subjected to 60 minutes of coronary occlusion followed by 24 hours of reperfusion (acute MI/R). Animals were randomized to receive vehicle or insulin 10 minutes before reperfusion. Consistent with previous findings, insulin administration significantly attenuated acute MI/R injury in ND mice, an effect lost in diabetic mice (12 weeks of HFD). Interestingly, the infarct sparing and functional improvement actions of insulin were significantly blunted in animals as early as after 4 weeks of HFD (−47.2% vs. −24.4% for infarct sparing; +32.2% vs. +25.1% for function improvement. Figures 1A/B), demonstrating that cardiac insulin resistance occurred long before type 2 diabetes development. To obtain direct evidence at a cellular level, left

ventricular cardiomyocytes were isolated from ND, prediabetic (4 weeks HFD), or diabetic hearts (12 weeks HFD). Cells were treated with insulin for 30 minutes. In cardiomyocytes from ND animals, insulin activated Akt and stimulated Glut4 membrane translocation. However, the response to insulin treatment was significantly blunted in prediabetic animal cardiomyocytes (Figure 1C) and abolished in the diabetic heart (Figure S1A).

The loss of cardioprotection mediated by insulin in the diabetic heart has been largely attributed to reduced expression levels of signaling molecules[11]. To determine whether similar mechanisms are responsible for the impairment of cardioprotective signaling during pre-diabetes, key molecules involved in insulin signaling were determined. Expression of insulin receptor-β (IRβ), insulin receptor substrate-1 (IRS-1), and Cav3 was significantly reduced in the diabetic heart (12 weeks HFD, Figures 1D/E). However, their expression levels remained unchanged in the prediabetic heart (4 weeks HFD, Figures 1D/E), despite significantly blunted insulin cardioprotective signaling. IRS-1 phosphorylation is a recognized mechanism of insulin resistance in diabetes. Consistent with the previous report, IRS-1 was phosphorylated in the diabetic heart (HFD 12 weeks). However, no IRS-1 phosphorylation was detected in the prediabetic heart (HFD 4 weeks) (Figure S1B). Taken together, these results demonstrated that short-period HFD (pre-diabetes) significantly impaired cardioprotective insulin signaling via mechanisms outside of signaling molecule downregulation or IRS-1 phosphorylation.

Cav3-centered signalsomes were dissociated in the prediabetic heart due to Cav3 nitrative modification, blocking transmembrane signaling

We and others previously demonstrated that direct interaction between Cav3 and the intracellular signaling molecules (including IRβ and AdipoR1) is necessary for transmembrane signaling[20]. To determine whether pre-diabetes may impair Cav3 signalsome integrity (thus blocking transmembrane signaling), the following experiments were performed. First, cardiac samples from ND or HFD (4 weeks) were immunoprecipitated with an antibody against IRβ and immunoblotted with an antibody against Cav3. IRβ and Cav3 expression remained unchanged at this time point (Figure 2A input). However, Cav3/IRβ complex was significantly reduced in the prediabetic heart (Figure 2A). Similarly, AdipoR1 expression remains unchanged 4 weeks after HFD (Figure S2A). However, Cav3/AdipoR1 complex was also significantly reduced in the prediabetic heart (Figure S2B), indicating that pre-diabetes broadly impairs Cav3-centered signaling complex formation.

Second, the mechanisms responsible for signaling complex dissociation were determined. Post-translational modification of proteins regulates their ability to interact with other molecules[25].

Since Cav3 is the central hub of cardioprotective signaling complexes, and Cav3/IRβ and Cav3/AdipoR1 associations are both significantly reduced in the prediabetic heart, we reasoned modifications to Cav3 are likely responsible for its dissociation from the signaling complex at this early time point. Currently known post-translational modifications altering protein structure were screened computationally or experimentally. Phosphorylation and palmitoylation have been identified in Cav1 but not in Cav3[26, 27]. A ubiquitination site is present in Cav3[28]. However, Cav3 protein expression levels were normal at 4 weeks

post-HFD. Phosphorylation, palmitoylation, and ubiquitination were therefore not further investigated. Neither acetylation nor sumoylation of Cav3 was detected 4 weeks after HFD (prediabetic model). Oxidative stress is the most recognized pathological alteration in the obesity/diabetes heart, and Cav3 oxidation has been previously reported[29], we next determined the effect of H_2O_2 upon the Cav3 signalsome in adult cardiomyocytes from ND mice. Surprisingly, H_2O_2 (at a concentration causing significant cell injury) had no effect on Cav3/IRβ signaling complex formation (Figure S2C).

Nitrative stress is a recently recognized pathological alteration in the diseased heart. Two forms of nitrative stresses exist. These include nitric oxide (NO) induced S-nitrosylation and peroxynitrite ($ONOO^-$, the reaction product between superoxide and NO) induced tyrosine nitration. SIN-1 is a unique molecule producing equal moles of superoxide and NO, serving as a peroxynitrite donor in the absence of superoxide dismutase (SOD) but a NO donor in the presence of excessive SOD[30]. Treating cardiomyocytes with a pathologically relevant concentration of SIN-1 (100 μM) resulted in multiple protein nitration, with a strong band at a molecular weight of 18 kDa. The addition of SOD significantly reduced SIN-1-induced protein nitration (Figure S3A). Moreover, SIN-1 virtually abolished Cav3 interaction with IRβ (Figure 2B). SOD addition rescued their interaction, suggesting $ONOO^-$ (not NO) is the responsible molecule for Cav3 signalsome dissociation. Consistently, treatment with SIN-1 virtually abolished insulin-induced Akt activation and Glut4 membrane translocation, an effect rescued by SOD co-treatment (Figure 2C).

Third, to obtain in vivo evidence that protein nitration plays a signifcnat pathologic role in prediabetic hearts, three experiments were performed. First, cardiomyocytes were isolated from ND or prediabetic hearts. Samples were immunoblotted with an antibody against 3-nitrotyrosine. 4 weeks of HFD significantly increased cardiomyocyte protein nitration (Figure S3B). Second, cardiac samples from ND or prediabetic hearts were immunoprecipitated with antibodies against Cav3, IRβ, or AdipoR1 and immunoblotted with antibodies against 3-nitrotyrosine. 4-weeks of HFD resulted in significant Cav3 nitration (Figure 2D). However, neither IRβ (Figure 2E) nor AdipoR1 (Figure S3C) nitration was detected at this time point. Finally, Cav3 (not IRβ) was nitrated in cardiac tissue from db/db mice (Figures S3D–3E), and Cav3/IRβ interaction was significantly blunted (Figure S3F). Collectively, these results demonstrated that HFD-induced peroxynitrite production and resultant Cav3 nitration are responsible for Cav3 signalsome dissociation, blocking transmembrane cardioprotective signaling.

Identifying $Cav3^{Y73}$ as the responsible site for nitrative dissociation of Cav3 signalsomes

To obtain direct evidence that Cav3 is nitrated in the diabetic heart and, more importantly, to identify specific tyrosine residue(s) nitratively modified, mass spectrometry was performed as previously reported[24]. Cardiac tissue from the left ventricular free wall of ND or prediabetic mice was removed and homogenized. Samples were immunoprecipitated with an antibody against Cav3, subjected to electrophoretic separation, and performed MS analysis. In ND heart samples, no tyrosine nitration was detected. However, in HFD heart samples, Tyr^{73} was nitratively modified (Figure 3A).

Next, we determined the biological significance of Tyr^{73} nitrative modification by replacing Tyr^{73} with phenylalanine ($Cav3^{Y73F}$). Neonatal cardiomyocytes were isolated from $Cav3^{-/-}$ and infected with adenovirus expressing either $Cav3^{WT}$ or $Cav3^{Y73F}$. Similar to that observed in WT cardiomyocytes, SIN-1 induced significant Cav3 nitration (Figure 3B) and reduced Cav3/IRβ complex formation (Figure 3C) in $Cav3^{-/-}$ neonatal cardiomyocytes re-expressing $Cav3^{WT}$. However, in $Cav3^{-/-}$ neonatal cardiomyocytes re-expressing $Cav3^{Y73F}$, SIN-1 did not induce significant Cav3 nitration (Figure 3B). The Cav3/IRβ signalsome was preserved (Figure 3C). To determine whether re-expressing a nitration resistant Cav3 may preserve transmembrane signaling, cardiomyocytes were cultured with normal or high glucose/high lipid (HG/HL) medium for 48 hours. In $Cav3^{-/-}$ cardiomyocytes re-expressing $Cav3^{WT}$, HG/HL significantly attenuated Akt activation (Figure 3D) and Glut4 membrane translocation (Figures 3E) in response to insulin treatment. However, insulin transmembrane signaling was preserved in $Cav3^{Y73F}$ cardiomyocytes (Figures 3D/E).

To determine whether nitrative modification of Cav3 at Tyr^{73} is also responsible for diabetic impairment of other cardioprotective signalings, the effect of SIN-1 treatment on Cav3/AdipoR1 complex formation and APN signaling was determined. SIN-1 virtually abolished Cav3/AdipoR1 complex formation and blocked APN-induced AMPK, Akt, and ERK1/2 activation in isolated cardiomyocytes. These pathologic effects were reversed by the addition of excessive SOD (Figures 4A/B). More importantly, re-expressing $Cav3^{Y73F}$ in $Cav3^{-/-}$ cardiomyocytes blocked SIN-1 induced Cav3/AdipoR1 dissociation (Figure 4C), and preserved APN transmembrane signaling in HG/HL cultured cardiomyocytes (Figures 4D/E and Figure S4). Collectively, we identified Tyr^{73} as the responsible site for Cav3 nitrative dissociation from its signaling partners, blocking transmembrane signaling.

Cardiomyocyte $Cav3^{Y73F}$ re-expression restored cardioprotective signaling in prediabetic heart, attenuating post-MI remodeling and ischemic HF

To obtain in vivo evidence that $Cav3^{Y73}$ is responsible for HFD-induced nitrative modification, Cav3 signalsome dissociation, and transmembrane signaling blockade, a cardiomyocyte-specific $Cav3^{Y73F}$ re-expressing mouse line was established (Figures S5). Animals were fed HFD for 4 weeks, and left ventricular tissue samples were prepared. Same as that observed in WT mice, HFD caused significant Cav3 nitration (Figure 5A) and Cav3 signalsome disassembly (Figures 5B) in $Cav3^{-/-}$ mice re-expressing $Cav3^{WT}$ ($Cav3KO^{Cav3WT}$). In contrast, HFD-induced cardiac Cav3 nitration was blocked (Figure 5A), and signalsomes (Cav3/IRβ and Cav3/AdipoR1) were preserved in $Cav3^{-/-}$ mice re-expressing $Cav3^{Y73F}$ ($Cav3KO^{Cav3Y73F}$, Figure 5B and Figure S6). More importantly, in cardiomyocytes isolated from HFD-fed $Cav3KO^{Cav3Y73F}$ mice, transmembrane protective signaling was significantly enhanced. Compared to $Cav3KO^{Cav3WT}$ cardiomyocytes, in vitro insulin treatment caused greater Akt activation (Figure 5C) and Glut4 membrane translocation (Figure 5D) in $Cav3KO^{Cav3Y73F}$ cardiomyocytes.

Next, we determined whether re-expressing a nitration-resistant Cav3 may enhance insulin cardioprotection in vivo. HFD-fed (4 weeks) $Cav3KO^{Cav3WT}$ or $Cav3KO^{Cav3Y73F}$ mice were subjected to 60 minutes MI and treated with either vehicle or insulin 10 minutes before reperfusion. The effect of insulin upon apoptosis (3 hours after reperfusion,

infarction border zone), infarct size, and cardiac function (24 hours after reperfusion) were determined. The anti-apoptotic effect of insulin (determined by caspase-3 cleavage and TUNEL staining, Figures 6A/B) was significantly increased in Cav3KO^{Cav3Y73F} mice compared to Cav3KO^{Cav3WT} mice. During ischemia/reperfusion, cardiac apoptosis is primarily mediated by the mitochondrial pathway[31]. In a consistent fashion, re-expressing Cav3^{Y73F} significantly increased insulin's inhibitory effect upon mitochondrial apoptotic markers, including suppressed caspase-9 cleavage, decreased Bax expression, and increased Bcl2 upregulation (Figure 6A). Consistently, insulin reduced infarct size to a greater extent in Cav3KO^{Cav3Y73F} mice compared to Cav3KO^{Cav3WT} mice (Figure 6C). Finally, re-expressing a nitration-resistant Cav3 significantly increased insulin-mediated cardiac function improvement, evidenced by increased LVEF, fractional shortening (Figures 7A/B), radial strain, and longitudinal strain (Figures 7C/D) in Cav3KO^{Cav3Y73F} mice compared to Cav3KO^{Cav3WT} mice. APN treatment decreased infarct size to a greater extent in Cav3KO^{Cav3Y73F} mice compared to Cav3KO^{Cav3WT} mice (Figure S7).

In a final attempt to determine whether blocking Cav3^{Y73} nitrative modification will exert a persistent cardioprotective effect against post-MI remodeling and HF progression, a chronic MI/R model was utilized. Cav3KO^{Cav3WT} and Cav3KO^{Cav3Y73F} mice were fed HFD for 4 weeks and subjected to 60 minutes of coronary occlusion. An insulin bolus was administered 10 minutes before reperfusion, followed by continuous osmotic pump peritoneal administration. 4 weeks after reperfusion (8 weeks after HFD), the following experiments were executed. First, Cav3 nitration and its interaction with IRβ and AdipoR1 were analyzed. Nitrative modification of Cav3 was detected in Cav3KO^{Cav3WT} heart but not in Cav3KO^{Cav3Y73F} heart (Figure S8A). Consistently, Cav3/IRβ and Cav3/AdipoR1 complexes were detected in Cav3KO^{Cav3Y73F} heart but not in Cav3KO^{Cav3WT} hearts (Figures S8B/C). Second, Akt and ERK phosphorylation and Glut4 membrane translocation were assessed. In insulin-treated mice, Akt and ERK activation and Glut4 membrane translocation were significantly increased in Cav3KO^{Cav3Y73F}. However, this response was absent in Cav3KO^{Cav3WT} (Figures S9A/B/C). Third, cardiac function was determined by echocardiography. Surprisingly, insulin administration failed to improve systolic function in Cav3KO^{Cav3WT} mice subjected to chronic MI/R (Figure 8). In contrast, insulin administration in Cav3KO^{Cav3Y73F} mice significantly improved systolic cardiac function, evidenced by enhanced LVEF and FS (Figures 8A/B, Figure S10A), and increased radial strain and longitudinal strain (Figures 8C–F, Figure S10B). Similarly, insulin treatment significantly reduced the ratio of heart/body weight (Figure 8G), and lung/body weight (Figure 8H), and decreased cardiac fibrosis in Cav3KO^{Cav3Y73F} mice (Figures 8I/J). These anti-remodeling effects were not observed in Cav3KO^{Cav3WT} mice. Finally, APN administration significantly increased AMPK and ERK1/2 phosphorylation, and decreased cardiac fibrosis in Cav3KO^{Cav3Y73F}, but not in Cav3KO^{Cav3WT} (Figures S11A/B/C). Collectively, these results demonstrated the loss of insulin and APN protective effect in prediabetic animals subjected to HFD after chronic MI/R, a pathological alteration rescued by re-expressing a nitration resistant Cav3.

DISCUSSION

In the present study, we have made four novel observations. First, we provide evidence that impairment of the cardioprotective response to insulin precedes HFD-inducing type 2 diabetes via mechanisms distinct from those previously identified. Strong clinical and experimental evidence demonstrates many cardioprotective signals become blunted or lost in the diabetic heart, exacerbating diabetic heart injury[6–11, 32]. The loss of cardioprotection in the diabetic heart has been largely attributed to reduced expression levels of signaling molecules, such as cell membrane receptors and intracellular molecules[7]. However, our current study demonstrated for the first time that attenuation of the cardiac response to cardioprotective interventions (4 weeks HFD) occurred long before type 2 diabetes development (12 weeks HFD). Moreover, expression levels of all signaling molecules, including IRβ, IRS-1, and Cav3, remained normal at this prediabetic stage. These results suggest that targeting deranged signaling mechanisms (outside of downregulation of relevant signaling molecules) may be therapeutic against cardiac injury during early type 2 diabetes development.

Second, we identified that Cav3-centered signalsomes dissociation is responsible for transmembrane signaling impairment in prediabetic cardiomyocytes. Insulin resistance is the hallmark of type 2 diabetes. Cardiac insulin resistance, characterized by impaired Akt activation in response to insulin and attenuated Glut4 membrane translocation, is a well-accepted risk factor in diabetic heart injury[33]. However, underlying molecular mechanisms remain elusive and effective intervention reversing cardiomyocyte insulin signaling is unavailable. APN is an adipocyte-derived cytokine exerting a strong cardioprotective effect primarily via AdipoR1-dependent activation of cell salvage kinases, including AMPK, Akt, and ERK1/2[34, 35]. Emerging evidence indicates that APN resistance develops in the liver, skeletal muscles, heart, and vascular endothelial cells in diabetic individuals[36–39]. Finally, preconditioning, an intervention that activates multiple cardioprotective signals and exerts strong cardioprotection in nondiabetics, is also significantly weakened in the diabetic heart[8, 9, 11]. The “universal” loss of the pro-survival response in the diabetic heart strongly suggests impairment of the molecule(s) required in broad pro-survival signaling cascades in the diabetic heart. We and others previously demonstrated that direct interaction between Cav3 and the intracellular signaling molecules (formation of Cav3-centered signalsomes) is requisite for transmembrane signaling[20]. Our current study provides direct evidence that the expression levels of individual signaling molecules are unchanged in the prediabetic heart. However, their interaction and signaling complex formation was significantly reduced. These results demonstrate that dissociation of the Cav3-centered signalsome (with resultant impairment of transmembrane protective signaling) is a novel mechanism responsible for cardiac insulin resistance, contributing to ischemic cardiac injury in the prediabetic heart. Preserving the Cav3 signalsome may be an effective early intervention to restore insulin signaling and protect against HFD-exacerbated ischemic injury.

Third, we identified that nitrative modification of Cav3 is responsible for Cav3/IRβ dissociation in the prediabetic heart. Emerging evidence indicates that protein nitration is a novel post-translational modification with a critical impact on physiologic and pathological signaling[40]. Protein nitration is primarily mediated by peroxynitrite ($ONOO^-$), a near-

diffusion limiting reaction product between nitric oxide and superoxide[41, 42]. Diabetes significantly upregulates iNOS expression, producing a high level of NO[43]. NADPH oxidase is the primary superoxide source in the failing heart[44, 45]. They are primarily located in caveolae[46]. As such, iNOS-derived NO may react with NADPH oxidase-derived superoxide, resulting in a high level of peroxynitrite in proximity to Cav3. Moreover, diabetes may result in caveolae-located eNOS uncoupling, locally producing a high level of peroxynitrite[47].

In the diabetic heart, peroxynitrite is significantly increased. However, the causative role of nitrative protein modification in diabetic exacerbation of ischemic HF has not been established. More importantly, whether cardiac Cav3 is nitratively modified in HFD-fed animals and whether this modification may impair transmembrane cardioprotective signaling has not been previously investigated. Our current study obtained several lines of evidence supporting Cav3 nitrative modification is responsible for Cav3 signalsome dissociation, blocking insulin transmembrane protective signaling. First, treatment of cardiomyocytes with SIN-1 virtually abolished Cav3/IRβ interaction. The addition of SOD rescued their interaction, indicating peroxynitrite (not nitric oxide) is responsible for Cav3 signalsome dissociation. Second, treatment with SIN-1 blocked insulin-induced cell salvage kinase activation, an effect rescued by SOD co-treatment. Previous studies have demonstrated that SOD and pharmacological SOD mimetics limit diabetic ischemic heart injury. Our current study showing nitrative modification of Cav3 blocks insulin/adiponectin activation of protective signaling and enhances ischemic injury in prediabetic animals provides a likely explanation for the protective effect of anti-oxidative therapy in diabetic cardiac injury. Third, in cardiac samples from prediabetic animals (4-weeks HFD), significant Cav3, not IRβ, nitration was detected. Fourth, MS analysis identified that $Cav3^{Y73}$ is nitratively modified in prediabetic cardiac tissue. Finally, re-expressing $Cav3^{Y73F}$ in $Cav3^{-/-}$ neonatal cardiomyocytes blocked SIN-1 induced Cav3 nitration, preserved Cav3 signalsomes, and restored insulin transmembrane protective signaling in HG/HL challenged cardiomyocytes.

Not all Tyr residues are identically modified by nitrative stress[42]. Tyr nitration selectivity is independent of protein abundance or Tyr residue number. Certain structural characteristics (such as paucity of reactive cysteine residues in the vicinity of the Tyr; proximity to a negatively charged residue; absence of steric hindrances; surface exposure and Tyr residues in loop structure) render some Tyr residues more susceptible to nitration[42]. Notably, Tyr^{73} is located within the Cav3 scaffolding domain, a region critically responsible for Cav3 interaction with signaling partners[13]. Importantly, Cav3 nitration broadly impairs its signalsome formation ability, blocking cardioprotective signaling in addition to insulin signaling. Specifically, treating cardiomyocytes with a chemical peroxynitrite donor (SIN-1) reduced Cav3/AdipoR1 complex formation and blocked APN transmembrane signaling. More importantly, re-expressing a nitration-resistant Cav3 in $Cav3^{-/-}$ cardiomyocytes inhibited SIN-1-induced Cav3/AdipoR1 dissociation and restored APN-induced AMPK and Akt activation in HG/HL challenged cardiomyocytes.

Finally, we proved that cardiomyocyte $Cav3^{Y73F}$ re-expression restored insulin cardioprotective signaling in prediabetic hearts, attenuating post-MI remodeling and ischemic HF. It is well known that Cav3 is critical for heart protection against ischemia/reperfusion injury. Cardiac-specific overexpression of Cav3 induces endogenous

cardiac protection[48], whereas Cav3 knockout causes insulin resistance[49], progressive cardiomyopathy[50], and blocks opioid and isoflurane-induced cardiac protection[51, 52]. To obtain the best possible evidence clarifying the role of nitrative Cav3 modification in prediabetic cardiac injury, cardiac-specific Cav3KO^{Cav3WT} and Cav3KO^{Cav3Y73F} mouse lines were established. Mice were fed HFD for 4 weeks and subjected to MI/R. We demonstrated that re-expressing Cav3^{Y73F} blocked HFD-induced Cav3 nitration, preserved Cav3/IRβ signalsome integrity, and restored insulin transmembrane signaling. Moreover, compared to Cav3KO^{Cav3WT} mice, Cav3KO^{Cav3Y73F} mice exhibited significantly increased response to insulin protection in an acute MI/R model, as evidenced by reduced cardiomyocyte apoptosis, decreased infarct size and improved cardiac function. Most interestingly, re-expressing Cav3^{Y73F} enabled insulin to exert a sustained protective effect in a chronic MI/R model (60-minute MI followed by 4 weeks reperfusion). Insulin treatment significantly improved systolic function, reduced cardiac fibrosis, and decreased heart/body weight and lung/body weight ratios in Cav3KO^{Cav3Y73F}. These chronic/sustained protective actions were not observed in Cav3KO^{Cav3WT} mice. Collectively, these results indicate that blocking Cav3 nitrative modification may achieve the best therapeutic outcome.

It should be noted that the cardioprotective effect of insulin was significantly reduced but not lost in HFD-fed (4 weeks) WT mice subjected to acute MI/R (60-minute MI and 24 hours reperfusion). In contrast, insulin failed to protect prediabetic animals subjected to chronic MI/R (60-minute MI and 4 weeks reperfusion), unless a nitrative-resistant Cav3 mutation was re-expressed. Several possibilities exist. First, in the chronic MI/R model, mice were subjected to a total of 8 weeks of HFD (4 weeks before MI and 4 weeks during reperfusion). Longer HFD duration likely has a stronger impact on Cav3-centered signalsome integrity and signaling. Second, HFD feeding after MI may have a more significant harmful impact on cardiac injury than HFD feeding before MI. This possibility is consistent with the current understanding that a healthy diet is critical in MI patients after successful reperfusion. Third, insulin treatment may delay, rather than block, MI/R injury in Cav3KO^{Cav3WT} animals. Therefore, the protective effect observed in an acute MI/R model (24 hours after reperfusion) may not sustain during prolonged reperfusion (4 weeks). Previous studies have demonstrated that infarct extension (hibernating myocardium with delayed cell death) continually develops beyond 24 hours (up to a week). Many other pathological alterations, including angiogenesis, cardiac fibrosis, and immunoregulation, develop chronically and significantly contribute to cardiac remodeling in addition to initial infarct size. Finally, we utilized an AAV9-mediated cardiac-specific gene expression strategy. Cav3 is only re-expressed in cardiomyocytes in Cav3KO mice. Insulin treatment in WT mice may activate protective signaling in other cell types, such as coronary endothelial cells, and indirectly protect the heart from MI/R injury. These indirect protective mechanisms are unavailable in Cav3KO mice with cardiac-specific Cav3^{WT} re-expression. All such possibilities warrant future direct investigation".

It is important to note a few limitations of our study, which all warrant future investigations. First, the initial size of MI is one of the most important determinants of cardiac remodeling. The acute infarct-sparing effect of insulin in Cav3KO^{Cav3Y73F} likely contributes to an attenuated chronic remodeling observed four weeks after reperfusion. Moreover, Cav3KO^{Cav3Y73F} may affect many other signaling mechanisms in addition to insulin.

Second, considering Tyr^{73} is located within the Cav3 scaffolding domain, which is very important to form signalsome with other proteins, we identify the nitration of Tyr^{73} is critical to signalsome forming in this study. However, Tyr^{73} may act independently of its nitration due to its crucial location and high conservation in mice and humans, particularly in human prediabetic hearts. Third, we cannot produce a Tyr73-specific nitrated Cav3 protein and assess in vitro binding due to technical limitations. Additional experiments utilizing the genetic code expansion approach are under consideration. Fourth, it should be indicated that the results presented in Figures 2D and 5A are relative, not an absolute quantification. Although repeated experiments with multiple samples consistently detect Y73 nitration, it is unfortunate that a consistent stoichiometry result with different samples was not observed due to technical challenges. However, our point mutation experiments provide clear evidence that a nitration-resistant mutation (i.e., Tyr^{Y73F}) preserved Cav3/IRβ interaction and restored insulin cardioprotection, indicating that Cav3 Tyr^{73} nitration plays a significant role in cardiac insulin resistance. Fifth, our present experiments provided clear evidence that preventing $Cav3^{Tyr73}$ nitration by genetic mutation approach preserved insulin signaling and cardioprotection in prediabetic hearts. Whether and how reversing HFD back to normal chow (allowing nitrated Cav3 to be replaced by de novo Cav-3) may restore insulin signaling is a clinically important question. Finally, the current study focused on the impact of Cav3 Tyr^{73} nitration on diabetes and MI/R injury. Whether the accumulation of nitrated Tyr^{73} may cause other cardiovascular dysfunction warrants future investigation.

In summary, we demonstrate for the first time that nitrative modification of Cav3 at Tyr^{73} and resultant signal complex dissociation is responsible for cardiac insulin/adiponectin resistance in the prediabetic heart, contributing to ischemic HF progression (Figure S12). Early interventions preserving Cav3-centered signalsome integrity are effective novel strategies against prediabetic exacerbation of ischemic HF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

AAR	Area-at-risk
AdipoR1	Adiponectin receptor-1
APN	Adiponectin
Cav3	Caveolin-3
FS	Fraction shortening

HF	Heart failure
HFD	High-fat diet
Inf	Infarction
IRβ	Insulin receptor-β
KO	Knockout
LVEF	Left ventricular ejection fraction
MI/R	Myocardial ischemia/reperfusion
ND	Normal diet
NMVM	Neonatal mouse ventricular cardiomyocytes
TTC	Triphenyltetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Clinical Perspective

What Is New?

- This study provides the first evidence that Cav3-centered signalsome is disassembled in prediabetic cardiomyocytes, blocking multiple cardioprotective signaling.
- This study identified that Cav3 nitration at Tyr73 is a novel post-translational modification of a molecule critical in organizing transmembrane signaling, contributing to prediabetic cardiac injury.
- This study demonstrated for the first time that preventing Cav3 nitration is cardioprotective against ischemic heart failure in predaibetic animals.

What Are the Clinical Implications?

- Protein post-translational modification, particularly protein nitration, plays a significant pathogenic role in cardiac insulin resistance in obesity/diabetes;
- Early interventions preserving Cav3-centered signalsome integrity may potentially protect against ischemic heart injury in prediabetics.

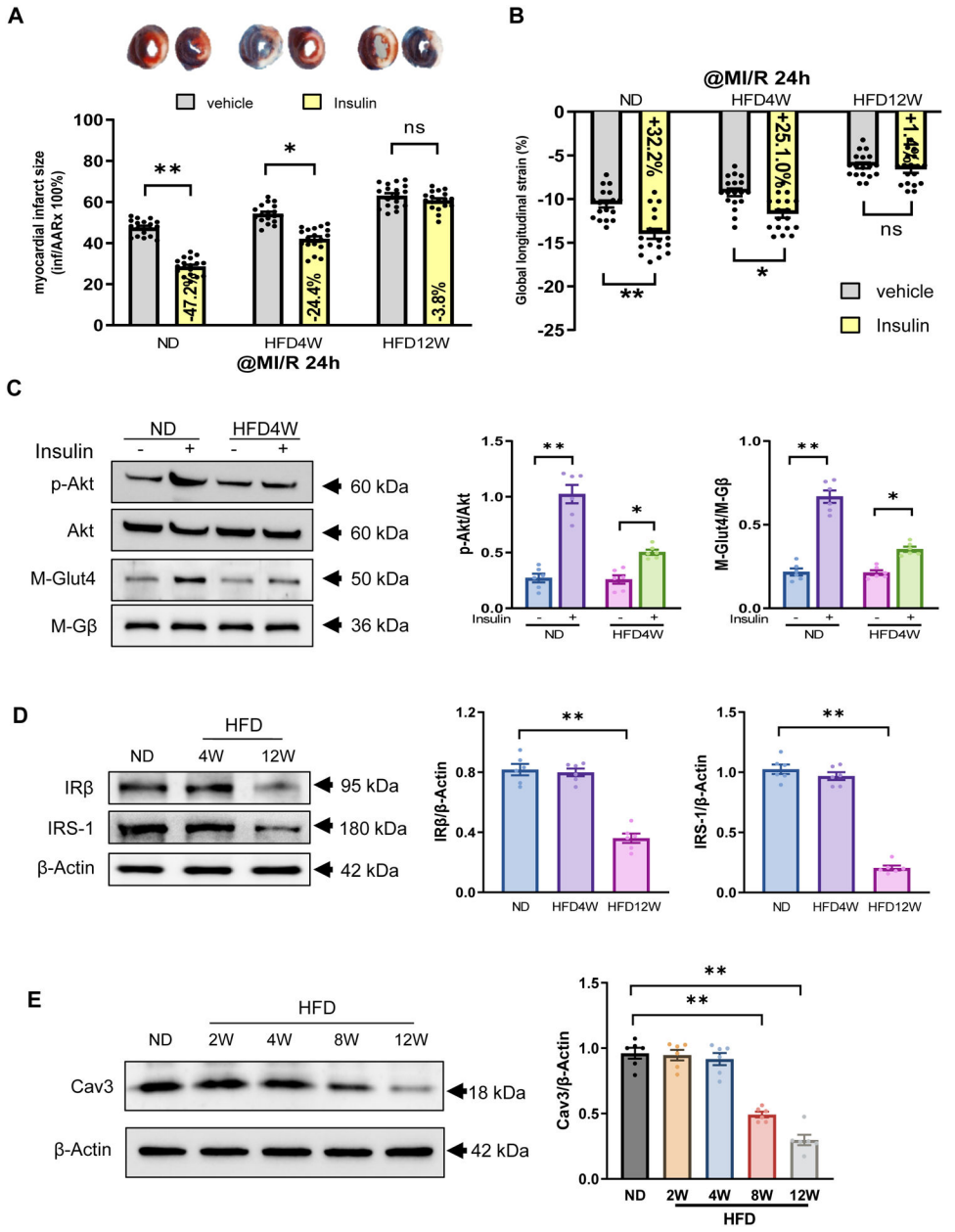

Figure 1. Impairment of cardioprotective response and transmembrane signaling occurred long before HFD-induced type 2 diabetes.

A. Infarct-sparing effect of insulin was detected by Evans blue-TTC staining in ND and HFD (4- and 12-weeks) mice. **B.** Global longitudinal strain (GLS) determined by echocardiography in ND and HFD (4- and 12-weeks) mice. n = 18–20/group. *p < 0.05, **p < 0.01 vs. vehicle, respectively. **C**. Transmembrane signaling was determined by Akt phosphorylation and Glut4 membrane translocation 30 min after insulin treatment in adult cardiomyocytes after HFD 4 weeks. n = 5–6/group, *p < 0.05, **p < 0.01 vs. vehicle, respectively. **D**. IRβ and IRS-1 expression in ND and HFD (4- and 12-weeks) heart. n = 6/group, **p < 0.01 vs. ND group. **E.** Cav3 expression in ND and HFD (2–12 weeks) heart. n = 5–6/group, **p < 0.01 vs. ND group. ND: normal diet; HFD: high-fat diet;

MI/R: myocardial ischemia/reperfusion; IRβ: insulin receptor β; IRS-1: Insulin receptor substrate-1.

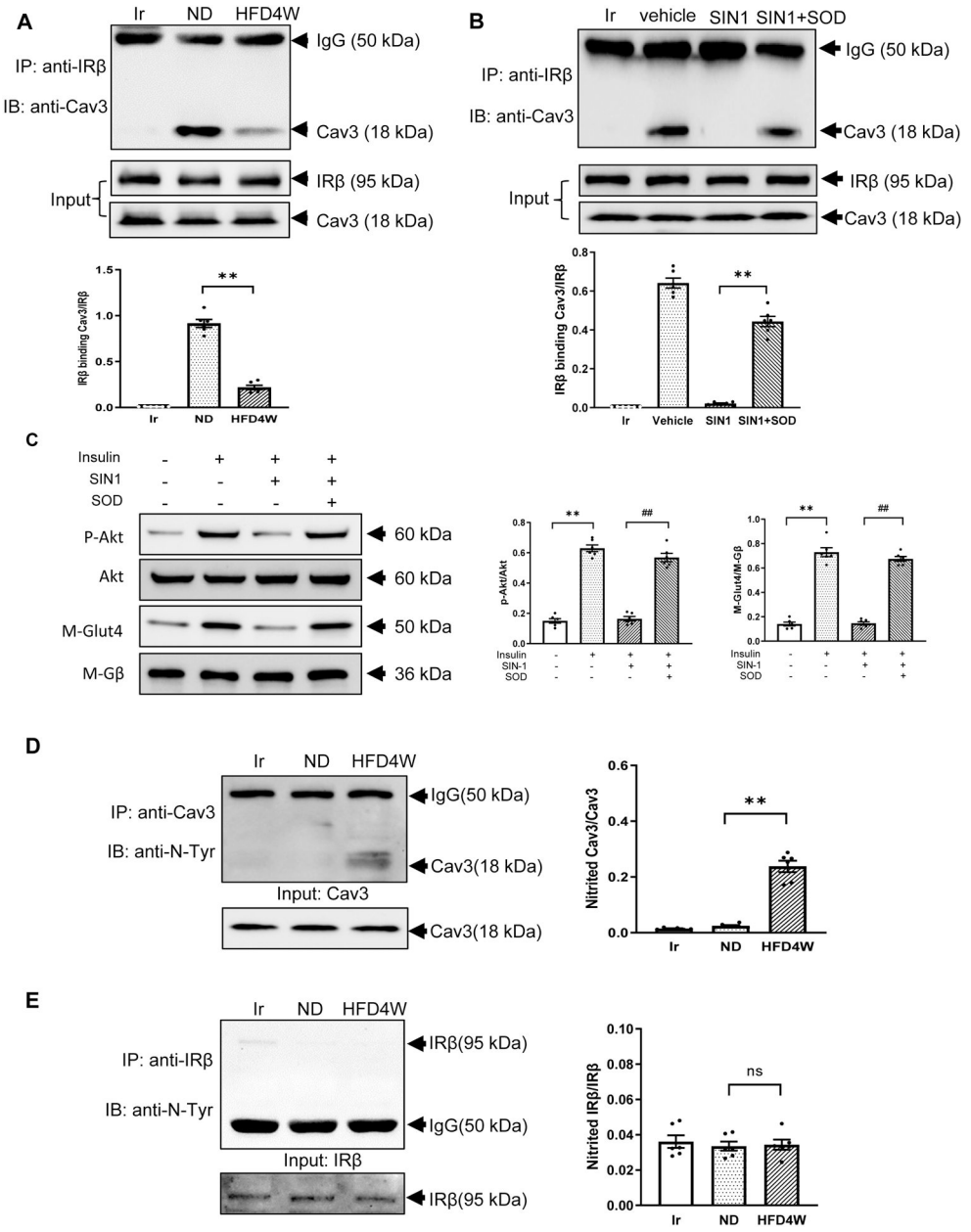

Figure 2. Cav3-centered signalsomes were dissociated in the prediabetic heart, a pathologic effect phenocopied by SIN1.

A. Detection of Cav3/IRβ complex interaction in ND or HFD (4 weeks) heart samples. n = 6/group, **p < 0.01 vs. ND. **B**. Detection of Cav3/IRβ association in SIN1-treated adult cardiomyocytes in the presence and absence of SOD. n = 6/group, **p < 0.01 vs. SIN1 group. **C.** Insulin-induced Akt phosphorylation and Glut4 membrane translocation in adult cardiomyocytes were blocked by SIN1, an effect rescued by SOD. n = 6/group, **p < 0.01 vs. vehicle, ##p < 0.01 vs. SIN1 without SOD. **D/E**. Cav3, not IRβ, was significantly nitrated 4 weeks post-HFD. The level of nitrated Cav3/total Cav3 was about 38.77±7.9%. n = 6/group. **p < 0.01 vs. ND. SIN1: 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride; SOD: Superoxide Dismutase.

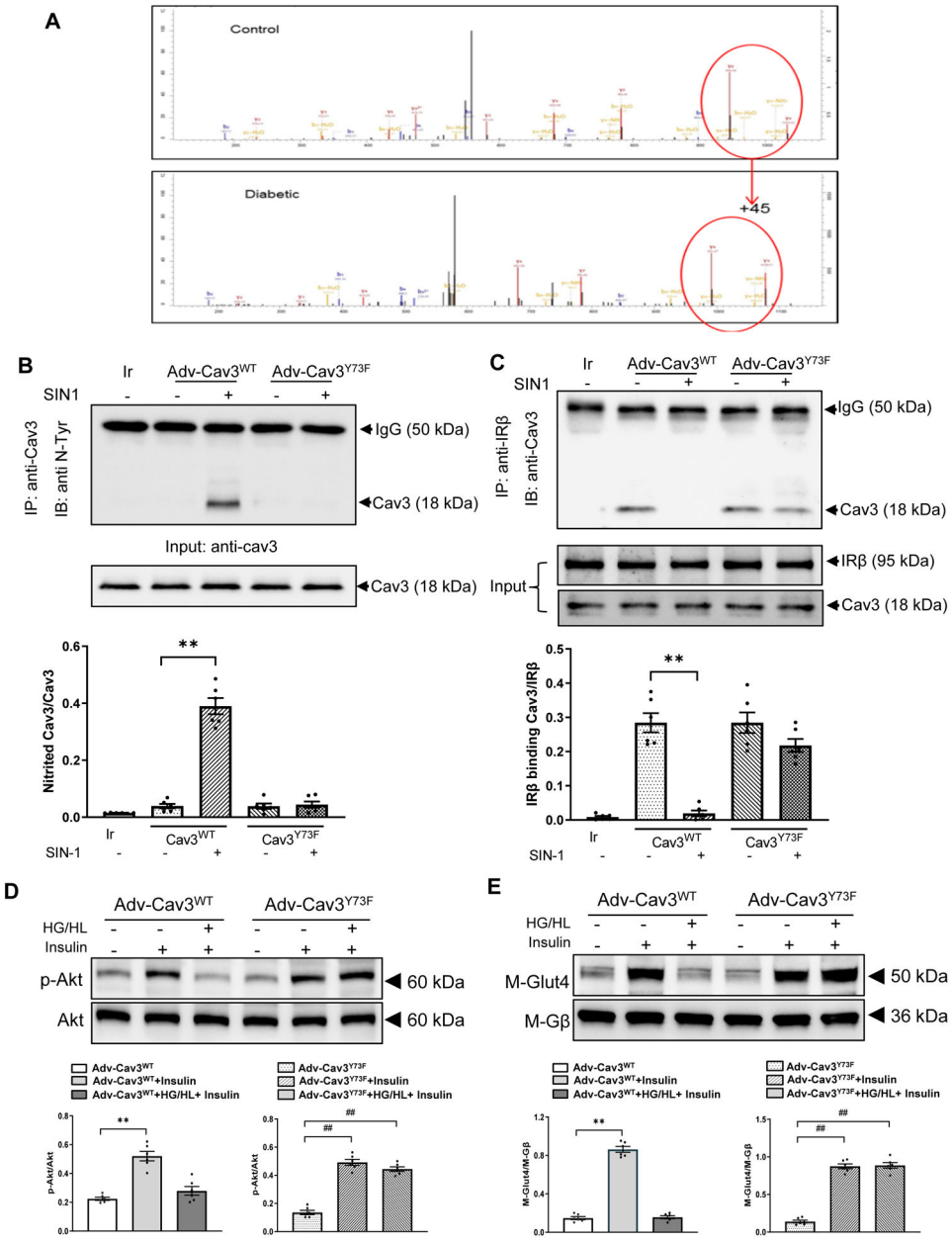

Figure 3. Identifying Cav3^{Y73} as the responsible site for nitrative dissociation of Cav3 signalsomes.

A. MS analysis identified Tyr73 nitration in prediabetic but not in normal control heart. **B.** SIN1-induced Cav3 nitration was detected in Cav3KO neonatal mouse cardiomyocytes re-expressing WT Cav3 (Adv-Cav3^{WT}) but not in Cav3KO neonatal mouse cardiomyocytes re-expressing Tyr73 mutated Cav3 (Adv-Cav3^{Y73F}). n = 6/group. **p < 0.01 vs. Adv-Cav3^{WT}. **C.** SIN1 caused Cav3/IRβ complex dissociation in Adv-Cav3^{WT} neonatal mouse cardiomyocytes, an effect blocked in Adv-Cav3^{Y73F} neonatal mouse cardiomyocytes. n = 6/group, **p < 0.01 vs. Adv-Cav3^{WT}. **D/E.** 48 hours HG/HL culture significantly attenuated insulin transmembrane signaling in Adv-Cav3^{WT} neonatal mouse cardiomyocytes (attenuated Akt phosphorylation and reduced Glut4 membrane translocation). Re-expressing

$Cav3^{Y73F}$ in Cav3KO neonatal mouse cardiomyocytes rescued insulin-induced Akt phosphorylation (D) and Glut4 membrane translocation (E) after HG/HL treatment. n = 6/group. **p < 0.01 vs. Adv-$Cav3^{WT}$ treated with vehicle. ##P<0.01 vs. Adv-$Cav3^{Y73F}$ treated with vehicle.

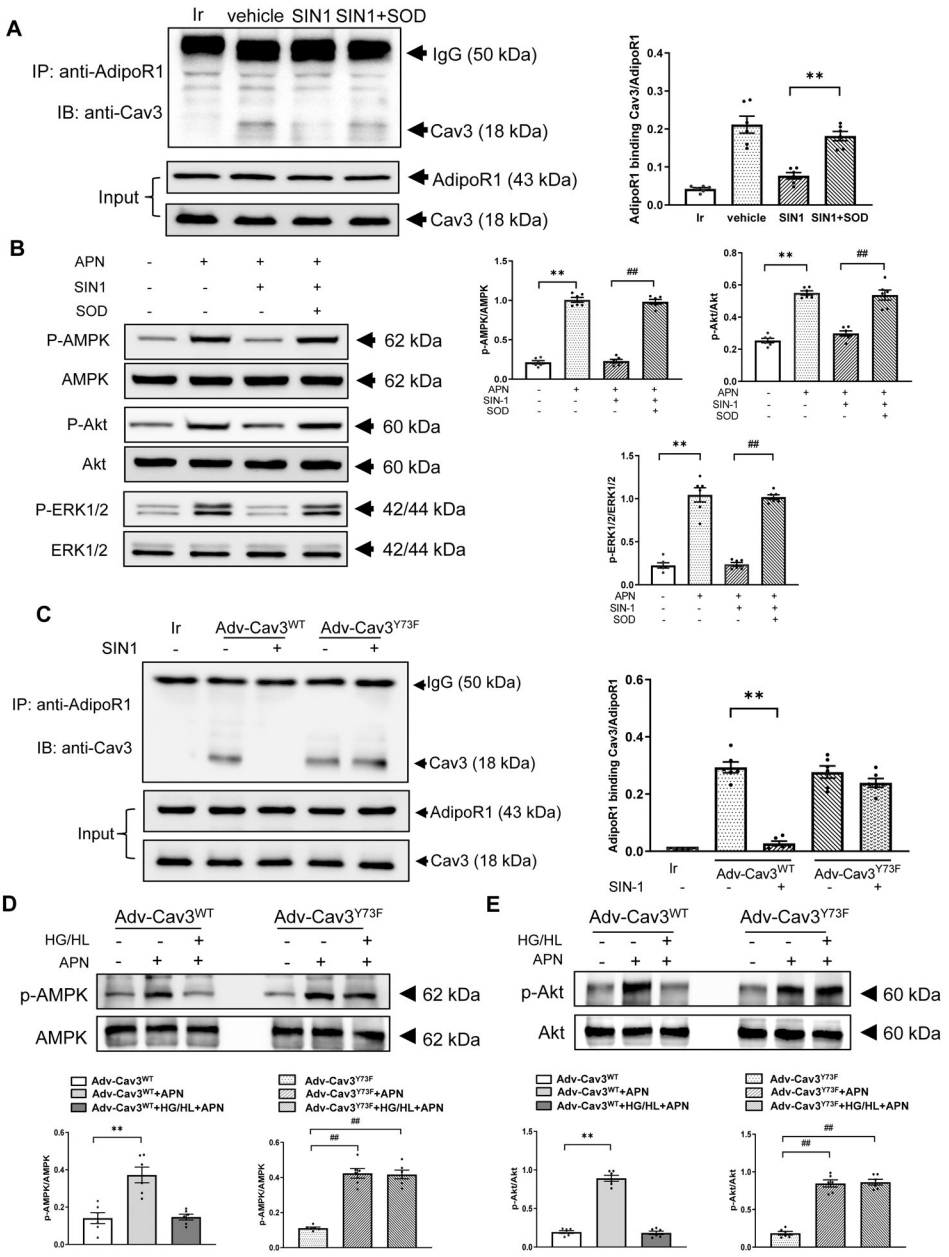

Figure 4. Tyr73 nitration dissociates Cav3/AdipoR1 and impairs APN transmembrane signaling. A/B.

Detection of Cav3/AdipoR1 association in SIN1-treated adult cardiomyocytes in the presence and absence of SOD. n = 6/group, **p < 0.01 vs. SIN1 group. **B.** APN-induced AMPK, Akt and ERK1/2 phosphorylation in adult cardiomyocytes were blocked by SIN1, an effect rescued by SOD. n = 6/group, **p < 0.01 vs. vehicle, ##p < 0.01 vs. SIN1 without SOD. **C.** SIN1 caused Cav3/AdipoR1 complex dissociation in Adv-Cav3^{WT} neonatal mouse cardiomyocytes, an effect blocked in Adv-Cav3^{Y73F} neonatal mouse cardiomyocytes. n = 6/group, **p < 0.01 vs. Adv-Cav3^{WT}. **D/E.** 48 hours HG/HL culture significantly attenuated APN transmembrane signaling in Adv-Cav3^{WT} neonatal mouse cardiomyocytes (attenuated AMPK and Akt phosphorylation). Re-expressing Cav3^{Y73F} in Cav3KO neonatal

mouse cardiomyocytes rescued APN-induced AMPK (D) and Akt phosphorylation (E) after HG/HL treatment. n = 6/group. **p < 0.01 vs. Adv-Cav3^{WT} treated with vehicle. $^{\#\#}$P<0.01 vs. Adv-Cav3^{Y73F} treated with vehicle.

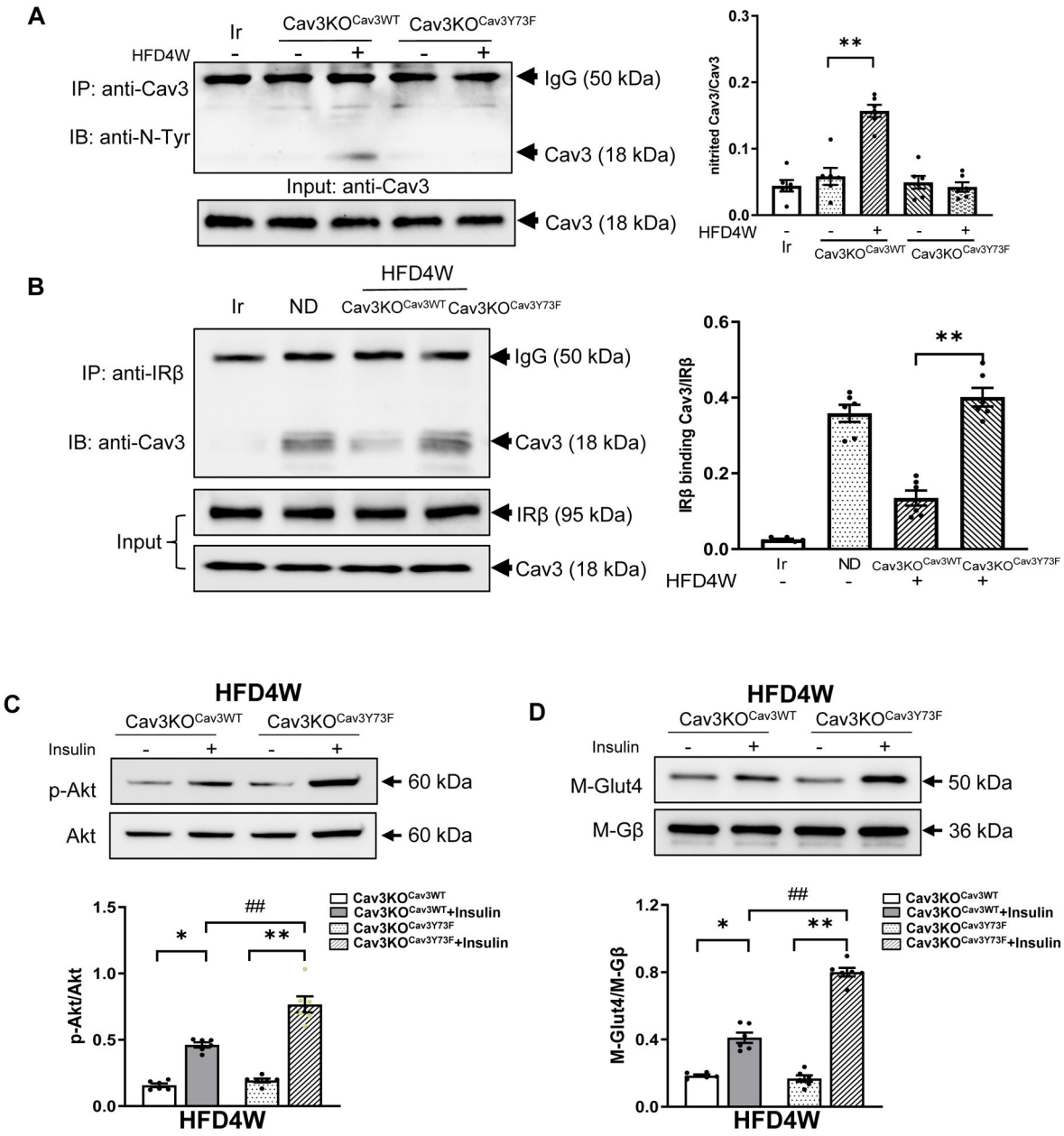

Figure 5. Re-expressing Cav3^{Y73F} in Cav3KO mice (Cav3KO^{Cav3Y73F}) blocked HFD-induced Cav3 nitration, preserved Cav3 signalsome integrity, and rescued transmembrane signaling.

A. HFD causes significant Cav3 nitration in cardiac tissue from Cav3KO mice re-expressing Cav3^{WT} (Cav3KO^{Cav3WT}), a pathologic effect blocked in Cav3KO^{Cav3Y73F} heart. The level of nitrated Cav3/total Cav3 was about 29.9±4.2% n = 6/group. **p < 0.01 vs. Cav3KO^{Cav3WT}. **B.** HFD dissociated Cav3/IRβ complex in cardiac tissue from Cav3KO^{Cav3WT} mice, a pathologic effect blocked in Cav3KO^{Cav3Y73F} heart. n = 6/group. **p < 0.01 vs. Cav3KO^{Cav3WT}. **C/D.** Insulin transmembrane signaling (Akt phosphorylation and Glut4 membrane translocation) was significantly improved in cardiomyocytes isolated from Cav3KO^{Cav3Y73F} heart subjected to HFD. n = 6/group, *p < 0.05 vs. Cav3KO^{Cav3WT}

treated with vehicle; **p < 0.01 vs. Cav3KO^{Cav3Y73F} treated with vehicle; $^{\#\#}$P<0.01 vs. Cav3KO^{Cav3WT} treated with insulin.

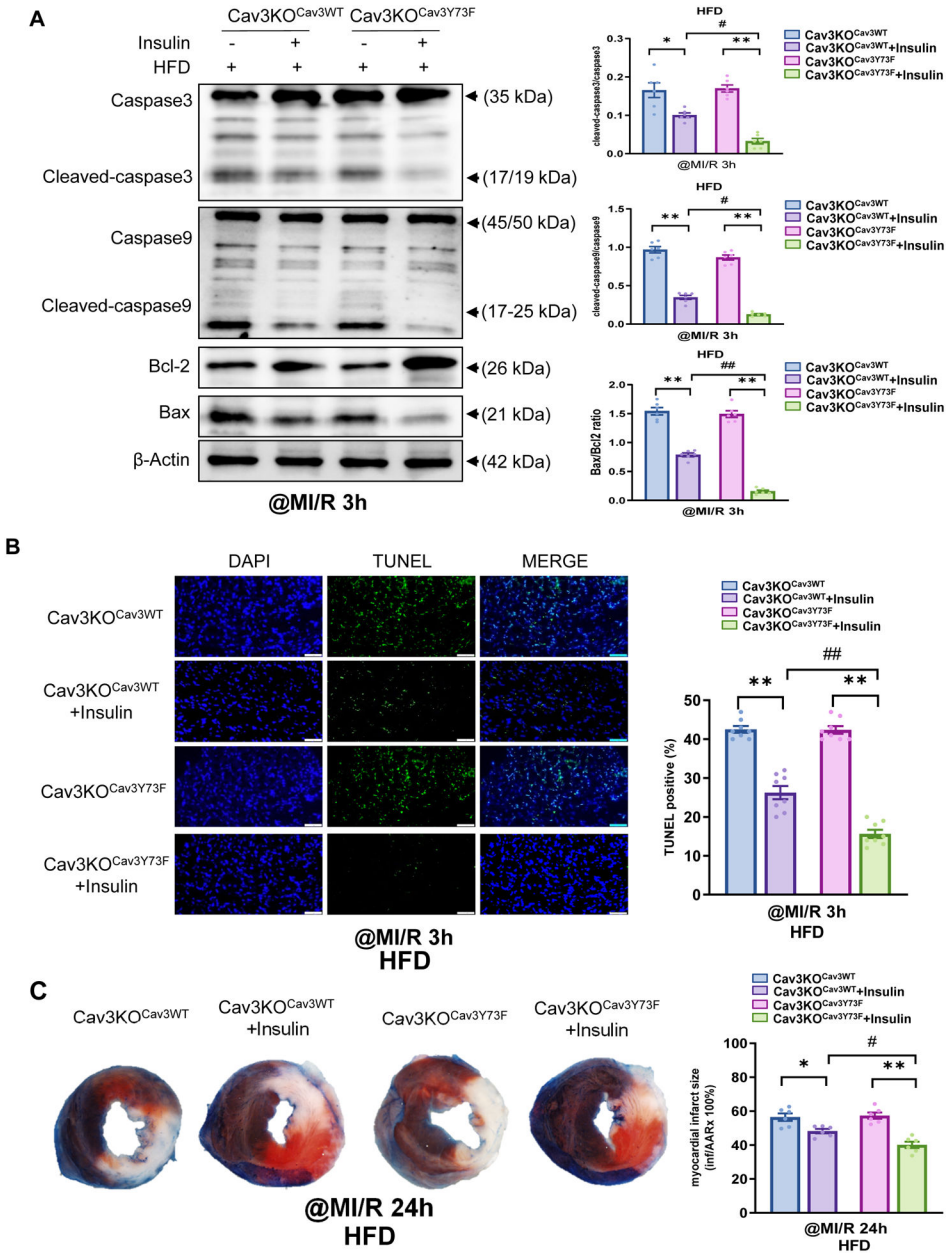

Figure 6. Anti-apoptotic and infarct sparing effect of insulin were enhanced in Cav3KO^{Cav3Y73F} mice. A/B.

Prediabetic mice (4-week HFD) were subjected to acute MI/R (60-minute MI followed by 3 hours reperfusion). Mice were treated with vehicle or insulin 10 minutes before reperfusion. Apoptosis was determined by caspase 3 activation, caspase 9 activation, Bax/Bcl2 ratio and TUNEL staining. n = 6–10/group. *p < 0.05 vs. Cav3KO^{Cav3WT} treated with vehicle; **p < 0.01 vs. Cav3KO^{Cav3Y73F} treated with vehicle; $^{\#}$P<0.05 & $^{\#\#}$P<0.01 vs. Cav3KO^{Cav3WT} treated with insulin. **C.** Pre-diabetic mice were subjected to 60-minute MI and treated with vehicle or insulin 10 minutes before reperfusion. Infarct size was determined 24 hours after reperfusion by Evans blue/TTC double stain. n = 8–10/group. *p < 0.05 vs.

Cav3KO^{Cav3WT} treated with vehicle; **p < 0.01 vs. Cav3^{Cav3Y73F} treated with vehicle; $^{\#}$P<0.05 vs. Cav3KO^{Cav3WT} treated with insulin.

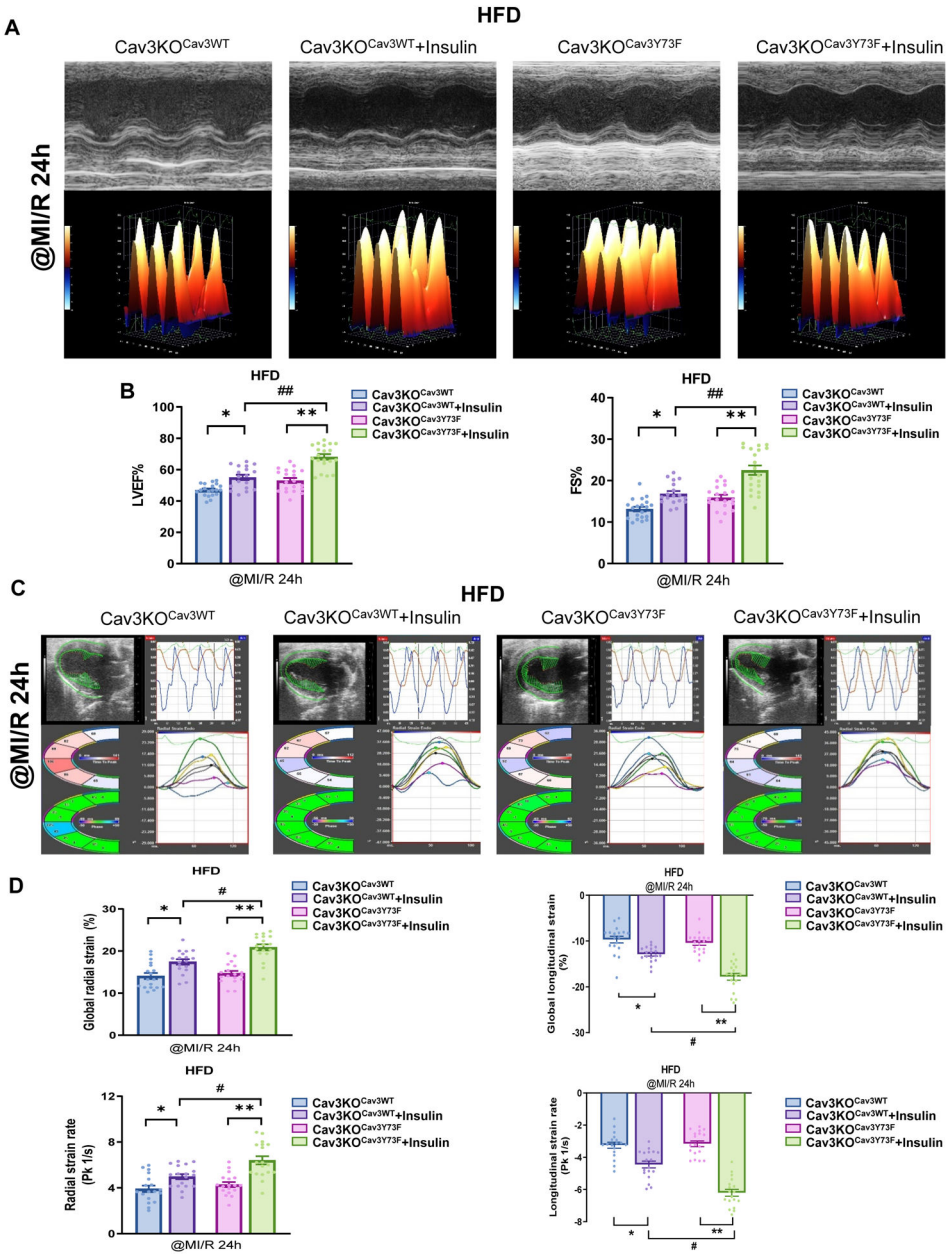

Figure 7. The acute cardioprotective effect of insulin was improved in Cav3KO^{Cav3Y73F} mice.

Prediabetic mice were subjected to 60-minute MI and treated with vehicle or insulin 10 minutes before reperfusion. Cardiac function was determined 24 hours after reperfusion by echocardiography. **A.** Typical images of three-dimensional regional wall velocity diagrams of radial strain showing contraction (orange/positive values) of 3 consecutive cardiac cycles. Vector diagrams showing the direction and magnitude of endocardial contraction at systole. **B.** Compared to Cav3KO^{Cav3WT} mice, insulin administration had a greater improvement effect upon LVEF and FS in Cav3KO^{Cav3Y73F} mice. n = 18–20 mice/group. *p < 0.05 vs. Cav3KO^{Cav3WT} treated with vehicle; **p < 0.01 vs. Cav3^{Cav3Y73F} treated with vehicle; $^{\#\#}$P<0.01 vs. Cav3KO^{Cav3WT} treated with insulin. **C.** Representative images of speckle-tracking analysis in the long-axis B-mode and radial segmental synchronicity of the LV.

D. Global strain and strain rate measured in the radial and longitudinal axes across the LV endocardium. Compared to Cav3KO^{Cav3WT} mice, insulin administration had a greater improvement effect on the global radial strain, radial strain rate, global longitudinal strain, and longitudinal strain rate in Cav3KO^{Cav3Y73F} mice. n = 18–20 mice/group. *$p < 0.05$ vs. Cav3KO^{Cav3WT} treated with vehicle; **$p < 0.01$ vs. Cav3^{Cav3Y73F} treated with vehicle; $^{\#}P<0.05$ vs. Cav3KO^{Cav3WT} treated with insulin.

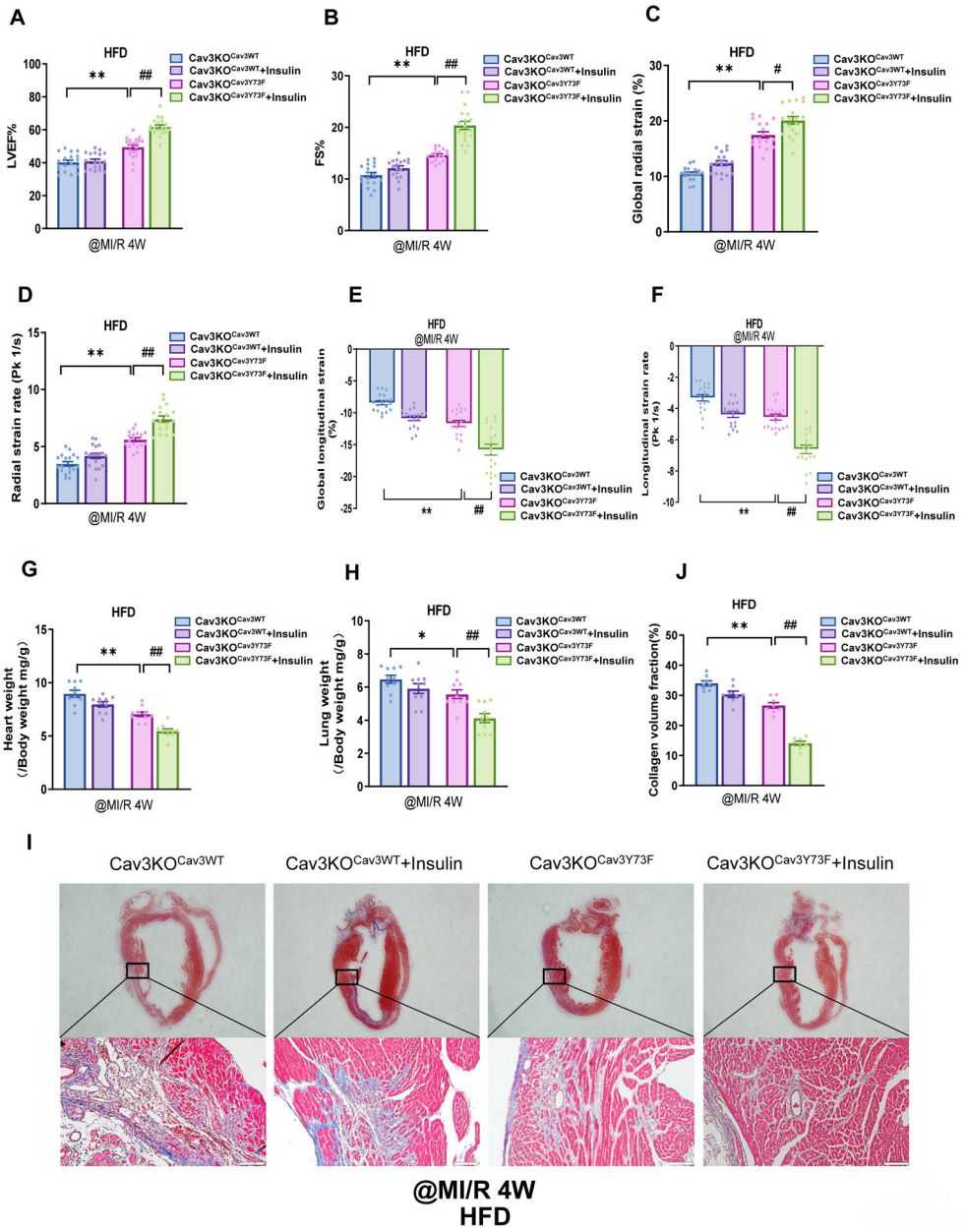

Figure 8. Chronic cardioprotective and anti-remodeling effects of insulin were preserved in Cav3KO^{Cav3Y73F} mice.

Prediabetic mice were subjected to 60-minute MI and treated with vehicle or insulin (bolus administration 10 minutes before reperfusion followed by continuous administration for 4 weeks via an osmatic pump). **A-F.** Insulin administration failed to improve cardiac function 4 weeks after reperfusion in Cav3KO^{Cav3WT} mice fed with HFD. In contrast, insulin significantly improved cardiac function in HFD Cav3KO^{Cav3Y73F} mice. n = 18–20/group. *p < 0.05 or **p < 0.01 vs. Cav3KO^{Cav3WT} treated with vehicle. #p < 0.05 or ##p < 0.01 vs. Cav3KO^{Cav3Y73F} treated with vehicle. **G/H.** Insulin treatment significantly reduced ratios of heart weight/body weight and lung weight/body weight in Cav3KO^{Cav3Y73F} mice, a protective effect absent in Cav3KO^{Cav3WT} mice. n = 10/group. *p < 0.05 vs.

Cav3KO^{Cav3WT} treated with vehicle. ##p < 0.01 vs. Cav3KO^{Cav3Y73F} treated with vehicle. **I-J.** Insulin treatment significantly reduced cardiac fibrosis in Cav3KO^{Cav3Y73F} mice, but not in Cav3KO^{Cav3WT} mice. n = 8–10/group. *p < 0.05 vs. Cav3KO^{Cav3WT} treated with vehicle. ##p < 0.01 vs. Cav3KO^{Cav3Y73F} treated with vehicle.