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ORIGINAL RESEARCH ARTICLE

Small Extracellular Vesicle External Surface Adiponectin-Mediated Adipocytes/ Cardiomyocytes Communication in Diabetic Ischemic Heart Failure

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BACKGROUND: Mortality from acute myocardial infarction (MI) has declined significantly in the past decade for nondiabetic patients. However, both morbidity and mobility of ischemic heart failure (IHF) persistently escalate in the diabetic population via incompletely understood mechanisms. Recent studies demonstrated that small extracellular vesicles (sEVs) released from nondiabetic and diabetic adipocytes (ADps) exert opposite effects on acute myocardial ischemia and reperfusion (MI/R) injury. However, whether and how ADp sEVs may protect against post-MI remodeling and IHF, and more important, whether and how diabetes may impair this protective effect, remain unknown.

METHODS: sEVs were isolated from epididymal fat pads of nondiabetic animals and intramyocardially injected in nondiabetic or diabetic hearts subjected to MI (90 minutes of MI followed by 4 weeks of reperfusion).

RESULTS: sEV treatment significantly attenuated post-MI cardiac remodeling and improved cardiac function in nondiabetic mice. However, the protection was not observed in diabetic hearts. In adult cardiomyocytes isolated from nondiabetic hearts, sEVs rapidly (15 minutes) activated cell salvage kinases (ERK [extracellular signal-regulated kinase], AMPK [AMP-activated protein kinase], and ACC [acetyl-CoA carboxylase]) and suppressed oxidative stress-induced cell death, suggesting sEV external surface molecules are responsible for the observed cytoprotection. The Exo-Flow (a technology detecting sEV external surface molecules) demonstrated that adiponectin (APN) is enriched on the sEV external surface. The sEVs from APN knockout mice or APN neutralization (NU) antibody pretreated sEVs from the WT mice failed to protect the heart against IHF. Moreover, the cardioprotective effects of sEVs were abolished in APN receptor-1 (AdipoR1)–deficient mice (the primary receptor for APN signaling in the heart) or in mice overexpressing GRK2 (G–protein-coupled receptor kinase 2, a kinase that phosphorylates and inactivates AdipoR1). Finally, diabetes significantly increased cardiac GRK2 expression and AdipoR1 phosphorylation, which prevented sEVs from exerting their beneficial effects. Restoring AdipoR1 function by knock-in a mutated phosphorylation-resistant AdipoR1 (AdipoR1^{S205A}) via AAV9 (adeno-associated virus 9)-mediated gene delivery to cardiomyocytes rescued ADp sEV cardioprotection in diabetic mice.

CONCLUSIONS: Our study reveals that APN is enriched on the ADp-derived external surface of sEVs and is biologically active, playing a critical role in ADp–cardiomyocyte communication. Diabetes disrupts this communication by enhancing GRK2-mediated AdipoR1 phosphorylation, impairing sEV signaling, and exacerbating IHF. These findings provide new insights into the pathophysiology and therapy of IHF in diabetes.

Key Words: adipocyte ■ adiponectin ■ diabetes ■ ischemic heart failure ■ small extracellular vesicle

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Clinical Perspective

What Is New?

- This study provides the first evidence that adiponectin enrichment on the external surface of adipocyte small extracellular vesicles (ADp sEVs) plays a crucial role in ADp–cardiomyocyte communication, activating cardioprotective signaling.
- This study identifies for the first time that adiponectin receptor-1 (AdipoR1) phosphorylation at Ser205 disrupts ADp sEV transmembrane signaling, contributing to myocardial ischemia and reperfusion injury in the diabetic heart.
- This study demonstrates for the first time that blocking AdipoR1 phosphorylation restores ADp sEV cardioprotection and mitigates ischemic heart failure (IHF) in diabetic animals.

What Are the Clinical Implications?

- By demonstrating that adiponectin enrichment on the surface of ADp sEVs plays a crucial role in cardioprotective signaling, this research could lead to the development of new therapeutic strategies aimed at enhancing cardioprotection, particularly in patients with diabetes.
- The finding that blocking AdipoR1 phosphorylation restores ADp sEV cardioprotection and mitigates IHF in diabetic animals suggests that similar strategies could be employed in clinical settings to improve outcomes for patients with diabetes with heart failure.

Nonstandard Abbreviations and Acronyms

AdipoR1	adiponectin receptor-1
ADp	adipocyte
APN	adiponectin
CM	cardiomyocyte
GPCR	G-protein–coupled receptor
Grk2	G-protein–coupled receptor kinase 2
HFD	high-fat diet
IHF	ischemic heart failure
ISK	injury salvage kinase
KO	knockout
LVEF	left ventricular ejection fraction
MI/R	myocardial ischemia and reperfusion
ND	normal diet
NMVM	neonatal mouse ventricular cardiomyocyte
NU	neutralization
PAMC	primary adult mouse cardiomyocyte
R1$^{-/-}$ mice	adipoR1 knockout mice
sEV	small extracellular vesicle
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	wild-type

This study investigates the role of diabetes in aggravating myocardial ischemia and reperfusion (MI/R) injury and the subsequent development of heart failure. For the first time, we identify phosphorylation at the S205 site of adiponectin (APN) receptor-1 (AdipoR1) on the cardiomyocyte membrane as a key mechanism contributing to resistance against APN-mediated cardioprotective signaling. Moreover, we demonstrate for the first time that membrane-bound APN on adipocyte (ADp) small extracellular vesicles (sEVs) effectively transmits cardioprotective signals. Given the excellent biocompatibility, safety, and delivery efficiency of sEVs, the use of ADp sEVs to supplement APN combined with pharmacological- and genetic-modification strategies to inhibit AdipoR1^{S205} phosphorylation may offer a promising therapeutic strategy for mitigating MI/R injury and preventing the progression to heart failure.

Cardiovascular disease is the leading cause of morbidity and mortality in patients with type 2 diabetes, a disease affecting >23 million people in the United States. Obesity, hyperglycemia, and hyperlipidemia are the most common metabolic disorders in diabetes and are established cardiovascular risk factors. However, recent large-scale clinical trials failed to demonstrate cardiovascular mortality benefit from strict glycemic control in patients with diabetes.[1–4] Novel strategies capable of protecting diabetic cardiomyocytes from exacerbated postmyocardial infarction (post-MI) remodeling and ischemic heart failure (IHF) are urgently needed.

Research in the past decade has increased understanding of the role of ADps in health and disease.[5–7] The ADps are traditionally considered an inert storage depot for nutrients. However, recent studies demonstrated that adipose tissue is the largest endocrine organ, producing a wide range of hormones and cytokines regulating remote organ functions.[8] Normal (lean) adipocytes are beneficial in maintaining systemic metabolic homeostasis, whereas dysfunctional (obese) ADps prominently affect the development/progression of type 2 diabetes and diabetic cardiovascular complications.[9–12] Understanding how normal ADps exert their cardioprotective effect and how diabetes adversely impacts ADp–cardiomyocyte communication will help identify novel effective therapies against MI injury in individuals with diabetes.

sEVs are released by all cells and have critical roles in homeostatic processes and intercellular communication.[13] Recent studies demonstrate that ADps are a significant source of circulation sEVs and play a critical role in systemic metabolic hemostasis.[14,15] Healthy adipose tissue–derived sEVs positively regulate remote organ functions, enhancing systemic insulin sensitivity[16] and promoting pancreatic insulin secretion.[17] In contrast,

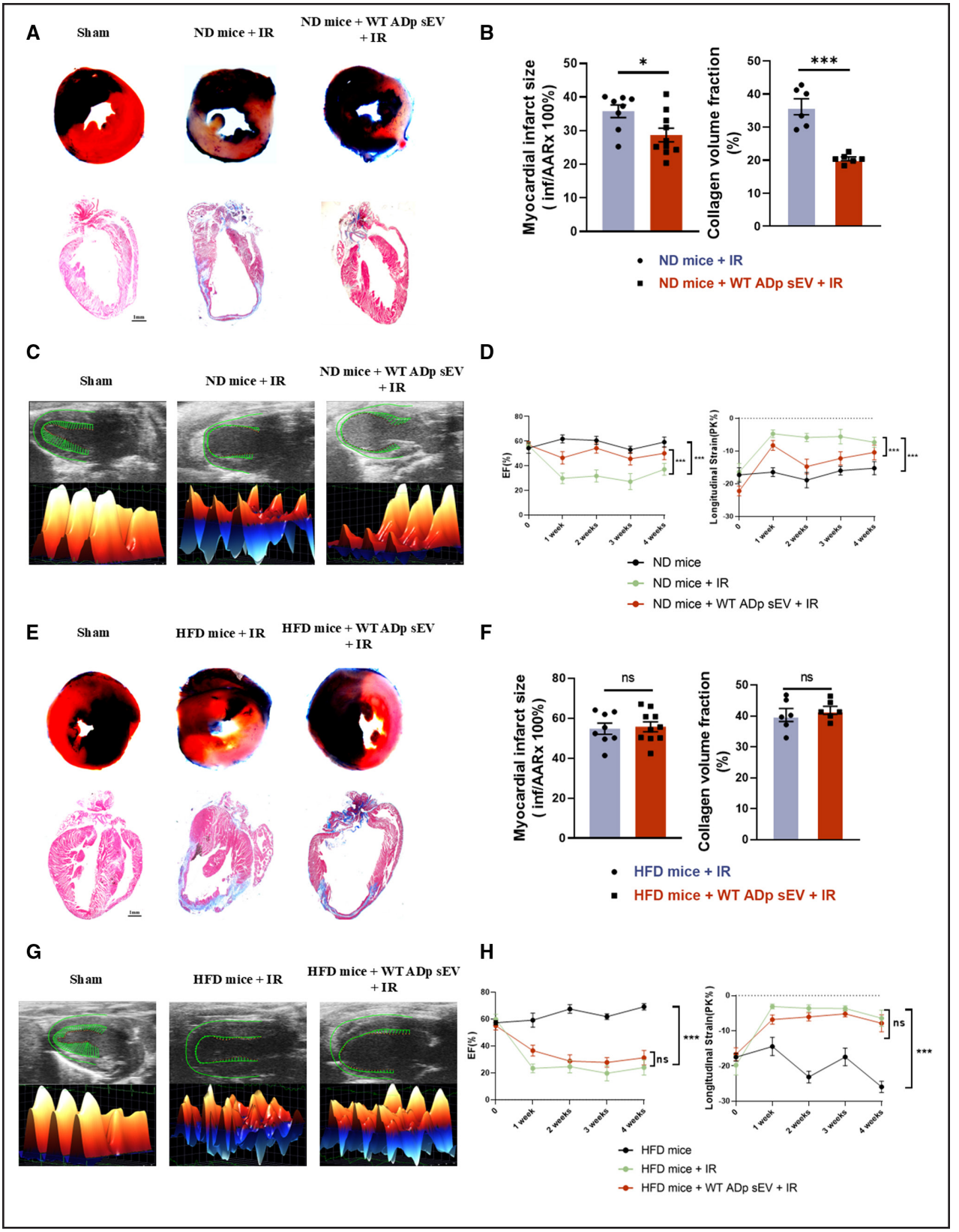

Figure 1. Adipocyte-derived sEVs protected against MI/R injury and IHF in nondiabetic mice, but failed to do so in diabetic mice.

A, Nondiabetic mice were subjected to 90-minute MI and reperfusion, with/without intramyocardial injection of ADp sEVs 48 hours before MI. Twenty-four hours after MI/R, hearts were collected, and Evans blue-triphenyltetrazolium chloride double stain was performed to determine the infarct size. Four weeks after MI/R, hearts were collected, and Masson's trichrome staining was performed to determine the (*Continued*)

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Figure 1 Continued. collagen volume fraction. Scale bar=1 mm. **B**, Quantification of infarct size (Inf/ AAR× 100%) and fibrotic area. For results of infarct size, n=8 in the "ND mice + IR" group and n=10 in the "ND mice + WT ADpsEV + IR" group. For results of fibrotic area, n=6/group, unpaired *t* test, **P*<0.05, ****P*<0.001 vs "ND mice + IR" group, respectively. **C**, Three-dimensional regional wall velocity diagrams (B-mode tracing) of LV endomyocardial strain showing contraction (orange) and relaxation (blue) of 3 consecutive cardiac cycles. **D**, Quantitative analysis of EF(%) and longitudinal strain (PK%) measured across the LV endocardium. For the "ND mice" sham group, n=8/group. For "ND mice + IR" and "ND mice + WT ADp sEV + IR" groups, n=10/group, mixed-effects 2-way ANOVA, ****P*<0.001 vs "ND mice + IR" group. **E**, After MI/R induced IHF in HFD mice, Evans blue-triphenyltetrazolium chloride staining determined myocardial infarct size 24 hours after reperfusion, and Masson's trichrome staining determined collagen volume fraction 4 weeks after reperfusion, with/without intramyocardially injection of ADp sEVs 48 hours before MI. Scale bar=1 mm. **F**, Quantification of infarct size (Inf/ AAR× 100%) and fibrotic area. For results of infarct size, n=8 in the "HFD mice + IR" group, and n=10 in the "HFD mice + WT ADp sEV + IR" group. For results of fibrotic area, n=6/group, unpaired *t* test, ns indicates not significant, vs "HFD mice + IR" group. **G**, LV endomyocardial was determined based on representative images generated from speckle-tracking analysis in the long-axis B-mode and radial segmental synchronicity. **H**, Global strain and strain rate measured in the radial and longitudinal axes across the LV endocardium. For the "HFD mice" sham group, n=8/group. For "HFD mice + IR" and "HFD mice + WT ADp-sEV + IR" groups, n=10/group, mixed-effects 2-way ANOVA, ****P*<0.001, ns indicates not significant, vs "HFD mice + IR" group. ADp indicates adipocyte; EF(%), ejection fraction; HFD, high-fat diet; IHF, ischemic heart failure; IR, ischemia/reperfusion; LV, left ventricular; MI, myocardial infarction; MI/R, myocardial ischemia and reperfusion; ND, normal diet; PK, peak; sEV, small extracellular vesicle; WT, wild-type; and WT ADp sEV, sEVs isolated form the primary ADps of epididymal fat tissue of WT mice.

dysfunctional ADp sEVs promote remote organ pathological remodeling, including systemic insulin resistance,[18–21] cancer cell metastasis,[22] and atherosclerosis.[19] Our recent study demonstrated for the first time that sEVs from nondiabetic ADps significantly attenuate acute MI/R injury. In contrast, diabetic ADp-generated sEVs are vehicles carrying cytotoxic molecules from diabetic ADps to cardiomyocytes, significantly exacerbating MI/R injury.[23] However, several important questions remain unanswered. First, the cardioprotective molecules carried by ADp sEVs remain unidentified. Second, whether ADp sEVs may attenuate chronic post-MI remodeling and IHF remains unknown. Third, and most important, whether and how diabetes may alter cardiomyocyte properties and impair protective signaling remain unclear. Clarifying the protective role of ADp sEVs in ADp–cardiomyocyte communication and their alterations by diabetes is the foundation for developing specific interventions that block sEV-mediated, diabetes-exacerbated post-MI remodeling and IHF.

Through a combination of in vitro molecular mechanistic analysis and in vivo concept demonstration, we provided the first evidence that APN is enriched on the external surface of ADp sEVs and confers cardioprotection against chronic post-MI remodeling and IHF through the activation of cardiomyocyte AdipoR1. Diabetes disrupts this communication by enhancing GRK2 (G–protein-coupled receptor kinase)–mediated AdipoR1 phosphorylation, impairing sEV signaling, and exacerbating IHF.

METHODS

Detailed methods for ADp sEV isolation, cell isolation and culture, plasmid constructions and transfections, adeno-associated virus 9 (AAV9) vector production and infection, cell viability and apoptosis, Western and coimmunoprecipitation, echocardiography and strain analysis, and immunofluorescent cellular and Masson's trichrome staining are provided in the Supplemental Material. The data that support the findings of this study are available from the corresponding author on 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), APN knockout (KO), and AdipoR1 knockout (AdipoR1-KO) mice were used in the study. The selection of typical photos for cellular and animal investigations, as well as representative Western blot images, was conducted to align closely with the mean values of the measured parameters. To generate a cardiomyocyte-specific AdipoR1 mutation mouse line, a previously reported method for administering AAV to neonatal mice[24] was utilized. In brief, a total of 1×10^{11} viral genome particles per mouse of AAV9–cTNT-eGFP (cardiac troponin T–enhanced green fluorescent protein), AAV9-cTNT-AdipoR1^{WT}, or AAV9–cTNT-AdipoR1^{S205A} were injected subcutaneously into the nape of 5- to 7-day-old AdipoR1-KO neonatal mice. Stable cardiac-specific protein expression was evidenced by cardiac-specific eGFP expression 24 weeks after AAV9-cTNT–eGFP injection (Figure S1).

Adult (8 weeks of age) WT C57BL/6J mice, AdipoR1-KO mice (no AdipoR1 was detected; Figure S2) re-expressing AdipoR1WT (AdipoR1KO^{AdipoR1WT}), or AdipoR1-KO mice re-expressing AdipoR1S205A (AdipoR1KO^{AdipoR1S205A}) mice were fed a high-fat diet (HFD; 60% kcal fat, 20% kcal protein, 20% kcal carbohydrate, catalogue No. D12492; Research Diets) for 12 weeks to establish an HFD-induced type 2 diabetic model.[23] Age-matched C57BL/6J mice on a normal diet (ND) serve 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 coronary artery as previously described.[25] Ninety minutes after MI, the slipknot was released, allowing reperfusion for 3 hours (apoptosis assay), 24 hours (infarct size assay), and 4 weeks (post-MI remodeling and cardiac function assays). ADp sEVs (diluted in 20 µL of phosphate-buffered saline [PBS], 2×10^{8}/mice) or PBS (vehicle) were intramyocardially injected into the left ventricle at 3 distinct points distal of the planned coronary ligation site 48 hours before MI/R.

Statistical Analysis

All numerical information is presented as the mean±SEM. Using the Shapiro-Wilk test, the normality of the data was determined. Using the unpaired *t* test, comparisons were made

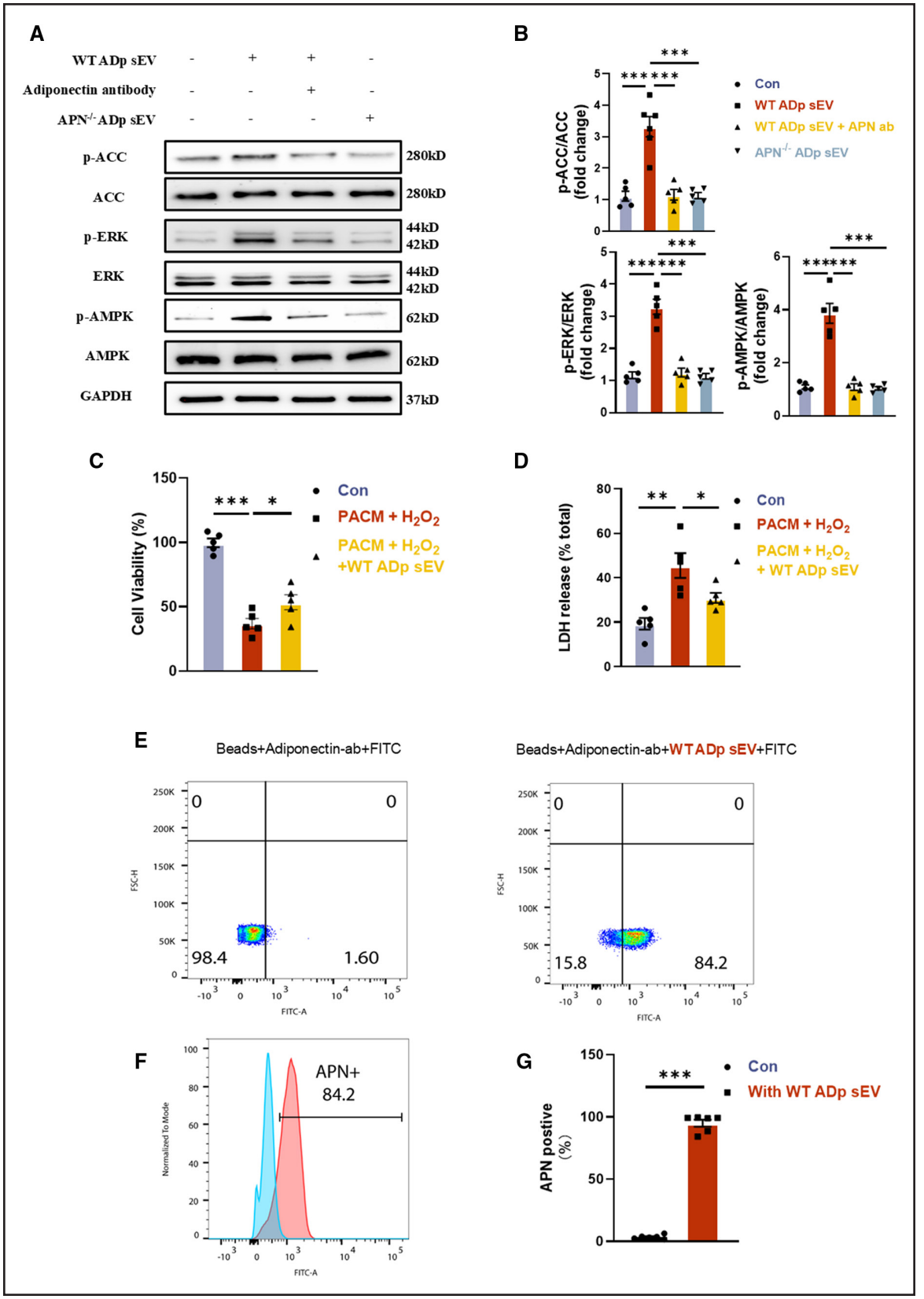

Figure 2. APN is located on the surface of ADp sEVs and activates injury salvage kinases in cardiomyocytes.
A, PAMCs were isolated from ND mice and incubated with ADp sEVs, ADp sEVs pretreated with an APN neutralization (NU) antibody, or APN-KO–ADp sEVs for 15 minutes. Effect of cell salvage kinase activation in cardiomyocytes induced by ADp sEVs, ADp sEVs pretreated with an APN neutralization antibody or APN-KO ADp sEVs. **B**, Quantification of the Western blot results normalized to GAPDH. n=5/group, one-way ANOVA, ***P<0.001 vs "WT ADp sEV" group, respectively. PAMC was isolated from ND mice and incubated with WT ADp sEVs for 24 hours, (*Continued*)

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Figure 2 Continued. followed by H_2O_2 treatment for 2 hours. **C**, PAMC viability was determined by MTT assay. **D**, PAMC injuries were tested by LDH assay. n=5/group, one-way ANOVA, *P<0.05, ** P<0.01, *** P<0.001 vs "PAMC + H_2O_2" group, respectively. **E** and **F**, The sEV-Flow experiment was performed to determine whether APN is located on the surface of ADp sEVs. **G**, Quantification of the Exo-Flow results; n=6, unpaired t test, *** P<0.001 vs Con group. ab indicates antibody; ADp indicates adipocyte; AMPK, AMP-activated protein kinase; APN, adiponectin; Con, control; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KO, knockout; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethythiazol-2-yl)2,5-diphenyltetrazolium bromide; ND, normal diet; PAMC, primary adult mouse cardiomyocyte; p-ACC, phosphorylated Acetyl-CoA carboxylase; p-AMPK, Phospho-5′-adenosine monophosphate-activated protein kinase; p-ERK, phosphorylated extracellular signal-regulated kinase; sEV, small extracellular vesicle; and WT, wild-type.

between the 2 groups; ≥3 groups were compared using 1- or 2-way ANOVA followed by the Tukey test for multiple comparisons. For the repeated measurements on the same mice in the echo analysis, mixed-effects 2-way ANOVA was used to account for the correlation of observations. GraphPad Prism 9.4.1 was used to conduct all statistical analyses. P values <0.05 were considered statistically significant.

RESULTS

ADp sEVs Attenuated Post-MI Remodeling and IHF in Nondiabetic but not in Diabetic Mice

We previously demonstrated that nondiabetic ADp-derived sEVs attenuate, whereas diabetic ADp-derived sEVs exacerbate, reperfusion injury in nondiabetic animals. To determine whether nondiabetic ADp sEVs may exert a sustained cardioprotective effect against post-MI remodeling and IHF, and more important, whether ADp sEVs might be an effective therapeutic intervention against post-MI remodeling in diabetic animals, 2×10^8/mice ADp sEVs or PBS was injected into the left anterior ventricle wall of nondiabetic or HFD mice. Similar to what we previously observed in the reperfusion model, administration of ADp sEVs significantly attenuated chronic post-MI remodeling and IHF in nondiabetic mice (Figure 1A through 1D). Unfortunately, intramyocardial injection of ADp sEVs failed to attenuate post-MI remodeling in diabetic mice (Figure 1E through 1H). These results indicated that use of ADp sEVs is an effective therapeutic intervention against chronic post-MI remodeling in nondiabetic mice but ineffective in diabetic mice.

ADp sEVs Rapidly Activated Injury Salvage Kinases and Attenuated Cardiomyocyte Oxidative Injury

Post-MI remodeling involves complex cellular mechanisms. To determine whether ADp sEVs exerted a direct protective effect against cardiomyocyte injury, we isolated primary adult mouse cardiomyocytes (PAMCs) and performed in vitro experiments. First, PAMCs were treated with ADp sEVs, and the time-dependent activation (5 to 30 minutes) of multiple injury salvage kinases (ISKs), including ERK (extracellular signal-regulated kinase), AMPK (AMP-activated protein kinase), and ACC (acetyl-CoA carboxylase), was determined. Interestingly, ADp sEVs rapidly (as early as 15 minutes) and significantly (P<0.01) activate these ISKs, as evidenced by increased phosphorylation forms of ISKs without altering their total protein levels (Figure 2A and 2B). To determine whether sEV activation of ISKs may be translated into a sustained cellular protective action, PAMCs were treated with H_2O_2 in the presence and absence of ADp sEVs. Cellular injury was determined by 3-(4,5-dimethythiazol-2-yl) 2,5-diphenyltetrazolium bromide and lactate dehydrogenase. Treatment with ADp sEVs significantly attenuated oxidative cell injury (Figure 2C and 2D). sEVs regulate recipient cell functions through 2 distinctive mechanisms: receptor-mediated signaling (rapid action in minutes) and endocytosis-dependent intracellular cargo delivery (slow but sustained action).[26–28] Collectively, these results suggest that cytoprotective molecules are present on the external surface of ADp sEVs, protecting cardiomyocytes from oxidative injury through receptor-mediated signaling.

APN Is Enriched on the External Surface of ADp sEVs and Is Responsible for sEV Cardioprotection

APN is an ADp-derived cytoprotective molecule. There was no significant difference in APN expression levels between ADps and ADp sEVs (Figure S3). Additionally, all 3 forms of APN (high, medium, and low molecular weights) were detected in ADp sEVs (Figure S4). Having demonstrated that ADp sEVs rapidly activate ISKs and attenuate cardiomyocyte injury, we reasoned that APN might be the external surface molecule of ADp sEVs responsible for their cardioprotective action. Several experiments were performed to test this novel hypothesis. First, we performed an in vitro experiment utilizing Exo-Flow (fluorescence-activated cell sorting), a recently developed technique that specifically detects sEV surface molecules (SBI and CSFLOWBASICA-1). The results showed that ADp sEVs carried high levels of APN on their external surface (Figure 2E through 2G). Second, ADp sEVs were pretreated with an APN neutralization (NU) antibody before their addition to PAMC. As summarized in Figure 2A and 2B, ADp sEVs pretreated with an APN-NU antibody (APN-Nu–ADp sEVs) lost their ability to activate ISKs. Third, ADp sEVs were isolated from APN-KO mice (in which APN expression was not detected; Figure S5), and their effect on ISK activation was determined. Similar to that observed with APN-NU antibody–pretreated sEVs, ADp sEVs from APN-KO mice (APN-KO–ADp sEVs)

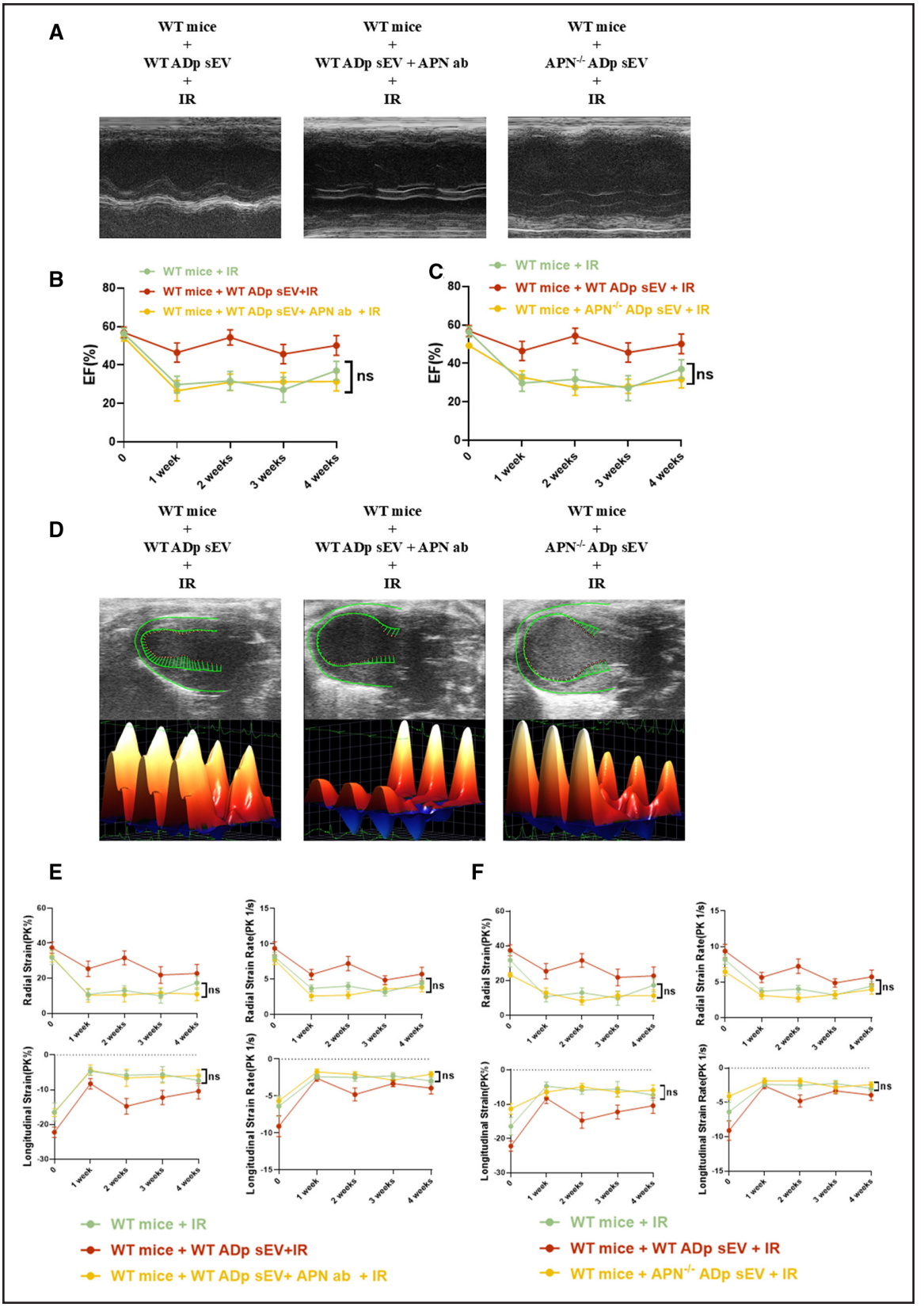

Figure 3. WT ADp sEVs protected MI/R injury and after IHF, but WT ADp sEVs pretreated with APN neutralization antibody or APN-KO ADp sEVs lost the cardioprotective effect.

WT mice were subjected to 90 minutes of MI and 4 weeks of reperfusion 48 hours after intramyocardial injection with WT ADp sEVs, WT ADp sEVs pretreated with APN neutralization antibody or APN-KO ADp sEVs. Echocardiography was performed each week after MI/R to determine the cardiac function. **A**, In M-mode, echocardiography was traced based on typical images. (*Continued*)

Figure 3 Continued. **B** and **C,** Quantitative analysis of cardiac function was performed by echocardiography after MI/R surgery and intramyocardial injection. n=10/group, mixed-effects 2-way ANOVA, ns indicates not significant, vs "WT mice + IR" group. **D**, In B-mode tracing, contraction (orange) and relaxation (blue) of left ventricular endomyocardial strain were analyzed based on 3-dimensional regional wall velocity diagrams. **E** and **F**, Quantitative analysis of strain and strain rate in the radial and longitudinal axes in long-axis B-mode. n=10/group, mixed-effects 2-way ANOVA. ns indicates not significant vs "WT mice + IR" group. ADp indicates adipocyte; APN, adiponectin; EF(%), ejection fraction; IHF, ischemic heart failure; IR, ischemia/reperfusion; KO, knockout; MI, myocardial infarction; MI/R, myocardial ischemia and reperfusion; PK%, peak; sEV, small extracellular vesicle; and WT, wild-type.

failed to activate ISK (Figure 2A and B). Finally, in vivo experiments were performed to determine whether ADp sEV cardioprotection is mediated by its external surface APN. ADp sEVs were isolated from WT mice or APN-KO mice. WT mice–derived sEVs were pretreated with either nonspecific or APN-NU antibodies. Three groups of ADps sEVs (ADp sEVs, APN-Nu–ADp sEVs, and APN-KO–ADp sEVs) were administered as described above. The quality control of sEVs was performed via Nanoparticle Tracking Analysis traces, Western blot anti–sEV-specific markers, and transmission electron microscopy (Figure S6). Their protective effect on post-MI remodeling was determined. Consistent with in vitro observations, administration of ADp sEVs significantly attenuated post-MI remodeling. However, neither APN-Nu–ADp sEVs nor APN-KO–ADp sEVs significantly protect against post-MI remodeling and IHF. Specifically, administration of APN-Nu–ADp sEVs or APN-KO–ADp sEVs had no significant effects on left ventricular ejection fraction (Figure 3A through 3C), left ventricular wall velocity (Figure 3D), the radial/longitudinal strain and their strain rates (Figure 3E and 3F), infarct size (Figure 4A and 4B), apoptotic death (Figure 4C through 4F), and cardiac fibrosis (Figure 4G and 4H). Collectively, these in vitro and in vivo experimental results demonstrated that ADp sEV surface–enriched APN is the molecule responsible for their cardioprotective property.

Cardiomyocyte AdipoR1 Mediates ADp sEV Cardioprotection, a Function Blocked by GRK2

Having demonstrated that APN is the critical molecule responsible for ADp sEV cardioprotection, we next investigated how diabetes may adversely impact cardiomyocytes and impair their response to ADp sEV cytoprotection. AdipoR1 is the dominant APN receptor expressed in cardiomyocytes. Neonatal cardiomyocytes were isolated from WT mice or AdipoR1-KO mice and treated with vehicle or ADp sEVs. ADp sEVs activated ACC, ERK, and APMK in WT neonatal cardiomyocytes but failed to do so in AdipoR1-KO neonatal cardiomyocytes (Figure 5A and 5B). Consistent with in vitro experimental results, the intramyocardial injection of ADp sEVs in AdipoR1-KO mice showed no protective effect against post-MI remodeling and the development of IHF (Figures 5 and 6). These results demonstrate that the ADp-expressed Exo surface–localized APN and cardiomyocyte-expressed AdipoR1 are essential for protective communication between ADps and cardiomyocytes. In addition to AdipoR1, the role of T-cadherin in mediating the signaling effects of APN presented on the surface of ADp sEVs was investigated. Knockdown of T-cadherin using short hairpin RNA significantly reduced ADp sEV–induced AMPK phosphorylation, a key event in APN-mediated cardioprotective signaling. These results indicate that T-cadherin partially contributes to the transmission of cardioprotective signals by ADp sEVs (Figure S7).

We recently reported that GRK2 causes AdipoR1 phosphorylation at Ser^{205}, promoting its endocytosis and blocking APN transmembrane signaling.[29] AdipoR1-KO neonatal cardiomyocytes were transfected with a plasmid expressing either a WT AdipoR1 ($AdipoR1^{WT}$) or mutant AdipoR1 ($AdipoR1^{S205A}$) with/without co-transfection with GRK2. Forty-eight hours after transfection, cardiomyocytes were treated with ADp sEVs. GRK2 overexpression blocked ADp sEV activation of ACC, AMPK, and ERK in $AdipoR1^{WT}$ neonatal cardiomyocytes. However, ADp sEV activation of ACC, AMPK, and ERK was not affected by GRK2 in $AdipoR1^{S205A}$ re-expression cardiomyocytes (Figure 7A through 7D). Moreover, GRK2 overexpression in $AdipoR1^{WT}$ cardiomyocytes enhanced oxidative stress–induced cell death, as evidenced by poorer cell viability and higher LDH release (Figure 7E and 7G, right). However, GRK2 overexpression in $AdipoR1^{S205A}$ cardiomyocytes did not increase oxidative stress–induced cell death (Figure 7E through 7H; Figure S8). Similar to the H_2O_2-induced oxidative stress model, WT ADp sEVs protected against the increased cell death and injury of adult mouse cardiomyocytes caused by the simulated ischemia/reoxygenation (SI/R) model. In the neonatal cardiomyocytes isolated from $AdipoR1^{KO}$ mice and reconstituted with $AdipoR1^{WT}$, WT ADp sEVs significantly attenuated simulated SI/R injury; however, this protective effect was abolished upon co-overexpression of GRK2. Notably, phosphorylation-resistant mutant $AdipoR1^{S205A}$ overexpression preserved the cardioprotective effect of ADp sEVs, even in the presence of GRK2 overexpression (Figure S9).

Diabetes Significantly Upregulated GRK2 Expression and Caused AdipoR1 Phosphorylation, and a Phosphorylation-Resistant AdipoR1 Knockin Rescued ADp sEV Cardioprotection in Diabetic Animals

The ischemic upregulation of GRK2 and its critical role in post-MI remodeling are well recognized. However, whether GRK2 is upregulated in diabetic hearts and contributes to diabetic exacerbation of IHF remains

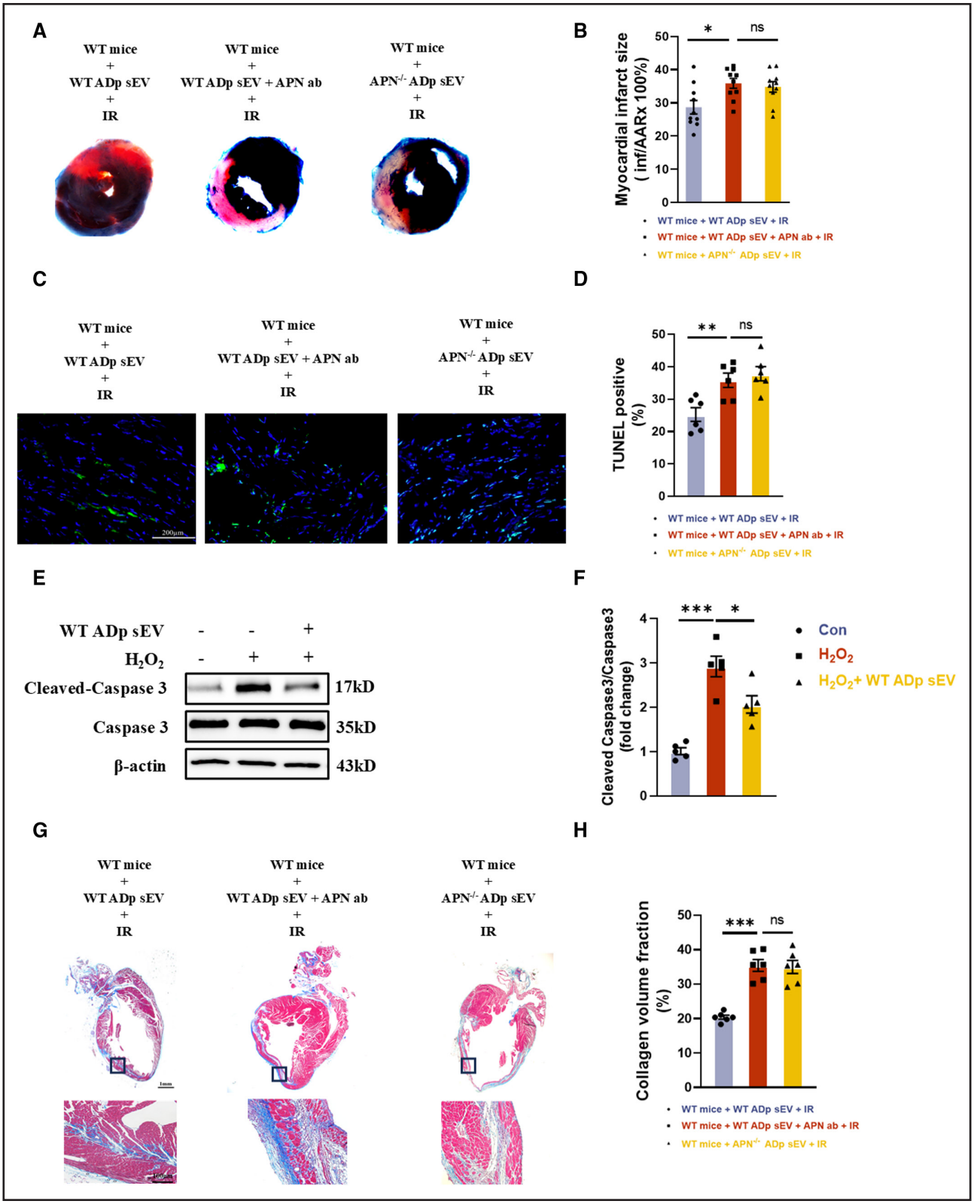

Figure 4. APN on the surface of WT ADp sEVs reduced heart injury and remodeling after MI/R.
WT ADp sEVs, WT ADp sEVs pretreated with APN neutralization antibody, or APN-KO ADp sEVs were intramyocardially injected into the heart. Forty-eight hours later, the mice received MI/R surgery. **A**, Evans blue-triphenyltetrazolium chloride double stain showed the infarct size of the heart 24 hours after reperfusion. **B**, Quantification of infarct size (Inf/ AAR× 100%). For the "WT mice + WT ADp sEV + IR" group, n=8/group. For "WT mice + WT ADp sEV + APN ab + IR" and "WT mice + APN$^{-/-}$ ADp sEV + IR" groups, n=10/group, one-way ANOVA, (*Continued*)

Figure 4 Continued. *P<0.05, ns indicates not significant, vs "WT mice + WT ADp sEV + APN ab + IR" group. **C**, Three hours after reperfusion, the heart was collected and TUNEL staining was performed based on the freezing microtome section of the heart. Scale bar=200 μm. **D**, Quantification of TUNEL-positive signal. n=6/group, one-way ANOVA, **P<0.01, ns indicates not significant, vs "WT mice + WT ADp sEV + APN ab + IR" group. **E**, PAMCs were isolated from WT mice and incubated with WT ADp sEVs for 48 hours. After that, PAMCs were treated with H_2O_2 for 2 hours, followed by Western blot anti–Cleaved-Caspase 3 or anti-Caspase 3 to determine the apoptosis of PAMCs. **F**, Quantitation of Western blot results based on **E**. n=5/group, one-way ANOVA, *P<0.05, ***P<0.001, vs "H_2O_2" group, respectively. **G**, Representative images of Masson's trichrome staining of the coronal plane. Scale bars=1 mm (**top**) and 100 μm (**bottom**). **H**, Quantification of fibrotic area. n=6/group, one-way ANOVA, ***P<0.001, ns indicates not significant, vs "WT mice + WT ADp sEV + APN ab + IR" group. ab indicates antibody; ADp, adipocyte; APN, adiponectin; Con, control; IR, ischemia/reperfusion; KO, knockout; MI/R, myocardial ischemia and reperfusion; sEV, small extracellular vesicle; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; and WT, wild-type.

unclear. Two in vivo experiments were conducted to address this critical question. AAV9-cTNT-AdipoR1^{WT} or AAV9-cTNT-AdipoR1^{S205A} were injected subcutaneously into the nape of 5- to 7-day-old AdipoR1-KO neonatal mice. At the age of 8 weeks, mice were fed HFD for 12 weeks. Cardiac GRK2 expression was significantly upregulated compared with nondiabetic hearts, and AdipoR1 phosphorylation was significantly increased in diabetic AdipoR1^{WT} re-expression hearts without coronary artery ligation, but was resisted in diabetic AdipoR1^{S205A} re-expression hearts (Figure 8A). In a separate cohort of animals, diabetic mice were randomized to receive vehicle or ADp sEV intramyocardial injection and subjected to MI/R, exhibiting enhanced uptake of ADp sEVs (Figure S10). Administration of ADp sEVs in AdipoR1^{WT} re-expression mice did not attenuate the diabetic exacerbation of ischemic heart injury. However, the cardioprotective effect of ADp sEVs was preserved in the AdipoR1^{S205A} re-expression heart. Specifically, administration of ADp sEVs in diabetic AdipoR1KO^{AdipoR1S205A} animals significantly reduced apoptosis (determined 3 hours after reperfusion; Figure 8B), reduced infarct size (determined 24 hours after reperfusion; Figure 8C), improved cardiac function, and reduced interstitial fibrosis (determined 4 weeks after reperfusion; Figure 8D through 8F). These results indicated that re-expression of a phosphorylation-resistant AdipoR1 rescued the cardioprotective effects of ADp sEVs in the diabetic heart. To further investigate the relationship between ADp sEVs and cardioprotective signaling, we focused on the AMPK and ERK pathways, given that ACC is a well-established downstream target of AMPK. Primary neonatal mouse cardiomyocytes were transfected with small interfering RNA targeting either AMPK or ERK, followed by treatment with ADp sEVs and H_2O_2-induced oxidative stress. Our MTT and LDH results showed that knockdown of either AMPK or ERK significantly reduced the protective effects of ADp sEVs, with AMPK knockdown producing a more pronounced inhibitory effect (Figure S11).

DISCUSSION

Cardiovascular complications, particularly ischemic heart disease, are the primary cause of death in patients with obesity and type 2 diabetes. Excessive and dysfunctional visceral ADps are the culprits of obesity-induced diabetes, whereas cardiomyocytes are the victims, most significantly contributing to diabetic cardiovascular death. A comprehensive understanding of the molecular mechanisms that mediate communication between ADps and cardiomyocytes may lead to the discovery of novel, effective therapies against diabetic cardiac injury. Our current study made several significant observations. First, we demonstrated for the first time that ADp sEVs carry cardioprotective molecules on their external surface. They activate multiple injury salvage kinases and significantly attenuate ischemic heart injury in nondiabetic but not in diabetic animals. Second, we provided the first direct evidence that sEV external–surface APN is biologically active and responsible for ADp–cardiomyocyte protective communication. Third, we demonstrated that GRK2 is significantly upregulated in diabetic cardiomyocytes, leading to AdipoR1 phosphorylation, which blocks ADp sEV cardioprotection and contributes to the exacerbation of IHF in diabetes (Figure 8G).

The pathological roles of diabetic ADp sEVs in the development of cardiovascular complications have been extensively investigated. Research from numerous investigators, including our group, has shown that ADp sEVs from obesity/diabetes lead to pathological remodeling of distant organs through complex mechanisms.[18,20–22,30] However, the physiological and protective roles of sEVs from nondiabetic ADps in regulating distant organs have only started to gain attention in recent years. sEVs from nondiabetic ADps[31] or adipose tissue macrophage[16] improves liver and skeletal muscle insulin sensitivity in diabetic mice. Additionally, nondiabetic ADp sEVs display pancreatic tropism, thereby improving pancreatic cell function.[17] We recently reported that diabetes switches ADp sEV phenotype from protective against acute reperfusion injury to harmful to reperfused cardiomyocytes.[23] However, whether and how sEVs from nondiabetic ADps may protect the heart from chronic post-MI remodeling and IHF, and whether and how this protective signaling is adversely altered by diabetes, have not been previously investigated.

sEV regulates recipient cell functions through 2 distinctive mechanisms: receptor-mediated signaling and endocytosis-dependent intracellular cargo delivery.[26–28] During the past 15 years, most sEV-mediated communications have been focused on the intercellular transfer of sEV cargoes, such as miRNAs, lipids, proteins, and organelles.[32,33] However, there is an increasing recognition

ORIGINAL RESEARCH ARTICLE

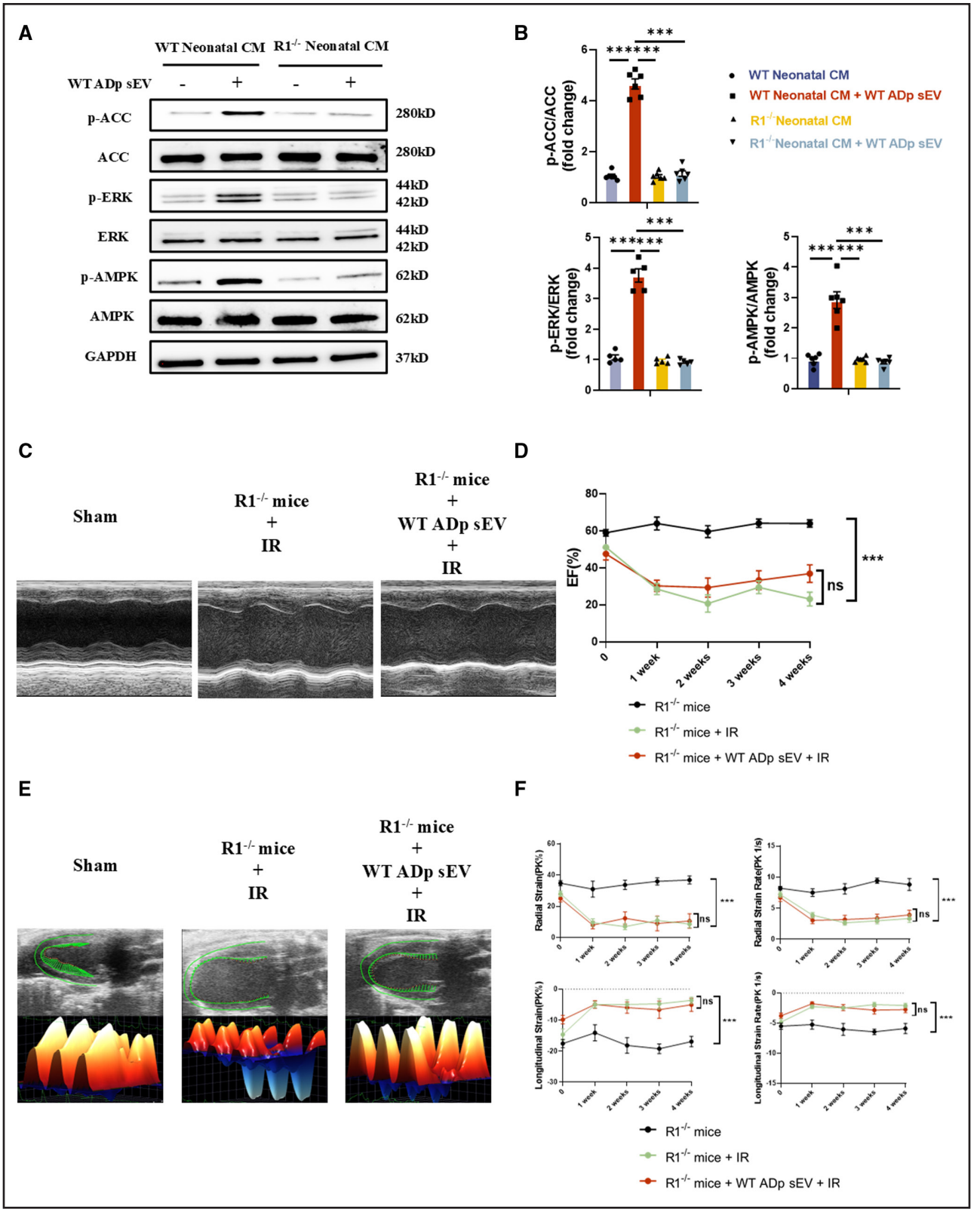

Figure 5. AdipoR1 knockout in cardiomyocytes lost the cardioprotective effect induced by APN located on the WT ADp sEVs. AdipoR1 in cardiomyocytes is responsible for the ADp sEV–induced cardioprotection. **A**, NMVMs were isolated from WT mice or AdipoR1 knockout mice and incubated with/without WT ADp sEVs for 15 minutes. Cell salvage kinases, including ACC, AMPK, and ERK, were determined in WT and AdipoR1-KO cardiomyocytes. **B**, Quantification of the Western blot results. **A**, Normalized to GAPDH; n=5/group; one-way ANOVA; ***$P<0.001$ vs "WT Neonatal CM + WT ADp sEV" group, respectively. Forty-eight hours after intramyocardial injection with PBS or WT ADp sEV, MI/R surgery was performed and followed by echocardiography every week. **C**, Representative echocardiographic images acquired (*Continued*)

Figure 5 Continued. from M-mode tracing. **D**, Quantitative analysis of LVEF to determine the cardiac function. For the "R1$^{-/-}$ mice" sham group, n=8/group. For "R1$^{-/-}$ mice + IR" and "R1$^{-/-}$ mice + WT ADp sEV + IR" groups, n=10/group, mixed-effects 2-way ANOVA, ***P<0.001, ns indicates not significant, vs "R1$^{-/-}$ mice + IR" group. **E**, Three consecutive cardiac cycles of left ventricular endomyocardial strain in 3-dimensional regional wall velocity diagrams were gained via B-model tracing after MI/R surgery. **F**, Quantitative analysis of longitudinal/radial strain and strain rate based on **E**. For the "R1$^{-/-}$ mice" sham group, n=8/group. For "R1$^{-/-}$ mice + IR" and "R1$^{-/-}$ mice + WT ADp sEV + IR" groups, n=10/group, mixed-effects 2-way ANOVA; ***P<0.001. CM indicates cardiomyocyte; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ns indicates not significant vs "R1$^{-/-}$ mice+IR" group; R1$^{-/-}$ mice, AdipoR1 knockout mice. ACC indicates acetyl-CoA carboxylase; AdipoR1, adiponectin receptor-1; ADp, adipocyte; AMPK, AMP-activated protein kinase; APN, adiponectin; EF(%), ejection fraction; ERK, extracellular signal-regulated kinase; IHF, ischemic heart failure; IR, ischemia/reperfusion; KO, knockout; LVEF, left ventricular ejection fraction; MI/R, myocardial ischemia and reperfusion; NMVM, neonatal mouse ventricular cardiomyocyte; p-ACC, phosphorylated Acetyl-CoA carboxylase; p-AMPK, Phospho-5′-adenosine monophosphate-activated protein kinase; PBS, phosphate-buffered saline; p-ERK, phosphorylated extracellular signal-regulated kinase; PK%, peak; sEV, small extracellular vesicle; and WT, wild-type.

that proteins located on the sEV external surface play a critical role in mediating sEV communication with remote organs.[34] sEVs derived from malignant osteosarcoma cells carries TGFβ on the external surface (associated with CD63^{+}), which binds to TGFβ receptors on mesenchymal stem cells and initiates pathological signaling.[35] In contrast, the sEVs from human placental-expanded stromal cells promotes angiogenesis, skin regeneration, and immunomodulation.[36] Removing the sEV external surface proteins abolishes these effects.[36] Our current study demonstrated that ADp sEVs rapidly activate multiple injury salvage kinases in nondiabetic cardiomyocytes, significantly attenuating oxidative stress–induced myocardial injury. These results indicate that the novel sEV external surface molecules–mediated intercellular communication is involved in ADp–cardiomyocyte protective signaling.

Compared with sEV cargo delivery regulation, sEV external molecule–mediated cell–cell communication has several advantages. First, the sEV external surface proteins activate their receptors on target cells, immediately triggering intracellular signaling. This allows sEVs to achieve their regulatory effects more rapidly than through intracellular cargo delivery by sEVs. Rapid action is crucial in protecting against acute cellular damage, such as that caused by myocardial ischemia/reperfusion injury. Second, sEV modification has been an area of intense research in recent years, with the goal of enhancing drug delivery and therapeutic efficacy. Bioengineering modifications to the molecules on the sEV external surface are likely more practical than modifications to the cargo content within the sEVs. Third, and more important, sEV external surface molecules may determine the cell/tissue selective recognition,[28,34,37] which not only ensures cell-selective signaling activation but may also regulate cell-selective cargo delivery (acting either as opsonins to facilitate or as dysopsonins to decrease the cellular uptake of the sEVs). In this regard, a recent study demonstrated that the integrin isoforms on the external surface of cancer cell sEVs determine organ-specific sEV cargo delivery.[38]

APN is an ADp-specific protein regarded as "ADp-derived insulin" with potent cardioprotective effects.[39,40] Considering that ADps are the primary cells of APN expression, it is unsurprising that ADp sEVs contain high levels of APN, which were significantly suppressed by diabetes (Figure S12). However, 2 unique properties of ADp sEV–carrying APN are exciting and important. First, ADp sEVs contain high levels of APN but deficient levels of leptin.[41] Given that leptin is an ADp-specific protein (the first identified ADp-specific cytokine), the absence of leptin in ADp sEVs suggests that APN is selectively packaged into ADp sEVs. We further corroborated this observation in the present study. After culturing primary ADps and isolating ADp sEVs, we collected protein samples from the primary ADps, purified the ADp sEVs, and the ADp sEV–depleted conditioned medium. Western blot analyses showed that leptin was readily detected in ADps and was abundant in the sEV-free conditioned medium, whereas it was extremely low (barely detectable) in the ADp sEV fraction (Figure S13). These findings support selective cargo packaging and indicate that ADp-derived leptin is secreted predominantly as a soluble extracellular protein with minimal loading into ADp sEVs. Accordingly, leptin carried by ADp sEVs is unlikely to be a major contributor to the cardiovascular effects observed in our study. This observation further highlights the potential for adipokine-specific sorting into ADp sEVs, suggesting that distinct adipokines may engage in cardiovascular signaling through different secretion and delivery modes. Second, chemical/biochemical experiments indicate that APN exists as an external surface protein in ADp-derived sEVs.[41] A very recent study demonstrated that ADp sEV external surface APN improves systemic insulin sensitivity, attenuates interleukin-1β and tumor necrosis factor-alpha release, and inhibits liver macrophage infiltration in HFD-induced diabetic mice.[31] Our current study provided more compelling and earliest evidence that ADp sEV external surface APN is responsible for ADp remote protection against ischemic cardiomyocyte injury. First, utilizing a recently developed flowcytometry method (Exo-Flow, System Biosciences), which specifically detects sEV external surface–located molecules,[42] we provide direct evidence that APN is present on the ADp sEV external surface. Second, we demonstrated that ADp sEVs isolated from APN-KO mice failed to protect the heart against post-MI remodeling and IHF.

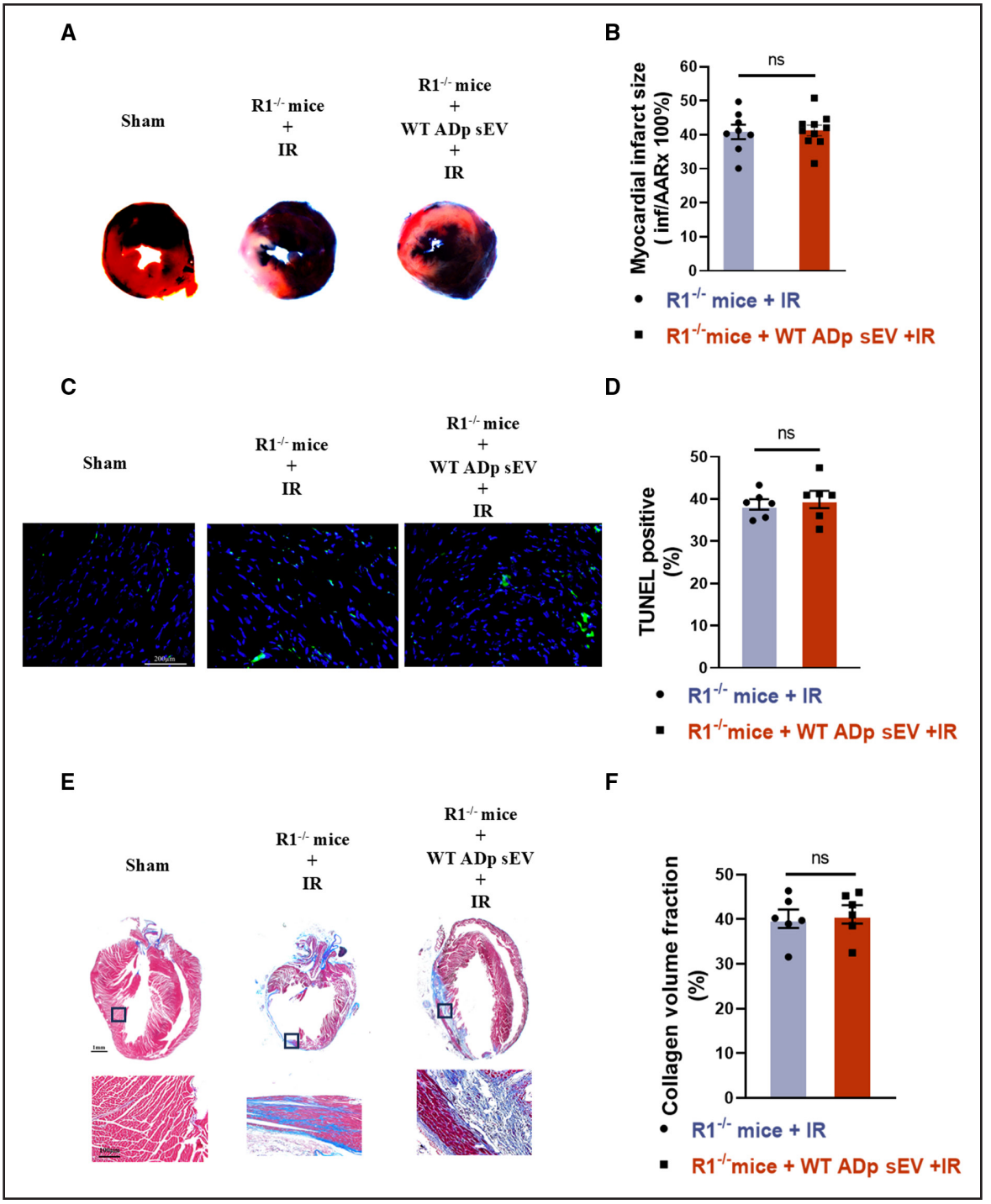

Figure 6. AdipoR1-KO in cardiomyocytes lost the response to the WT ADp sEV cardioprotective effect, which enhanced heart injury and remodeling after MI/R.

WT ADp sEVs were intramyocardially injected into the heart of AdipoR1-KO mice. After 48 hours, the mice received MI/R surgery. **A**, Twenty-four hours after reperfusion, heart was collected and Evans blue-triphenyltetrazolium chloride double stain was performed to determine the infarct size. **B**, Quantification of infarct size (inf/AAR× 100%) was analyzed based on **A**. For the "R1$^{-/-}$ mice + IR" group, n=8/group. For the "R1$^{-/-}$ mice + WT ADp sEV + IR" group, n=10/group, unpaired *t* test, ns indicates not significant, vs "R1$^{-/-}$ mice + IR" group. **C**, Three hours after reperfusion, the heart was removed for the freezing microtome section followed by TUNEL staining to show the cardiomyocytes apoptosis after MI/R. Scale bar=200 μm. **D**, Quantification of TUNEL-positive signal. n=6/group, unpaired *t* test, ns indicates not significant, vs "R1$^{-/-}$ mice + IR" group. **E**, Representative images of Masson's trichrome staining of the coronal plane were acquired after 4 weeks of reperfusion. Scale bars=1 mm (**top**) and 100 μm (**bottom**). **F,** Quantification of fibrotic area. n=6/group, unpaired *t* test, ns indicates not significant, vs "R1$^{-/-}$ mice + IR" group. AdipoR1 indicates adiponectin receptor-1; ADp, adipocyte; IR, ischemia/reperfusion; KO, knockout; MI/R, myocardial ischemia and reperfusion; sEV, small extracellular vesicle; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; and WT, wild-type.

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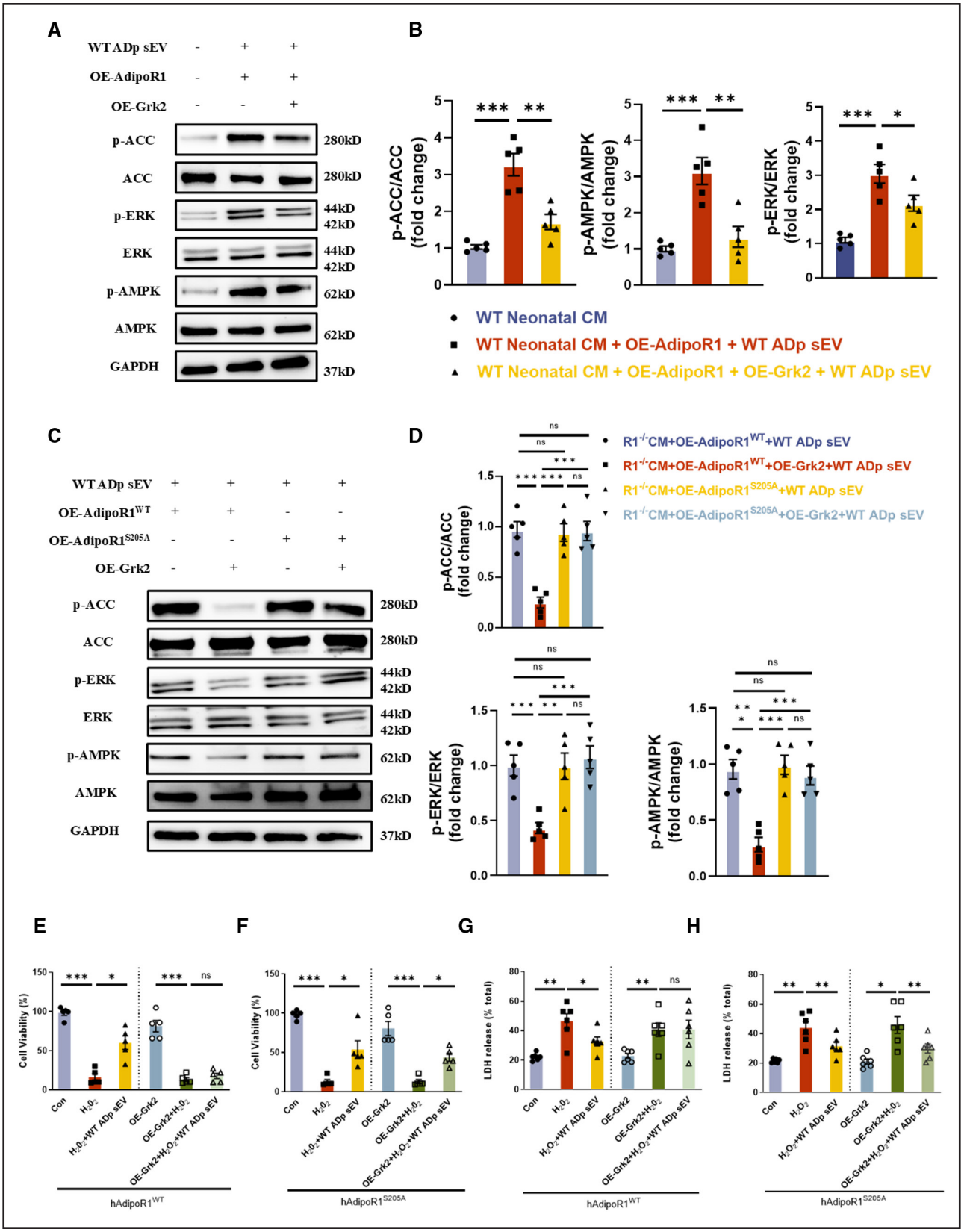

Figure 7. AdipoR1^{S205} phosphorylation induced by Grk2 in cardiomyocytes is responsible for the resistance of WT ADp sEV cardioprotection.

A, NMVMs were isolated from AdipoR1 knockout mice and transfected with plasmids overexpressing AdipoR1 with/without plasmids overexpressing Grk2. 48 hours later, WT ADp sEVs were added for a 15-minute incubation. Western blot was performed to determine the activation of ISKs (pACC, pAMPK, and pERK). **B**, Quantitative analysis of the Western bolt results in **A**. n=5/group, one-way ANOVA, (*Continued*)

Figure 7 Continued. *P<0.05, **P<0.01, ***P<0.001 vs "WT Neonatal CM + OE-AdipoR1 + WT ADp sEV" group, respectively. **C**, AdipoR1^{WT} or AdipoR1^{S205A} was overexpressed in AdipoR1-KO NMVMs, at the same time, Grk2 overexpression plasmid was transfected (**right**) or not (**left**). Forty-eight hours later, WT ADp sEVs were used to incubate with NMVMs for 15 minutes, followed by Western blot to determine the activation of ISKs (pACC, pAMPK, and pERK). **D**, Quantitative analysis of the Western blot results in **C**. n=5/group, one-way ANOVA, **P<0.01, ***P<0.001, ns indicates not significant. **E** and **F**, AdipoR1-KO NMVMs, overexpressed with AdipoR1^{WT} or AdipoR1^{S205A}, were incubated with WT ADp sEVs for 24 hours and then treated with H_2O_2 for 2 hours. The MTT assay was performed to test the viability of the NMVMs. **G** and **H**, AdipoR1^{WT} or AdipoR1^{S205A} overexpressed AdipoR1-KO NMVMs were treated with WT ADp sEVs and H_2O_2 as in **E** and **F**; NMVM injuries were tested by LDH assay. n=5/group, one-way ANOVA, *P<0.05, **P<0.01, ***P<0.001, ns indicates not significant, vs "H_2O_2" group (**left**) or "OE-Grk2 + H_2O_2" group (**right**), respectively. AdipoR1 indicates adiponectin receptor-1; ADp, adipocyte; CM, cardiomyocyte; ERK, extracellular signal-regulated kinase; Grk2, G-protein–coupled receptor kinase 2; ISK, injury salvage kinase; KO, knockout; LDH, lactate dehydrogenase; MTT, MTT, 3-(4,5-dimethythiazol-2-yl)2,5-diphenyltetrazolium bromide; NMVM, neonatal mouse ventricular cardiomyocyte; OE, overexpression; pACC, phosphorylated Acetyl-CoA carboxylase; pAMPK, Phospho-5′-adenosine monophosphate-activated protein kinase; pERK, phosphorylated extracellular signal-regulated kinase; sEV, small extracellular vesicle; and WT, wild-type.

Third, and most interesting, we showed for the first time that incubation of ADp sEVs with an APN-NU antibody blocked their cardioprotective function. Finally, we demonstrated that the AdipoR1-deficient hearts lose their protective response to ADp sEVs. To determine whether ADp sEVs may also modulate angiogenic signaling, we performed additional in vitro studies using primary cardiac microvascular endothelial cells (PCMECs) isolated from adult mouse ventricles. Hearts were enzymatically digested, and the cell suspension was sequentially filtered. Red blood cells were lysed, and endothelial cells were enriched by anti-CD31 magnetic bead selection, then plated on collagen I–coated dishes in endothelial growth medium. PCMECs were then treated with ADp sEVs or vehicle for 48 hours, followed by Western blot analysis of the pro-angiogenic markers VEGFA and VEGFR2. As shown in Figure S14, ADp sEV treatment markedly increased vascular endothelial growth factor A (VEGFA) and vascular endothelial growth factor receptor 2 (VEGFR2) expression in PCMECs, indicating activation of a pro-angiogenic program in vitro that likely contributes, together with the direct cardiomyocyte-protective effects, to the overall cardioprotection observed in vivo. To the best of our knowledge, this is the first study to clarify the molecular mechanism responsible for sEV-mediated protective signaling between ADps and cardiomyocytes, as well as cardioprotection.

Compared with soluble APN, sEV external surface APN has several advantages. First, APN association with sEVs significantly extends its half-life, thus intensifying its biological activity.[31] Second, the APN association with sEVs may prevent their pathological modification by circulating molecules, particularly under diabetic conditions.[43] Third, APN association with sEVs may facilitate their crossing of biological barriers and interaction with targeting cells. Finally, the external surface APN of sEVs may play a critical role in cell-specific communication between ADps and cells expressing high levels of APN receptors (such as cardiomyocytes, hepatocytes, and skeletal muscle cells), promoting the targeted delivery of sEV cargoes.

Two evolutionarily acquired specific APN receptors (AdipoR1 and AdipoR2) have been cloned.[44] They belong to a new family of membrane receptors (PAQRs) predicted to contain 7 transmembrane domains, similar to G-protein–coupled receptors (GPCRs), but structurally and topologically distinct.[45] APN can also bind to T-cadherin, in an interaction that only tethers APN to the cell surface without transmembrane signaling, as T-cadherin lacks an intracellular domain.[46] Activation of AdipoR1 (primarily expressed in muscular cells) and AdipoR2 (primarily expressed in hepatocytes) increases glucose and free fatty acid utilization, stimulates mitochondrial biogenesis, and inhibits inflammatory response.[22–26] We recently demonstrated that AdipoR1, but not AdipoR2, is a potent substrate of GRK2.[47] GRK2 phosphorylates AdipoR1 at S205, promoting its endocytosis and blocking APN cardioprotection.[29] Although the upregulation of cardiac GRK2 in the ischemic heart and its critical contribution to IHF are well-recognized, the role of GRK2 in diabetic exacerbation of IHF remains unexplored. Our current study demonstrated that HFD-induced type 2 diabetes significantly upregulated cardiac GRK2 and caused AdipoR1 phosphorylation before MI, blocking ADp sEV cardioprotection and contributing to diabetic exacerbation of IHF. Notably, the re-expression of a mutant, phosphorylation-resistant AdipoR1 (AdipoR1^{S205A}) preserves the activation of cardiomyocyte AdipoR1 by ADp sEVs, protecting diabetic IHF.

A limitation of this study is that ADp sEVs were isolated exclusively from epididymal (visceral) adipose tissue, without direct comparison with sEVs derived from other fat depots such as subcutaneous adipose tissue. Our focus on epididymal fat was driven by its central role as the principal visceral depot implicated in diabetic cardiac complications. Future studies will evaluate the efficacy of ADp sEV administration after MI/reperfusion, using alternative delivery routes or animal models that permit postreperfusion dosing, and will systematically compare sEVs from visceral versus subcutaneous and other adipose depots with more fully defined depot-specific contributions to diabetic IHF. Another limitation of this study is that ADp sEVs were delivered by pre-MI intramyocardial injection in a preventive paradigm, rather than as postreperfusion therapy, and thus do not fully recapitulate the clinical scenario in which treatment is initiated after reperfusion. This timing reflects the technical constraints of the Gao/Koch ischemia–reperfusion model, in which intramyocardial injection can be safely performed only during

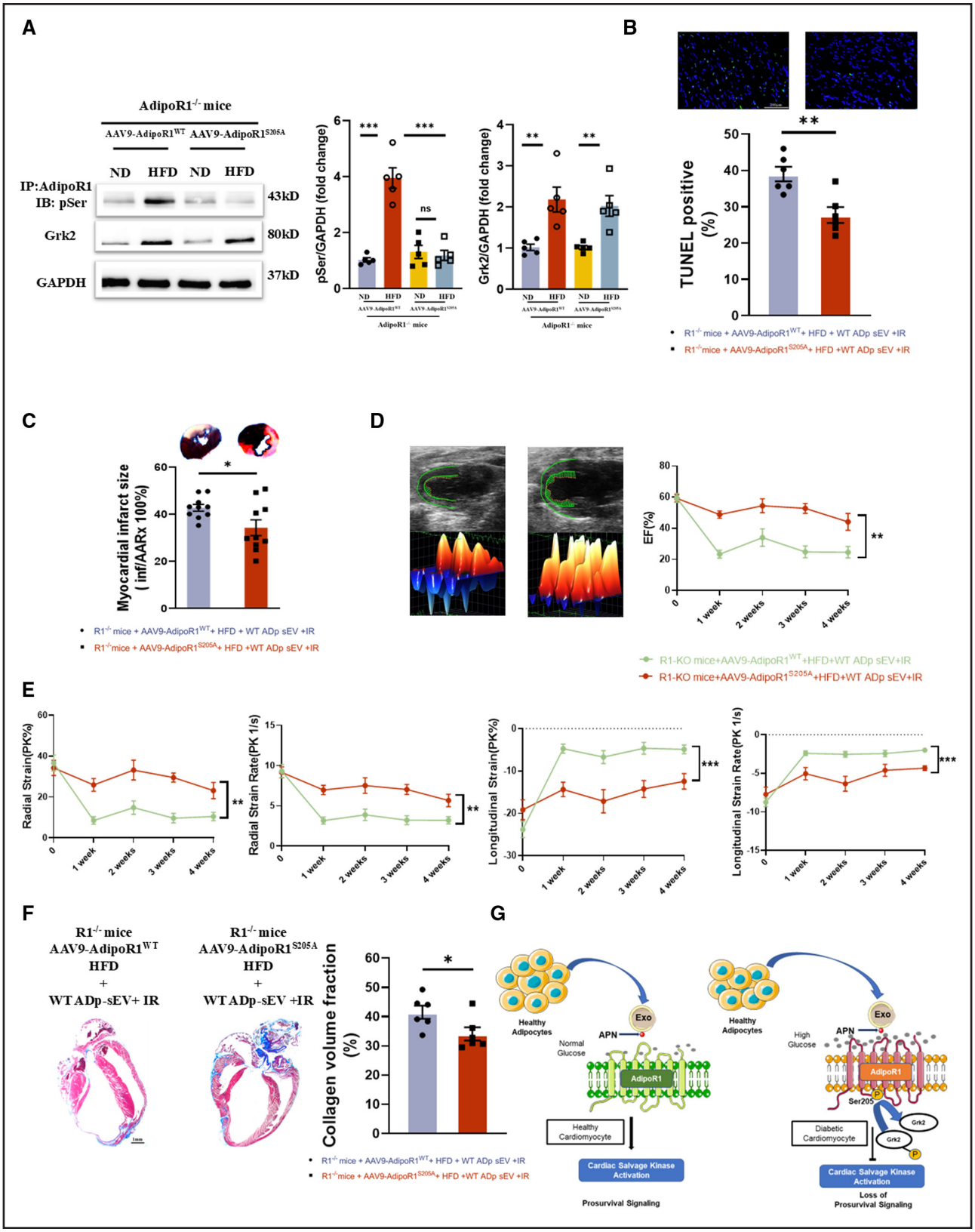

Figure 8. AdipoR1^{S205A} overexpression in cardiomyocytes resisted AdipoR1 phosphorylation and rescued WT ADp sEV cardioprotection in HFD mice.

AdipoR1^{WT} or AdipoR1^{S205A} was overexpressed in the heart of AdipoR1-KO mice, followed by 12 weeks of HFD. After that, WT ADp sEVs were intramyocardially injected; 48 hours later, the mice were subjected to 90-minute MI and reperfusion. **A**, AAV9-cTNT–AdipoR1^{WT} or AAV9-cTNT–AdipoR1^{S205A} was injected into AdipoR1-KO mice to generate AdipoR1^{WT} or AdipoR1^{S205A} heart-specific expression mice. (*Continued*)

Figure 8 Continued. After 12 weeks of HFD, the hearts were harvested, and CoIP was performed to determine the Ser phosphorylation in AdipoR1 protein, and a Western blot was performed to determine the expression of AdipoR1 and Grk2. In HFD mice, Grk2 was overexpressed in CM, which induced phosphorylation of AdipoR1. However, overexpression of the AdipoR1^{S205A} mutant protein in CM resisted phosphorylation in HF mice. n=5/group, one-way ANOVA, $^{**}P<0.01$, $^{***}P<0.001$, ns indicates not significant. **B**, Three hours after MI/R surgery, the heart was removed and frozen-microtome sectioned. Scale bar=200 μm. TUNEL staining was used to show cardiomyocyte apoptosis. n=6/group, unpaired *t* test, $^{**}P<0.01$ vs "R1$^{-/-}$ mice + AAV9-AdipoR1^{WT} + HFD + WT ADp sEV + IR" group. **C**, Twenty-four hours after reperfusion, Evans blue-triphenyltetrazolium chloride double stain was performed in the heart to determine the infarct size (Inf/ AAR× 100%), and the quantification of infarct size was analyzed. n=10/group, unpaired *t* test, $^{*}P<0.05$ vs "R1$^{-/-}$ mice + AAV9-AdipoR1^{WT} + HFD + WT ADp sEV + IR" group. **D**, Via B-mode tracing, 3 consecutive cardiac cycles from 3-dimensional regional wall velocity diagrams of LV endomyocardial strain showed the contraction (orange) and relaxation (blue) of the heart. And the quantitative analysis of EF% across the LV endocardium. n=10/group, mixed-effects 2-way ANOVA, $^{**}P<0.01$ vs "R1$^{-/-}$ mice + AAV9-AdipoR1^{WT} + HFD + WT ADp sEV + IR" group. **E**, Quantitative analysis of longitudinal/radial strain and strain rate. n=10/group, mixed-effects 2-way ANOVA, $^{**}P<0.01$, $^{***}P<0.001$ vs "R1$^{-/-}$ mice + AAV9-AdipoR1^{WT} + HFD + WT ADp sEV + IR" group. **F**, AdipoR1^{S205A} overexpression in cardiomyocytes responded to WT ADp sEV cardioprotection and resisted fibrotic area in HFD mice. Scale bar=1 mm. n=6/group, unpaired *t* test, $^{*}P<0.05$ vs "R1$^{-/-}$ mice + AAV9-AdipoR1^{WT} + HFD + WT ADp sEV + IR" group. **G**, Graphic illustration. APN located on the surface of ADp sEV acts as the critical executor of ADp-CM communication, mediating ADp sEV cardioprotection. GRK2-induced CM AdipoR1 phosphorylation blocks ADp sEV protective action, contributing to IHF progression. AdipoR1 indicates adiponectin receptor-1; ADp, adipocyte; CM, cardiomyocyte; CoIP, coimmunoprecipitation; EF%, ejection fraction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grk2, G-protein–coupled receptor kinase 2; HFD, high-fat diet; IB, immunolotting; IHF, ischemic heart failure; IP, immunoprecipitation; IR, ischemia/reperfusion; KO, knockout; LV, left ventricular; ND, normal diet; PK%, peak; sEV, small extracellular vesicle; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; and WT, wild-type.

the brief initial thoracotomy. A second thoracotomy at the time of reperfusion would be expected to cause substantial additional trauma, inflammation, and mortality, thereby confounding post-MI remodeling and heart failure readouts. Future studies will evaluate the efficacy of ADp sEVs administered after MI/reperfusion, using alternative delivery routes or animal models that permit postreperfusion dosing without repeat thoracotomy to more closely mirror clinical practice.

In conclusion, our study provides the first evidence that APN on the external surface of ADp sEVs and AdipoR1 in cardiomyocytes are critical players in sEV-mediated protective communication between ADps and cardiomyocytes. The upregulation of GRK2 in diabetes and the subsequent phosphorylation of AdipoR1 disrupt this crucial protective signaling, exacerbating post-MI pathological remodeling and IHF in diabetic animals. Therapeutic administration of sEVs and reactivation of sEV-mediated communication between ADps and cardiomyocytes (by inhibiting GRK2-induced phosphorylation of AdipoR1) may represent a promising strategy for treating IHF in patients with diabetes.

ARTICLE INFORMATION

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Author Contributions

Z.Z., B.L., T.C., Y.W., and X.M. conceived and designed the research; Z.Z., D.Z., L.G., and J.Z. performed experiments and acquired data; Z.Z., D.Z., Z.M., and P.Y. analyzed and interpreted the data; Z.Z. drafted the manuscript; Z.Z., D.L., G.G., Y.W., and X.M. critically revised the manuscript for important intellectual content; Z.Z. performed statistical analyses; Y.W. and X.M. obtained funding; Y.W. and X.M. supervised the study. All authors reviewed and approved the final manuscript.

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Disclosures

None.

Supplemental Material

Expanded Methods
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