Published in final edited form as:

Circ Res. 2022 January 21; 130(2): 252–272. doi:10.1161/CIRCRESAHA.121.319044.

Activated Protein C Strengthens Cardiac Tolerance to Ischemic Insults in Aging

Di Ren[1], **Julia Fedorova**[1], **Kayla Davitt**[1], **Tran Ngoc Van Le**[1], **John H. Griffin**[2], **Patricia C. Liaw**[3], **Charles T. Esmon**[4], **Alireza R. Rezaie**[4], **Ji Li**[1]

[1]Department of Surgery, Morsani College of Medicine, University of South Florida, Tampa, FL 33612

[2]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037

[3]Thrombosis and Atherosclerosis Research Institute, Department of Medicine, McMaster University, Hamilton, ON, Canada

[4]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

Abstract

Background: Activated protein C (APC) is a plasma serine protease with anticoagulant and anti-inflammatory activities. Endothelial protein C receptor (EPCR) is associated with APC's activity and mediates its downstream signaling events. APC exerts cardioprotective effects during ischemia and reperfusion (I/R). This study aims to characterize the role of the APC-EPCR axis in ischemic insults in aging.

Methods: Young (3–4 months) and aged (24–26 months) wild type C57BL/6J mice, as well as EPCR point mutation ($EPCR^{R84A/R84A}$) knock-in C57BL/6J mice incapable of interaction with APC and its wild type of littermate C57BL/6J mice, were subjected to I/R. Wild type APC, signaling-selective APC-2Cys, or anticoagulant-selective APC-E170A were administrated before reperfusion.

Results: The results demonstrated that cardiac I/R reduces APC activity, and the APC activity was impaired in the aged versus young hearts possibly attributable to the declined EPCR level

Address correspondence to: Dr. Ji Li, Department of Surgery, University of South Florida, Tampa, FL 33612, jili@usf.edu.

AUTHORSHIP CONTRIBUTIONS

Contributions: D. R. and J. L. designed and conducted the study; D. R., J. F., K. D., V. L. performed data collection and analysis; D. R., J. F., J.H. G., P. L., C.T. E., A.R. R., and J. L. interpreted the data; J.H. G. provided $PAR1^{R46Q/R46Q}$ mice and edited the manuscript; C.T.E. provided anti-APC antibody; P.L. provided the $EPCR^{R84A/R84A}$ mice and edited the manuscript; A.R.R. provided APC, APC-2Cys and APC E170A, and edited the manuscript; and D. R. and J. L. wrote the manuscript.

DISCLOSURES

None.

SUPPLEMENTAL MATERIALS

Expanded Methods

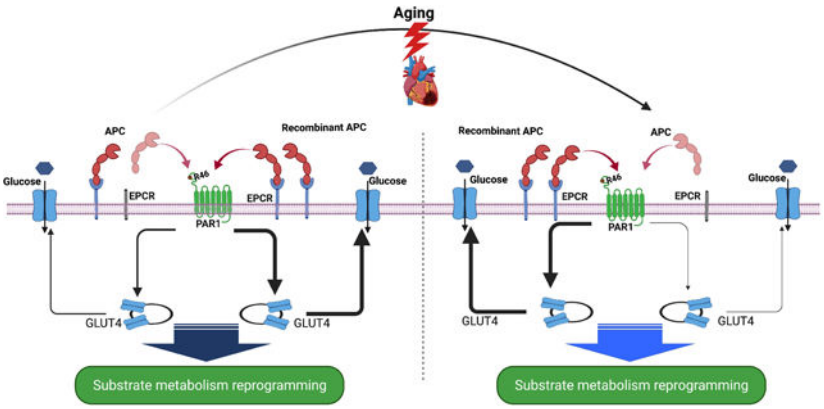
Data Supplement Tables S1–S8

Data Supplement Figures S1–S4

with aging. Serum EPCR measurement showed that I/R triggered the shedding of membrane EPCR into circulation, while administration of APC attenuated the I/R-induced EPCR shedding in both young and aged hearts. Subsequent echocardiography showed that APC and APC-2Cys but not APC-E170A ameliorated cardiac dysfunction during I/R in both young and aged mice. Importantly, APC elevated the resistance of the aged heart to ischemic insults through stabilizing EPCR. However, all these cardioprotective effects of APC were blunted in the EPCR$^{R84A/R84A}$ mice versus its wild-type littermates. The *ex vivo* working heart and metabolomics results demonstrated that AMP-activated protein kinase (AMPK) mediates acute adaptive response while protein kinase B (AKT) is involved in chronic metabolic programming in the hearts with APC treatment.

Conclusions: I/R stress causes shedding of the membrane EPCR in the heart, and administration of APC prevents I/R-induced cardiac EPCR shedding that is critical for limiting cardiac damage in aging.

Graphical Abstract



Keywords

Animal Models of Human Disease; Basic Science Research; Cell Signaling/Signal Transduction; Ischemia

INTRODUCTION

Ischemic heart disease is associated with a breakdown of blood flow to the myocardium because of coronary atherosclerosis, and coronary thrombosis in the heart.[1–3] The current treatment for myocardium ischemia is called reperfusion, which restores blood flow to the heart *via* recanalizing the occluded coronary artery with percutaneous coronary intervention, thrombolytic, or anticoagulants.[3, 4] While reperfusion reduces the mortality of ischemic heart disease, the restoration of blood flow is found paradoxically to inflict further damage named reperfusion injury by inducing myocardium apoptosis or necrosis.[5–9] Myocardial cell death caused by ischemia and reperfusion (I/R) injury is a major cause of morbidity and mortality globally, especially in aged population.[5] The aged heart sustains greater injury during I/R due to multiple factors, which include decreased oxidative phosphorylation and defective mitochondrial composition leading to maladaptive metabolic response to I/R stress

in both clinical settings and animal models of myocardial infarction.[5, 10–12] Unfortunately, there is no effective treatment for myocardial I/R injury in aging.[4, 13] Therefore, there is an urgent need for novel therapeutic strategies capable of limiting myocardial I/R injury in aging patients.

Activated protein C (APC) is a vitamin-K dependent plasma serine protease, which functions as a natural anticoagulant to downregulate thrombin generation in the clotting cascade.[14,15–19] In addition to its anticoagulant activity, APC also exerts potent cytoprotective and anti-inflammatory signaling activities through modulating cellular homeostasis.[20–23] It is demonstrated that APC generation is increased approximately 20 fold when its zymogen protein C (PC) binds to endothelial protein C receptor (EPCR) through its Gla-domain.[14, 17] Furthermore, APC cleaves protease-activated receptor 1 (PAR1) at Arg46 to initiate its cytoprotective signaling.[24] It has been established that the cytoprotective and anti-inflammatory effects of APC requires its binding to EPCR and subsequently cleaving PAR1 on endothelial cells.[14, 23,24, 25]

However, whether APC exerts its cardioprotective effects against I/R in aging and if EPCR and PAR1 are critical for these APC's cardioprotective properties remain unknown. In this study, we show that EPCR function is impaired in aged hearts leading to their increased sensitivity to I/R stress in a mouse model. Compared to its wild type littermates, EPCR$^{R84A/R84A}$ mice, with abolished APC binding activity, exhibit aging-like diastolic dysfunction and cardiac intolerance to I/R. Administration of APC increases resistance of the aged heart to ischemic insults through stabilizing EPCR from I/R-induced shedding in a mouse model. The activation of AMP-activated protein kinase (AMPK) by APC mediates energy metabolism to keep contractile properties of cardiomyocytes under stress. In addition, APC triggers protein kinase B (AKT) activation to control metabolic homeostasis in the hearts undergoing I/R. Thus, this study unveils a novel age-related APC-EPCR signaling axis in heart in response to stress, and administration of APC preserves cardiac tolerance to I/R in aging through stabilizing the receptor, EPCR, in a mouse model.

METHODS

Data Availability.

Data supporting the findings of this study are available from the corresponding author upon reasonable request. The metabolomics datasets used in this study are available at the National Metabolomics Data Repository under accession number: 2842.

Mice.

Young (3–4 months old) and aged (24–26 months old) wild type (WT) C57BL/6J mice were supplied from NIA contracted Charles River Laboratories (Wilmington, MA). The EPCR point mutation (EPCR$^{R84A/R84A}$) knock-in C57BL/6J mice (3–4 months old) and its wild-type littermates (EPCR$^{WT/WT}$) (3–4 months old) were prepared as described.[26] The PAR1 point mutation (PAR1$^{R46Q/R46Q}$) C57BL/6J mice (3–4 months old) and its wild-type littermates (PAR1$^{WT/WT}$) (3–4 months old) were prepared as described.[27] Both male and female mice were subjected to the experiments in this study. For animal use, the sample

size calculations were performed Power Analysis with PASS16 and the proposed analyses were performed with GraphPad Prism 9.0 statistical software. All animal protocols in this study were approved by the Institutional Animal Care and Use Committee of the University of South Florida as well as conform to the NIH Guide for the care and use of laboratory animals.

An expanded Materials and Methods section can be found in the Data Supplement.

RESULTS

Impaired APC signaling sensitizes heart to ischemic insults.

We found that EPCR mediates the cytoprotective effects of APC in a mouse model.[14, 28] To test the hypothesis that APC exerts cardioprotection through EPCR during I/R stress, EPCR point mutation (EPCR$^{R84A/R84A}$) knock-in mice and its wild type littermates, young-WT and aged-WT mice were subjected to ligation of left anterior descending coronary artery (LAD) to induce an *in vivo* regional ischemia of 45 min followed by release of the ligation for reperfusion of 24 hours. Cardiac functions were measured by echocardiography. The results showed that the cardiac systolic dysfunctions were observed in all groups during I/R (Figure 1A, Tables S1 and S2). Cardiac dysfunction was exacerbated in both aged-WT versus young-WT mice and EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$ during I/R (Figure 1A, Tables S1 and S2). Myocardium necrosis was accessed by dual staining with TTC and Evan's blue dye to measure the infarction size from the ratio of area at risk (AAR) to whole myocardial area. The myocardium infarction size was significantly larger in both aged-WT and young EPCR$^{R84A/R84A}$ mice in comparison to the young-WT and young EPCR$^{WT/WT}$ mice, respectively (Figure 1B), suggesting that the intolerance of aged-WT and young EPCR$^{R84A/R84A}$ heart to I/R stress could be due to a defect in EPCR-mediated signaling in aged-WT and EPCR$^{R84A/R84A}$ mice (Figure 1C).

Aging is known to be associated with echocardiographic indices of left ventricular diastolic dysfunction and lower mitral E/A wave ratio.[29] Intriguingly, the diastolic dysfunction with lower E/A ratio was observed in aged-WT and young EPCR$^{R84A/R84A}$ mice hearts but not in young-WT and young EPCR$^{WT/WT}$ mice hearts under sham and I/R conditions, and I/R did not affect diastolic functions (Figure 1D, Tables S1 and S2). The results suggest that an impaired APC signaling due to EPCR binding deficiency contributes to alteration of the cardiac phenotype in this mouse model, thereby rendering it susceptible to diastolic dysfunctions as shown by echocardiographic indices (Figure 1D).

Age-related EPCR deficiency in the heart.

Considering aging-like cardiac diastolic dysfunction, we hypothesized that a defective APC signaling in aged mice may cause intolerance to I/R. The plasma APC activity in young-WT, aged-WT, EPCR$^{WT/WT}$ and EPCR$^{R84A/R84A}$ mice undergoing sham or 45 min of ischemia followed by 24 h of reperfusion, was determined using a monoclonal anti-APC antibody.[30] The results showed that there are no statistical differences in plasma APC activity of young-WT and aged-WT groups under physiological condition (Figure 2A). However, the plasma APC activity was reduced during I/R in both young-WT and aged-WT mice (Figure

2A). Moreover, the decline in APC activity was exacerbated in aged-WT *vs.* young-WT mice during I/R (Figure 2A). There was no detectable APC activity in EPCR$^{R84A/R84A}$ mice (Figure 2A).

The receptor EPCR is expressed in various cell types, including endothelial cells and cardiomyocytes.[28] EPCR plays critical roles in promoting the generation of APC from protein C and mediating APC's anti-inflammatory effects under stress.[14, 28, 31] We thus examined the cardiac expression of EPCR. Immunoblotting studies showed that EPCR expression was significantly decreased in aged-WT versus young-WT hearts, while I/R did not alter EPCR levels in young or aged mouse hearts (Figure 2B). Moreover, we measured the EPCR expression in the left ventricular tissues collected from human hearts. The results showed that there is also a decreased trend of EPCR expression with aging in human heart (Figure 2B). In addition, no statistical differences were observed in EPCR mRNA levels in young-WT and aged-WT mouse heart but an upregulated trend in EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$ mice hearts (Figure S2).

EPCR can be shed from cell surface by tumor necrosis factor-a (TNFa) converting enzyme/ADAM17 (TACE), and proinflammatory factors could also induce EPCR shedding.[32 33] Both ischemic stress and aging can induce inflammatory responses.[1, 34] To determine whether I/R can cause EPCR shedding, we measured soluble EPCR (sEPCR) levels in young-WT and aged-WT under both sham and I/R conditions. Intriguingly, sEPCR was increased by about 30-fold in I/R versus sham (Figure 2C). Increased sEPCR could compete with membrane EPCR for binding PC/APC that could attenuate APC generation. The ratio of sEPCR over myocardial EPCR (mEPCR) was used as an indicator of the APC signaling in response to I/R stress. The ratio of sEPCR/mEPCR in aged-WT versus young-WT hearts was higher during I/R (Figure 2C). This could be a reason for lower APC activity in aged-WT versus young-WT plasma during I/R (Figure 2A). These data indicate that age-related EPCR deficiency can cause an impaired APC signaling in response to I/R stress in this mouse model.

Administration of APC attenuates EPCR shedding during I/R.

To rescue the impaired APC signaling in aging, we administrated recombinant APC. Intriguingly, administration of APC derivatives 5 min before reperfusion reduced the excessive I/R-induced sEPCR levels in both young-WT and aged-WT by two-fold (Figure 2D, upper panel). However, administration of APC did not affect I/R-triggered sEPCR in the EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$ mice (Figure 2D, lower panel), indicating that EPCR-APC complex formation is crucial for preventing EPCR shedding under I/R. Furthermore, the immunoblotting of EPCR in the left ventricular tissue showed that administration of the APC derivatives significantly increased myocardial EPCR levels in wild type hearts, whereas this effect was eliminated in EPCR$^{R84A/R84A}$ hearts (Figure 2E, and Figure S1). These results suggest that the APC-EPCR complex preserves the EPCR integrity under I/R, thereby preserving its downstream protective functions in response to stress in the heart. The RT-PCR data showed that no statistical difference was observed in EPCR mRNA levels in hearts (Figure S2). Therefore, APC did not transcriptionally affect cardiac EPCR expression but stabilized the myocardial EPCR through direct interaction. Furthermore, the

immunofluorescence staining of EPCR in the heart tissues demonstrated that I/R induced 32.81 ± 2.01% and 55.52 ± 2.21% reductions of EPCR in cardiomyocytes (CM) and endothelial cells (EC) of young-WT hearts, respectively. In the aged-WT group, I/R caused 36.08 ± 2.67% EPCR reduction in CM and 69.49 ± 5.05% EPCR reduction in EC (Figure 2F). Interestingly, APC administration attenuated the I/R-induced EPCR reduction in CM and EC of both young-WT and aged-WT hearts (Figure 2F). APC treatment did not affect I/R-induced EPCR reduction in EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$ hearts (Figure 2F). Therefore, these results indicate that administration of recombinant APC can prevent the I/R-triggered shedding of EPCR from myocardium through direct interaction with EPCR in this mouse model (Figure S5).

Administration of APCs increase cardiac tolerance to I/R insults.

The cardioprotective effects of APC derivatives were then assessed. Wild-type APC (0.2 μg/g), signaling-selective APC-2Cys (0.2 μg/g) or anticoagulant-selective APC-E170A (0.2 μg/g) was injected through the jugular vein 5 min before reperfusion after 45 min of regional ischemia. Echocardiography showed that I/R caused cardiac systolic dysfunctions as shown by a decrease of ejection fraction (EF) and fractional shortening (FS) in all groups (Figure 3A, and Table S1). APC and APC-2Cys but not APC-E170A improved the cardiac systolic functions of young-WT and aged-WT heart (Figure 3A, upper panel). In addition, myocardial infarction measurements showed that both APC and APC-2Cys significantly reduce the myocardial infarction in both young-WT and aged-WT (Figure 3B). These results demonstrated that the cytoprotective signaling rather than the anticoagulant property of APC plays a critical role in limiting cardiac damage by I/R insults. APC treatment ameliorated cardiac systolic functions and reduced infarction size of EPCR$^{WT/WT}$ but not of EPCR$^{R84A/R84A}$ hearts caused by I/R (Figures 3A and 3B, Table S2). This suggests that the interaction of APC with EPCR is required for APC's cardioprotective events in this mouse model. Intriguingly, the beneficial effect of APC on ischemic heart was abolished in PAR1$^{R46Q/R46Q}$ versus PAR1$^{WT/WT}$ mice, suggesting that the PAR1 activation triggered by APC cleavage at R46 site mediates APC's cardioprotection against ischemic injury by I/R stress (Figures 3A and 3B, Table S3) as it does for APC's neuroprotection and sepsis protection. [27]

Cardiac diastolic dysfunction is associated with hypertension, obesity, metabolic syndrome and aging independent of the systolic function.[35] To determine whether APC affects the diastolic function in aging under I/R, echocardiographic mitral Doppler flow velocity was measured in young/aged-WT, EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$, PAR1$^{WT/WT}$/PAR1$^{R46Q/R46Q}$ hearts under sham or I/R conditions with or without APC treatment. The results demonstrated that neither APC nor I/R influences cardiac diastolic functions (Figure 3C). Intriguingly, diastolic dysfunction was observed in aged-WT versus young-WT, EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$, and PAR1$^{R46Q/R46Q}$ versus PAR1$^{WT/WT}$ mice, indicating a crucial role for APC signaling in preserving cardiac diastolic function (Figure 3C). In addition, PAR1$^{R46Q/R46Q}$ versus PAR1$^{WT/WT}$ mice show an impaired cardiac systolic function (Figure 3A). It could be due to causing an imbalance between the cytoprotective property and the detrimental property of PAR1 occurring due to the Arg46 site mutation that causes cardiac systolic dysfunction, since PAR1 cleavage at Arg46 plays

a critical role in mediating downstream cytoprotective signaling.[27] Interestingly, lack of APC binding affinity in EPCR$^{R84A/R84A}$ mice did not affect cardiac systolic functions under physiological conditions (Figure 3A). Thus, the interaction of Gla domains of APC, APC-2Cys, and APC-E170A with EPCR accounts for stabilizing the myocardial EPCR and preserving its function in aging under I/R in our mouse model (Figure 2E, Figure S1). However, the anticoagulant selective APC-E170A cannot normally cleave PAR1 and trigger PAR1 activation, thus it did not show cardioprotective effects on ischemic injury caused by I/R in a mouse model (Figure 3D).

APC maintains the cardiac metabolic homeostasis in aging.

To determine the mechanism by which the APC-EPCR axis protects the heart from I/R insults, the metabolomics was performed in myocardium of young/aged-WT heart under sham or 45 min of ischemia and 24 h of reperfusion (I/R) conditions with or without APC. The results showed that several amino acid metabolism pathways were down-regulated in aged-WT versus young-WT heart (Figure 4A). The amino acid reservoir plays an important role in movements of skeletal and heart muscles.[36] The down-regulation of amino acid metabolism in aging could impair resistance to ischemic insults. The carbohydrate related pathways, such as glutamate metabolism, mitochondrial electron transport chain, starch and sucrose metabolism, and amino sugar metabolism, were all down-regulated during I/R in young-WT heart (Figure 4B). However, there were up-regulations in fatty acid metabolism and glycolysis besides the alterations in carbohydrate metabolism in aged-WT heart (Figure 4C). Therefore, an adaptive metabolic response to I/R stress in the mouse heart could be impaired in aging.

With APC administration before reperfusion, the glycolysis, Warburg effect, and branched fatty acid oxidation pathways were down-regulated in young-WT heart (Figure 4D). Moreover, several citrate cycle components, like fumarate and malate accumulation were reduced by APC treatment, indicating that APC promotes the utilization of carbohydrate through citrate acid cycle to generate energy rather than glycolysis or fatty acid metabolism (Figure 4D). Intriguingly, the equiform of alteration was found in aged-WT heart with APC treatment, i.e., glycolysis, Warburg effect, and branched fatty acid oxidation pathway were inhibited, while the carbohydrate and citrate cycles were up-regulated during I/R with APC (Figure 4E). The heatmap of myocardium metabolites alterations in young/aged hearts under sham or I/R conditions with or without APC demonstrated that APC treatment can rescue the maladaptive metabolic response occurring in the aged heart (Figure 4F). The heart is an energy dependent organ, which mainly utilizes fatty acid and glucose as substrate source for energy production through the tricarboxylic acid (TCA) cycle. The energy machine mitochondria in myocardium could be more susceptible to damage in aging during I/R, thus the TCA cycle was reduced, and glycolysis was upregulated in aging. This could lead to a maladaptive metabolic response during I/R stress in the aged heart. The administration of APC restores metabolic homeostasis, increasing TCA cycle and reducing glycolysis, thereby leading to a preserved cardiac function during I/R in this mouse model (Figure 4G).

EPCR mediates the APC-triggered metabolic remodeling.

Considering the findings that APC modulates the TCA cycle and arginine pathway, we investigated the possible role of the APC-EPCR-AKT cascade in modulating substrate metabolism in response to I/R stress in the heart. Immunoblotting showed that phospho-PDK1 (Ser^{241}) and phospho-AKT (Ser^{473}) were augmented in APC treated young/aged-WT (Figure 5A). Moreover, APC treatment elevated phospho-eNOS (Ser^{1177}) levels in young/aged-WT heart (Figure 5A). eNOS as a downstream target of AKT is responsible for generating nitric oxide (NO) through utilizing arginine as a substrate in response to stress.[37] AKT-eNOS axis regulates the translocation of glucose transporter 4 (GLUT4) from intracellular vesicles to the plasma membrane under physiological and pathological conditions.[38] To determine whether APC-AKT cascade modulates GLUT4 translocation in the heart, we employed glucose transporter surface labelling technique.[39] The results demonstrated that APC triggered translocation of GLUT4 to cell membranes in young/aged WT heart (Figure 5B). However, the GLUT4 translocation by APC was blunted in $EPCR^{R84A/R84A}$ versus $EPCR^{WT/WT}$ heart (Figure 5B), indicating that EPCR is required for the AKT regulation of GLUT4 trafficking by APC in myocardium. Intriguingly, the AKT activation by APC was abolished in $PAR1^{R46Q/R46Q}$ versus $PAR1^{WT/WT}$ hearts under sham and I/R conditions (Figure 5B), indicating that PAR1 activation by APC's cleavage at R46 is required to mediate the APC-AKT signaling cascade in the heart. Moreover, pre-treated with the pan-AKT inhibitor (ARQ-092) inhibited AKT activation and blocked GLUT4 translocation by APC (Figure 5B). Taken together, these results indicate that the APC-EPCR-PAR1 axis activates AKT signaling and promotes GLUT4 translocation to modulate glucose metabolism, thereby contributing to maintenance of metabolic homeostasis in the heart under I/R stress in this mouse model.

To determine the beneficial effects of APC-AKT signaling on cardiac functions under I/R, the AKT inhibitor was administrated by tail vein one hour before surgery and APC was administrated by jugular vein 5 min before reperfusion. Echocardiography showed that pretreatment with the ARQ-092, attenuated the cardioprotective effects of APC as shown by the EF and FS and by myocardial infarction size (Figures 5C, 5D and 5E). We did not observe statistical alterations in cardiac diastolic dysfunction by APC or ARQ-092 treatment under sham or I/R (Figure 5C). These results for the first time demonstrated that administration of APC can stabilize the myocardial EPCR and trigger AKT activation, thereby leading to translocation of GLUT4 to cell surface, modulating downstream substrate metabolism and benefiting cardiac functions under I/R in our mouse model (Figure 5F).

APC-EPCR modulates cardiac glucose and fatty acid oxidation.

We previously reported APC stimulates AMPK activation and modulates glucose and fatty acid metabolism in the heart.[28, 40] To determine whether EPCR is required for APC to regulate the cardiac glucose and fatty acid metabolism in response to I/R stress, the isolated hearts from young/aged-WT, and $EPCR^{WT/WT}$/$EPCR^{R84A/R84A}$ mice were perfused in the *ex vivo* working heart system for 20 min of ischemia and 30 min of reperfusion with or without APC. Glucose oxidation was analyzed by measuring [^{14}C]-glucose incorporation into $^{14}CO_2$ released from the post-reperfusion. Fatty acid oxidation was measured by the production of $^{3}H_2O$ from [9, 10-^{3}H]-oleate in the reperfusion perfusate. The results

demonstrated that APC administration significantly increased the rate of glucose oxidation in young/aged-WT heart and EPCR$^{WT/WT}$ but not in EPCR$^{R84A/R84A}$ heart during I/R (Figures 6A and 6B). On the contrary, fatty acid oxidation rate was significantly reduced by APC in young/aged-WT and EPCR$^{WT/WT}$ heart, whereas the effect was minimal in EPCR$^{R84A/R84A}$ heart (Figures 6A and 6B). Moreover, I/R leads to cardiac systolic dysfunction in terms of the heart rate and aortic flow in all groups while APC administration significantly improved the systolic function in young/aged-WT and EPCR$^{WT/WT}$ but not the EPCR$^{R84A/R84A}$ heart (Tables S5 and S6). This suggests that EPCR is required for APC to modulate the cardiac substrate metabolism and improve cardiac function through increasing glucose oxidation and decreasing fatty acid oxidation rate during I/R in our mouse model. The immunoblotting showed that APC triggers AMPK phosphorylation in the isolated cardiomyocytes from young/aged-WT and EPCR$^{WT/WT}$ but not from EPCR$^{R84A/R84A}$ heart (Figure 6C), indicating that EPCR is required for APC-AMPK signaling in mouse cardiomyocytes.

APC improves the contractile properties of cardiomyocytes under stress.

Energy supply and sufficient calcium flux are critical to maintain proper cardiomyocyte contractile function. Our previous study showed that APC can activate AMPK *via* the upstream kinase Ca^{2+}/calmodulin-dependent protein kinase kinase b (CaMKKβ) in cardiomyocytes.[28] AMPK is an energy sensor, mediating the majority of the ATP generation in the heart.[41] In light of the stimulatory effect of APC on AMPK activation in cardiomyocytes, we evaluated the effect of APC on the contractile properties of isolated cardiomyocytes from young/aged-WT and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or hypoxia and reoxygenation (H/R) conditions. The contractile functions of isolated cardiomyocytes determined by sarcomere shortening length, the percentage of shortening, and the rate of shortening were impaired in aged-WT versus young-WT and EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$ cardiomyocytes under H/R conditions (Figure 6D). APC treatment restored the contractile functions of young/aged-WT and EPCR$^{WT/WT}$ but not EPCR$^{R84A/R84A}$ cardiomyocytes under H/R conditions (Figure 6D). The analysis of the transient calcium flux in cardiomyocytes by fura-2 staining demonstrated that EPCR$^{R84A/R84A}$ but not young/aged-WT and EPCR$^{WT/WT}$ cardiomyocytes display upregulated transient calcium flux as evidenced by calcium shortening peak, the percentage of shortening, and the rate of shortening (Figure 6E). Moreover, H/R stress induced reduction in calcium flux of young/aged-WT and EPCR$^{WT/WT}$ but not EPCR$^{R84A/R84A}$ cardiomyocytes (Figure 6E). Interestingly, APC attenuated the calcium flux alterations in young/aged-WT and EPCR$^{WT/WT}$ but not in EPCR$^{R84A/R84A}$ cardiomyocytes during H/R (Figure 6E). These results indicate that EPCR is crucial to mediate the beneficial effects of APC in improving the energy metabolism and cardiomyocyte contractile properties under H/R stress conditions (Figure 6F).

APC stabilizes EPCR on cardiomyocytes under stress.

To determine whether administration of APC affects the EPCR on cardiomyocytes through direct binding interactions, immunofluorescence staining was performed to analyze the association and colocalization of EPCR and APC in isolated cardiomyocytes. Intriguingly, APC treatment for 40 mins significantly increased the levels of EPCR in young/

aged-WT and EPCR$^{WT/WT}$ under both normoxia and hypoxia/reoxygenation conditions but not in EPCR$^{R84A/R84A}$ cardiomyocytes (Figure 7), indicating that the APC-EPCR interaction is critical to preserve EPCR protein stability in cardiomyocytes. Moreover, the elevated colocalization of EPCR and APC in young/aged-WT and EPCR$^{WT/WT}$ but not EPCR$^{R84A/R84A}$ cardiomyocytes indicated that APC administration promotes EPCR accumulation through direct interaction and stabilization of the APC-EPCR complexes in response to H/R stress (Figures 7B and 7C, and Figure S3). When we examined the mRNA level of EPCR in the isolated cardiomyocytes from young/aged WT and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts, a reduction of EPCR mRNA was observed with APC treatment was seen in young-WT and EPCR$^{WT/WT}$ but not EPCR$^{R84A/R84A}$ cardiomyocytes (Figure S4). Transcriptional inhibition of EPCR could be due to a feedback response triggered by APC-induced EPCR stabilization observed in mouse cardiomyocytes.

DISCUSSION

In this study, we revealed that APC signaling is a critical factor in preserving cardiac function in ischemic heart disease in a mouse model. This was demonstrated by the observation that an impaired APC signaling in EPCR$^{R84A/R84A}$ mice is associated with more severe cardiac injury after I/R stress. The results implicate that the APC/EPCR axis is essential in facilitating an age-related adaptive response to I/R stress, as aged-WT and EPCR$^{R84A/R84A}$ mice exhibited similar impaired post-ischemic cardiac function and aggravated myocardial infarction. Intriguingly, similar cardiac diastolic dysfunction with significantly reduced E/A ratio was found in both aged-WT and EPCR$^{R84A/R84A}$ mice under physiological conditions, indicating that APC signaling is age-related in preserving the vascular and myocardial relaxation process in mice. Furthermore, the findings in the aged mice showing an impaired plasma APC activity and downregulated EPCR expression in the heart under I/R stress suggest that an impaired EPCR-dependent APC signaling in aging contributes to the intolerance of aged heart to ischemic insults.

EPCR can be cleaved from the cell surface by metalloproteases and TNFa converting enzyme/ADAM17 (TACE).[32, 42] Multiple pro-inflammatory cytokines, like interleukin 1-β and TNFa could induce EPCR shedding.[43] Chronic systemic pro-inflammatory responses, termed senoinflammation, may be part of a pervasive state for aging due to the dysregulation of immune response.[34] In this study, we found that there was significantly increased level of sEPCR in aging during I/R stress, indicating that an excessive EPCR shedding in aged heart may impair APC activity in aging in response to ischemic insults. The circulating sEPCR levels are reduced by administration of APC in EPCR$^{WT/WT}$ but not in EPCR$^{R84A/R84A}$ mice during I/R, implying that APC preserves myocardial EPCR through their interactions in mice. In addition, the immunoblotting and immunofluorescence staining showed that myocardial EPCR is significantly increased with administration of all APC derivatives, implying that the APC complexation with EPCR prevents EPCR shedding induced by I/R stress in mice. Immunofluorescence staining also showed that EPCR was expressed in both cardiomyocytes (CM) and endothelial cells (EC). I/R stress induced EPCR reduction in both CM and EC but with higher ratio in the EC of both young and aged hearts. Since all APC derivatives, i.e., APC-WT, APC-2Cys and APC-E170A, have Gla domains, their associations with EPCR stabilize and preserve EPCR under I/R stress in the mice heart.

However, the APC-E170A/EPCR complex cannot activate PAR1 through cleavage of R46 site, thus it did not show cardioprotective effects during I/R. The experimental data from EPCR$^{R84A/R84A}$ versus EPCR$^{WT/WT}$ and PAR1$^{R46Q/R46Q}$ versus PAR1$^{WT/WT}$ provides clear evidence that EPCR/PAR1 axis is required for the beneficial activities of APC in mice ischemic hearts.

APC initiates PAR1 biased cytoprotective signaling through β-arrestin-2 and the PI3K/AKT pathway in brain and endothelial cells.[44] In this study, we showed that APC markedly triggered AKT signaling and promoted GLUT4 trafficking to the cell membranes. The observation that in the genetic mutations of EPCR or PAR1 mice the AKT-mediated GLUT4 translocation was blocked strongly suggest that the APC-EPCR-PAR1 axis is required for the APC's cardioprotective activities through AKT-GLUT4 cascade mediated substrate metabolism in mice. The metabolomics analysis revealed that I/R stress caused downregulation of carbohydrate metabolism and mitochondrial oxidative phosphorylation of the mice heart. The maladaptive metabolic adaptive response in the heart with aging could lead to greater susceptibility of aged hearts to ischemic insults. APC treatment rescued the maladaptive metabolic response in aging and improved cardiac functions during I/R stress through modulating the glycolysis and TCA cycle in the mice heart. Furthermore, we showed that APC stimulates AMPK signaling and increases glucose oxidation but reduces fatty acid oxidation in response to the ischemic stress in the acute post-ischemia, reperfusion state in WT mice hearts. This effect was diminished in EPCR$^{R84A/R84A}$ mice heart, indicating that the APC-EPCR axis augments glucose oxidation, instead of fatty acid oxidation, to generate ATP during reperfusion. AMPK signaling is essential for regulating energy generation for the contractile function of the heart, especially under pathological conditions. When we measured the effects of APC treatment on contractile function of the isolated mouse cardiomyocytes under H/R conditions, we found that APC treatment improves cardiomyocyte contractility and calcium flux in WT but not EPCR$^{R84A/R84A}$ cardiomyocytes under stress, indicating that EPCR mediates APC-AMPK signaling to improve mice cardiomyocyte contractile function. The activation of AMPK is an acute adaptive response for modulating energy metabolism and cardiomyocyte contractility during ischemia and reperfusion stress.

In summary, decreased APC activity and augmented EPCR shedding in aging mice heart during I/R stress leads to an intolerance of aged hearts to I/R insults. APC interaction with the cell surface EPCR prevents I/R-induced receptor shedding. APC treatments improves cardiac function and ameliorates myocardial ischemic injury through modulation of both acute and chronic metabolic pathways. Further studies are warranted to determine whether other mechanisms that direct interaction of APC with EPCR contribute to preventing excessive receptor shedding under I/R stress. These studies could advance our understanding of the role of APC-EPCR-AMPK signaling in I/R stress and potentially lead to discovery of better approaches to prevent/cure ischemic insults in the elderly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We would like to thank Tea Reljic and The University of South Florida Research Methodology and Biostatistics Core for assistance with statistical review. We appreciated the *LifeLink* Foundation, Inc and its *LifeLink* of Florida Organ Procurement Organization for providing the human non-transplantable donor hearts.

SOURCES OF FUNDING

This work was supported by NIH R01GM124108, R01HL158515 (J. Li), R01HL142975 (J.H. Griffin), and R01HL101917 (A. R. Rezaie).

Nonstandard Abbreviation and Acronyms

AMPK	AMP-activated protein kinase
APC	activated protein C
CaMKKb	Ca^{2+}/calmodulin-dependent protein kinase kinase b
E/A	Early diastole peak velocity (the E wave)/the peak velocity of the late diastole caused by atrial contraction (the A wave)
EF	ejection fraction
EPCR	endothelial protein C receptor
FS	fractional shortening
LAD	left anterior descending coronary artery
LC-MS/MS	liquid chromatography with tandem mass spectrometry
PAR1	protease activated receptor 1
TACE	TNFa converting enzyme
TCA	tricarboxylic acid

REFERENCES

1. Ren D, Quan N, Fedorova J, Zhang J, He Z, Li J. Sestrin2 modulates cardiac inflammatory response through maintaining redox homeostasis during ischemia and reperfusion. Redox Biol. 2020;34:101556 [PubMed: 32447260]
2. Morrison A, Chen L, Wang J, Zhang M, Yang H, Ma Y, Budanov A, Lee JH, Karin M, Li J. Sestrin2 promotes lkb1-mediated ampk activation in the ischemic heart. FASEB J. 2015;29:408–417 [PubMed: 25366347]
3. Perricone AJ, Vander Heide RS. Novel therapeutic strategies for ischemic heart disease. Pharmacol Res. 2014;89:36–45 [PubMed: 25193582]
4. Turer AT, Hill JA. Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. Am J Cardiol. 2010;106:360–368 [PubMed: 20643246]
5. Buja LM. Myocardial ischemia and reperfusion injury. Cardiovasc Pathol. 2005;14:170–175 [PubMed: 16009313]
6. de Groot H, Rauen U. Ischemia-reperfusion injury: Processes in pathogenetic networks: A review. Transplant Proc. 2007;39:481–484 [PubMed: 17362763]
7. Hoffman JWGT Jr, Poston RS, Silldorff EP. Myocardial reperfusion injury: Etiology, mechanisms, and therapies. J Extra Corpor Technol. 2004;36:391–411 [PubMed: 15679285]

8. Hsieh YH HS, Wei FC, Hung LM. Resveratrol attenuates ischemia-reperfusion-induced leukocyte-endothelial cell adhesive interactions and prolongs allograft survival across the mhc barrier. Circulation Journal. 2007;71:423–428 [PubMed: 17322646]
9. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev. 2008;88:581–609 [PubMed: 18391174]
10. Quan N, Sun W, Wang L, Chen X, Bogan JS, Zhou X, Cates C, Liu Q, Zheng Y, Li J. Sestrin2 prevents age-related intolerance to ischemia and reperfusion injury by modulating substrate metabolism. FASEB J. 2017;31:4153–4167 [PubMed: 28592638]
11. Strait JB, Lakatta EG. Aging-associated cardiovascular changes and their relationship to heart failure. Heart Fail Clin. 2012;8:143–164 [PubMed: 22108734]
12. Lesnefsky EJ, Hoppel CL. Ischemia-reperfusion injury in the aged heart: Role of mitochondria. Arch Biochem Biophys. 2003;420:287–297 [PubMed: 14654068]
13. Morrison A, Yan X, Tong C, Li J. Acute rosiglitazone treatment is cardioprotective against ischemia-reperfusion injury by modulating ampk, akt, and jnk signaling in nondiabetic mice. Am J Physiol Heart Circ Physiol. 2011;301:H895–902 [PubMed: 21666107]
14. Ren D, Giri H, Li J, Rezaie AR. The cardioprotective signaling activity of activated protein c in heart failure and ischemic heart diseases. Int J Mol Sci. 2019;20, 1762
15. Nicolaes GA, Thomassen MC, Tans G, Rosing J, Hemker HC. Effect of activated protein c on thrombin generation and on the thrombin potential in plasma of normal and apc-resistant individuals. Blood Coagul Fibrinolysis. 1997;8:28–38 [PubMed: 9105635]
16. Esmon CT. Molecular events that control the protein c anticoagulant pathway. Thromb Haemost. 1993;70:29–35 [PubMed: 8236111]
17. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein c receptor augments protein c activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci U S A. 1996;93:10212–10216 [PubMed: 8816778]
18. Griffin JH, Fernandez JA, Gale AJ, Mosnier LO. Activated protein c. J Thromb Haemost. 2007;5 Suppl 1:73–80 [PubMed: 17635713]
19. Dahlback B The protein c anticoagulant system: Inherited defects as basis for venous thrombosis. Thromb Res. 1995;77:1–43 [PubMed: 7701473]
20. Mizutani A, Okajima K, Uchiba M, Noguchi T. Activated protein c reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation. Blood. 2000;95:3781–3787 [PubMed: 10845910]
21. Hirose K, Okajima K, Taoka Y, Uchiba M, Tagami H, Nakano K, Utoh J, Okabe H, Kitamura N. Activated protein c reduces the ischemia/reperfusion-induced spinal cord injury in rats by inhibiting neutrophil activation. Ann Surg. 2000;232:272–280 [PubMed: 10903607]
22. Laffan MA. Activated protein c resistance and myocardial infarction. Heart. 1998;80:319–321 [PubMed: 9875103]
23. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein c pathway. Blood. 2007;109:3161–3172 [PubMed: 17110453]
24. Mosnier LO, Sinha RK, Burnier L, Bouwens EA, Griffin JH. Biased agonism of protease-activated receptor 1 by activated protein c caused by noncanonical cleavage at arg46. Blood. 2012;120:5237–5246 [PubMed: 23149848]
25. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein c pathway. Science. 2002;296:1880–1882 [PubMed: 12052963]
26. Pepler L, Yu P, Dwivedi DJ, Trigatti BL, Liaw PC. Characterization of mice harboring a variant of epcr with impaired ability to bind protein c: Novel role of epcr in hematopoiesis. Blood. 2015;126:673–682 [PubMed: 26045607]
27. Sinha RK, Wang Y, Zhao Z, Xu X, Burnier L, Gupta N, Fernandez JA, Martin G, Kupriyanov S, Mosnier LO, Zlokovic BV, Griffin JH. Par1 biased signaling is required for activated protein c in vivo benefits in sepsis and stroke. Blood. 2018;131:1163–1171 [PubMed: 29343482]
28. Wang J, Yang L, Rezaie AR, Li J. Activated protein c protects against myocardial ischemic/reperfusion injury through amp-activated protein kinase signaling. J Thromb Haemost. 2011;9:1308–1317 [PubMed: 21535395]

29. Campbell DJ, Somaratne JB, Jenkins AJ, Prior DL, Yii M, Kenny JF, Newcomb AE, Schalkwijk CG, Black MJ, Kelly DJ. Diastolic dysfunction of aging is independent of myocardial structure but associated with plasma advanced glycation end-product levels. PLoS One. 2012;7:e49813 [PubMed: 23189164]
30. Liaw PC, Ferrell G, Esmon CT. A monoclonal antibody against activated protein c allows rapid detection of activated protein c in plasma and reveals a calcium ion dependent epitope involved in factor va inactivation. J Thromb Haemost. 2003;1:662–670 [PubMed: 12871399]
31. Li W, Zheng X, Gu J, Hunter J, Ferrell GL, Lupu F, Esmon NL, Esmon CT. Overexpressing endothelial cell protein c receptor alters the hemostatic balance and protects mice from endotoxin. J Thromb Haemost. 2005;3:1351–1359 [PubMed: 15978090]
32. Qu D, Wang Y, Esmon NL, Esmon CT. Regulated endothelial protein c receptor shedding is mediated by tumor necrosis factor-alpha converting enzyme/adam17. J Thromb Haemost. 2007;5:395–402 [PubMed: 17155946]
33. Gur-Cohen S, Itkin T, Chakrabarty S, Graf C, Kollet O, Ludin A, Golan K, Kalinkovich A, Ledergor G, Wong E, Niemeyer E, Porat Z, Erez A, Sagi I, Esmon CT, Ruf W, Lapidot T. Par1 signaling regulates the retention and recruitment of epcr-expressing bone marrow hematopoietic stem cells. Nat Med. 2015;21:1307–1317 [PubMed: 26457757]
34. Chung HY, Kim DH, Lee EK, Chung KW, Chung S, Lee B, Seo AY, Chung JH, Jung YS, Im E, Lee J, Kim ND, Choi YJ, Im DS, Yu BP. Redefining chronic inflammation in aging and age-related diseases: Proposal of the senoinflammation concept. Aging Dis. 2019;10:367–382 [PubMed: 31011483]
35. Schnelle M, Catibog N, Zhang M, Nabeebaccus AA, Anderson G, Richards DA, Sawyer G, Zhang X, Toischer K, Hasenfuss G, Monaghan MJ, Shah AM. Echocardiographic evaluation of diastolic function in mouse models of heart disease. J Mol Cell Cardiol. 2018;114:20–28 [PubMed: 29055654]
36. Timmerman KL, Volpi E. Amino acid metabolism and regulatory effects in aging. Curr Opin Clin Nutr Metab Care. 2008;11:45–49 [PubMed: 18090658]
37. Javanmard SH, Nematbakhsh M, Mahmoodi F, Mohajeri MR. L-arginine supplementation enhances enos expression in experimental model of hypercholesterolemic rabbits aorta. Pathophysiology. 2009;16:9–13 [PubMed: 19168337]
38. van Dam EM, Govers R, James DE. Akt activation is required at a late stage of insulin-induced glut4 translocation to the plasma membrane. Mol Endocrinol. 2005;19:1067–1077 [PubMed: 15650020]
39. Li J, Hu X, Selvakumar P, Russell RR 3rd, Cushman SW, Holman GD, Young LH. Role of the nitric oxide pathway in ampk-mediated glucose uptake and glut4 translocation in heart muscle. Am J Physiol Endocrinol Metab. 2004;287:E834–841 [PubMed: 15265762]
40. Costa R, Morrison A, Wang J, Manithody C, Li J, Rezaie AR. Activated protein c modulates cardiac metabolism and augments autophagy in the ischemic heart. J Thromb Haemost. 2012;10:1736–1744 [PubMed: 22738025]
41. Bairwa SC, Parajuli N, Dyck JR. The role of ampk in cardiomyocyte health and survival. Biochim Biophys Acta. 2016;1862:2199–2210 [PubMed: 27412473]
42. Xu J, Qu D, Esmon NL, Esmon CT. Metalloproteolytic release of endothelial cell protein c receptor. J Biol Chem. 2000;275:6038–6044 [PubMed: 10681599]
43. Ku SK, Yang EJ, Song KS, Bae JS. Rosmarinic acid down-regulates endothelial protein c receptor shedding in vitro and in vivo. Food Chem Toxicol. 2013;59:311–315 [PubMed: 23774263]
44. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein c, protease activated receptor 1, and neuroprotection. Blood. 2018;132:159–169 [PubMed: 29866816]
45. Ma H, Wang J, Thomas DP, Tong C, Leng L, Wang W, Merk M, Zierow S, Bernhagen J, Ren J, Bucala R, Li J. Impaired macrophage migration inhibitory factor-amp-activated protein kinase activation and ischemic recovery in the senescent heart. Circulation. 2010;122:282–292 [PubMed: 20606117]
46. Ren D, He Z, Fedorova J, Zhang J, Wood E, Zhang X, Kang DE, Li J. Sestrin2 maintains oxphos integrity to modulate cardiac substrate metabolism during ischemia and reperfusion. Redox Biol. 2021;38:101824 [PubMed: 33316744]

47. Lindhurst MJ, Yourick MR, Yu Y, Savage RE, Ferrari D, Biesecker LG. Repression of akt signaling by arq 092 in cells and tissues from patients with proteus syndrome. Sci Rep. 2015;5:17162 [PubMed: 26657992]

48. Quan N, Wang L, Chen X, Luckett C, Cates C, Rousselle T, Zheng Y, Li J. Sestrin2 prevents age-related intolerance to post myocardial infarction via ampk/pgc-1alpha pathway. J Mol Cell Cardiol. 2018;115:170–178 [PubMed: 29325933]

49. Li X, Liu J, Hu H, Lu S, Lu Q, Quan N, Rousselle T, Patel MS, Li J. Dichloroacetate ameliorates cardiac dysfunction caused by ischemic insults through ampk signal pathway-not only shifts metabolism. Toxicol Sci. 2019;167:604–617 [PubMed: 30371859]

50. He L, Li F, Yin X, Bohman P, Kim S, McClain CJ, Feng W, Zhang X. Profiling of polar metabolites in mouse feces using four analytical platforms to study the effects of cathelicidin-related antimicrobial peptide in alcoholic liver disease. J Proteome Res. 2019;18:2875–2884 [PubMed: 31188604]

51. Wei X, Shi X, Kim S, Patrick JS, Binkley J, Kong M, McClain C, Zhang X. Data dependent peak model based spectrum deconvolution for analysis of high resolution lc-ms data. Anal Chem. 2014;86:2156–2165 [PubMed: 24533635]

52. Wei X, Shi X, Kim S, Zhang L, Patrick JS, Binkley J, McClain C, Zhang X. Data preprocessing method for liquid chromatography-mass spectrometry based metabolomics. Anal Chem. 2012;84:7963–7971 [PubMed: 22931487]

53. Wei X, Sun W, Shi X, Koo I, Wang B, Zhang J, Yin X, Tang Y, Bogdanov B, Kim S, Zhou Z, McClain C, Zhang X. Metsign: A computational platform for high-resolution mass spectrometry-based metabolomics. Anal Chem. 2011;83:7668–7675 [PubMed: 21932828]

54. Lopez-Ibanez J, Pazos F, Chagoyen M. Mbrole 2.0-functional enrichment of chemical compounds. Nucleic Acids Res. 2016;44:W201–204 [PubMed: 27084944]

NOVELTY AND SIGNIFICANCE

What Is Known?

- Activated protein C (APC) downregulates thrombin generation in the clotting cascade.
- Activation of AMP-activated protein kinase (AMPK) by APC mediates cardioprotection against ischemia and reperfusion injury.
- An impaired cardiac AMPK signaling is responsible for vulnerability to ischemic insults in aging.

What New Information Does This Article Contribute?

- APC receptor EPCR shedding occurs in the heart during ischemia and reperfusion stress.
- Administration of APC rescues cardiac intolerance to ischemic insults in aging *via* maintaining membrane EPCR.
- AMPK mediates acute adaptive response and AKT modulates metabolic response by APC during ischemia and reperfusion stress.

We studied how that APC exerts cardioprotective effects during ischemia and reperfusion and found that administration of recombinant APC derivatives reduced shedding of the APC receptor EPCR in the heart during ischemia and reperfusion stress. Administration of APC strengthens the tolerance of the aged heart to ischemic insults through stabilizing the EPCR on the membrane. This study addresses a knowledge gap regarding the cardiac APC-EPCR-PAR1 signaling cascade in aging hearts and provides mechanistic insights about the cardioprotective effects of APC derivatives for ischemic insults. Therefore, appropriate APC derivatives can be considered as pharmaceutical drugs for increasing the heart’s resistance to ischemic insults in the elderly.

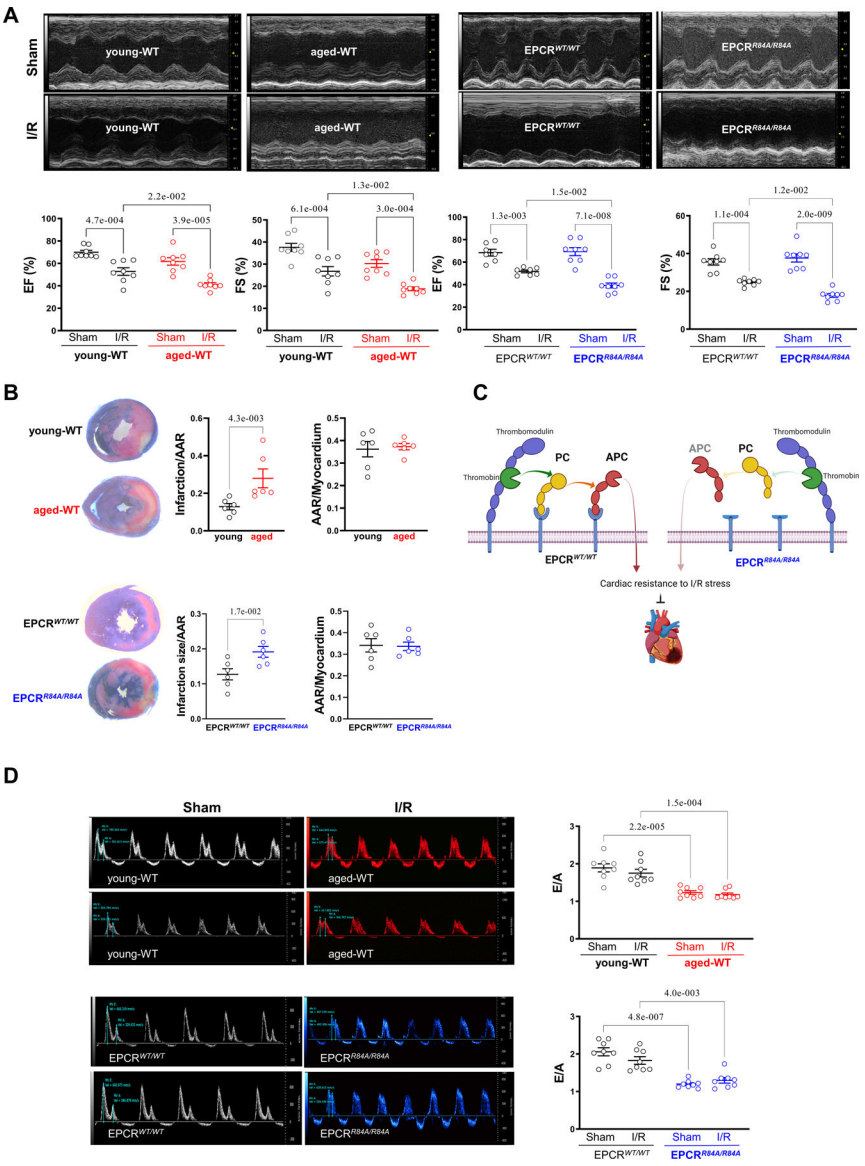
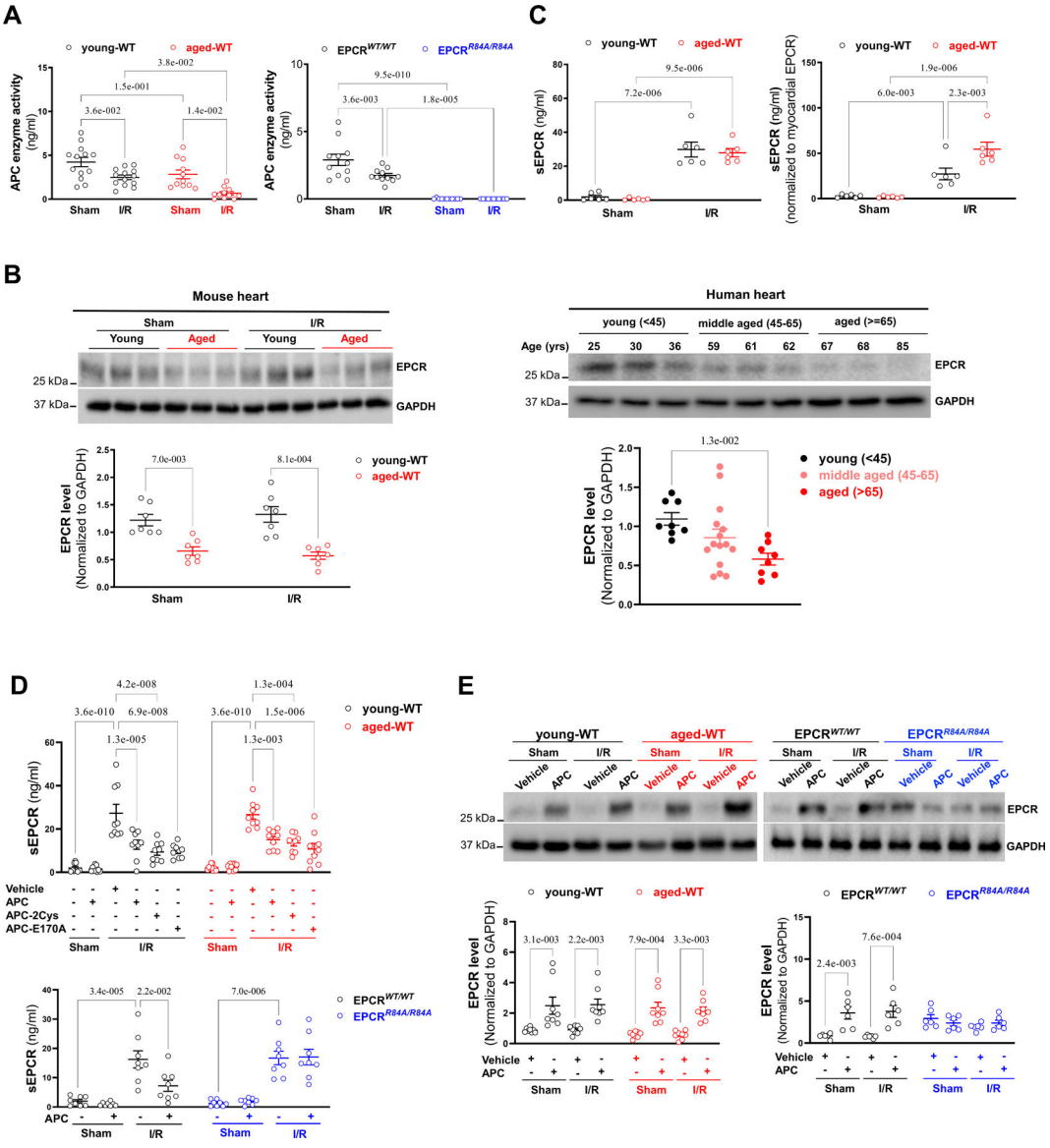


Figure 1. Cardiac function assessment of young/aged-WT and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ mice.

(A) Echocardiography showed that aged-WT *vs.* young-WT and EPCR$^{R84A/R84A}$ *vs.* EPCR$^{WT/WT}$ were vulnerable to I/R stress as shown by ejection fraction (EF) and fractional shortening (FS). Upper: Reprehensive images of M-mode echocardiography. Lower: Quantification of echocardiography measurements for EF and FS. Biological replicates N=8 for each group. P value was determined by two-way ANOVA with Tukey's *post-hoc* test. **(B)** Young-WT, aged-WT, EPCR$^{WT/WT}$ and EPCR$^{R84A/R84A}$ mice were subjected to *in vivo* regional ischemia for 45 min, followed by 24 h of reperfusion. Left: Representative sections of the extent of myocardial infarction were presented. Right: TTC staining showed larger infarct area in aged-WT *vs.* young-WT and EPCR$^{R84A/R84A}$ *vs.* EPCR$^{WT/WT}$ heart. Ratio of the area at the risk (AAR) to the total myocardial area refers to the area affected by ischemia, and ratio of the infarcted area to AAR is used to access to the myocardium injury. Biological replicates N=6 for each group. P value was determined by Mann-Whitney two

tailed test. **(C)** The scheme depicts that the impaired APC signaling in EPCR point mutation ($EPCR^{R84A/R84A}$) knock-in *vs.* $EPCR^{WT/WT}$ mice results in hypersensitive to ischemia reperfusion (I/R) injury. **(D)** Diastolic dysfunction was shown by E/A ratio in aged-WT *vs.* young-WT and $EPCR^{R84A/R84A}$ *vs.* $EPCR^{WT/WT}$ hearts under sham or I/R conditions. Left: Reprehensive images of Doppler echocardiography. The blue lines were the analysis performed on Vevo Image software to obtain the parameters for diastolic function analysis. Right: Quantification analysis of E/A ratio. Biological replicates N=8 for each group. P value was determined by two-way ANOVA with Tukey's *post-hoc* test.



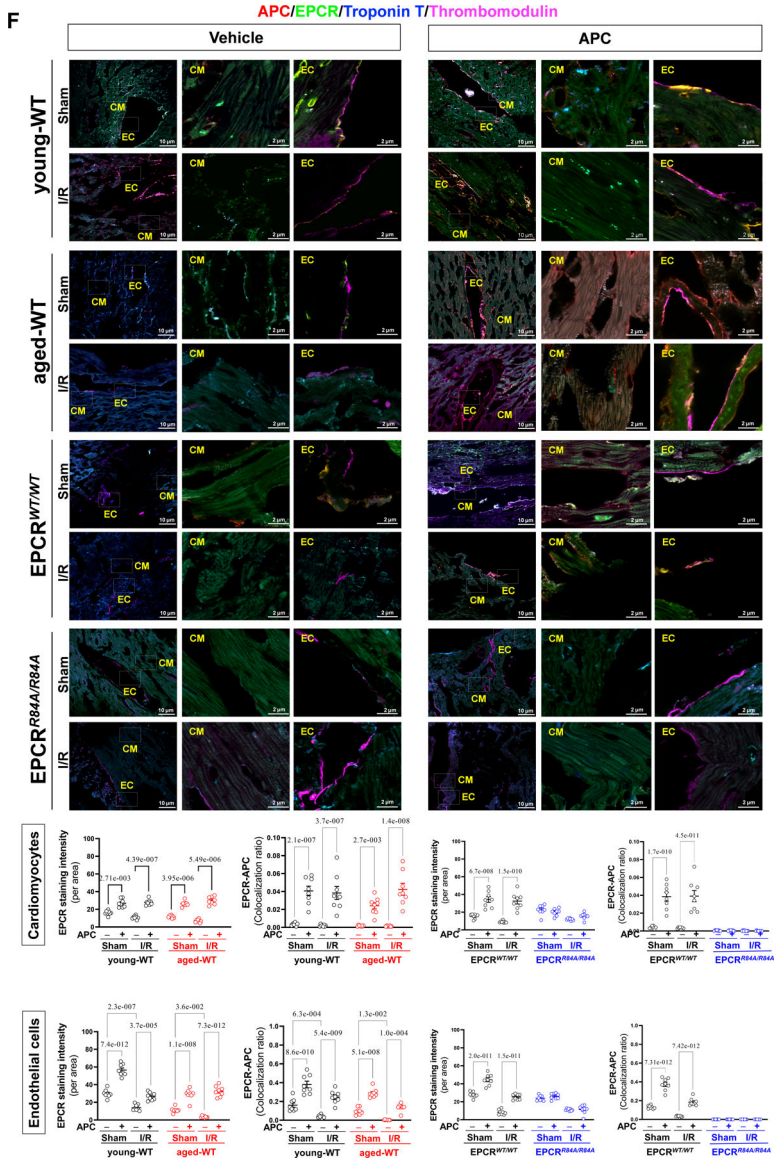
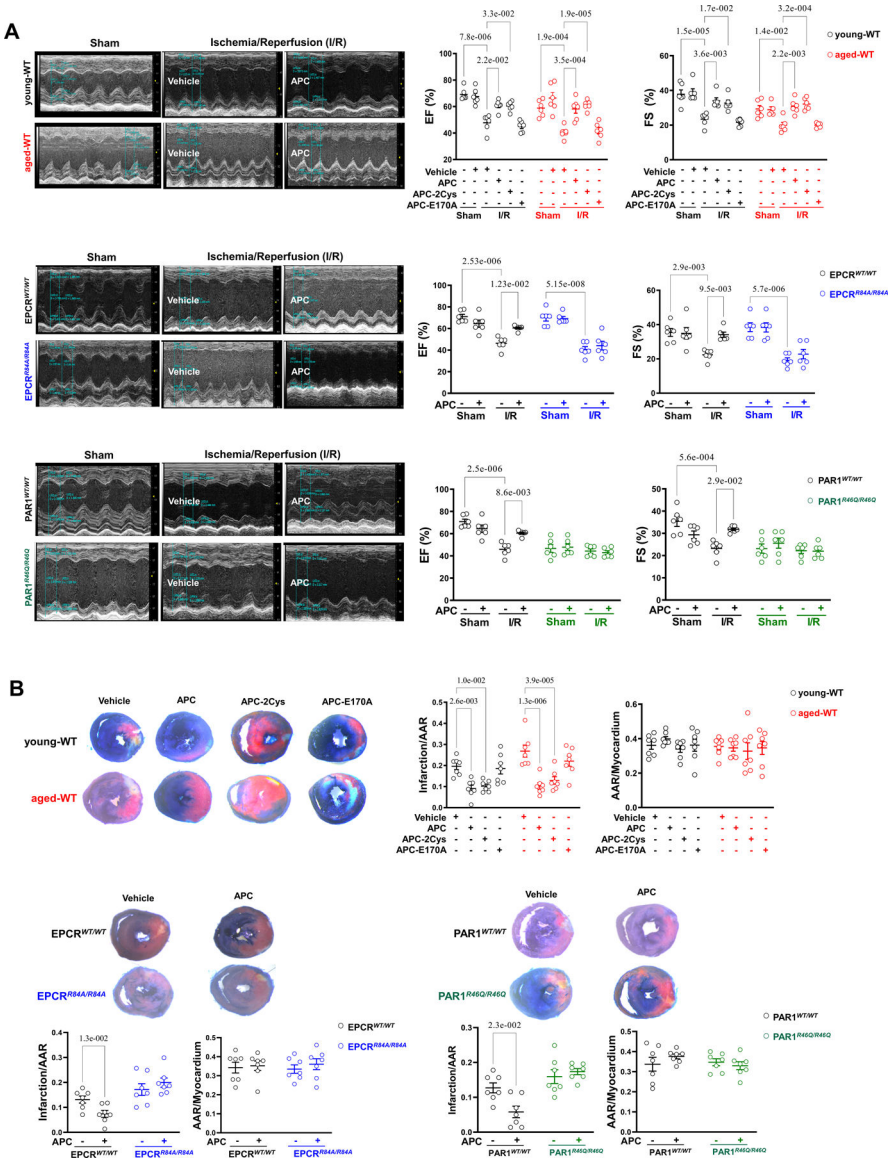


Figure 2. APC activity and EPCR integrity measurement in young/aged-WT and EPCR$^{WT/WT}$/ EPCR$^{R84A/R84A}$ mice in response to I/R stress.

(A) APC enzyme activity showed that I/R induced the exacerbated APC activity impairment in aged-WT *vs.* young-WT and null activity in EPCR$^{R84A/R84A}$ *vs.* EPCR$^{WT/WT}$ mice. Biological replicates N=13 for young groups and N=11 for aged groups and P value was determined by two-way ANOVA with Tukey's *post-hoc* test. Biological replicates N=11 for EPCR$^{R84A/R84A}$ and EPCR$^{WT/WT}$ groups and P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. **(B)** Left, Cardiac EPCR levels are defected in aged mice. Biological replicates N=7 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. Right, Cardiac EPCR levels are declined with aging in human. Biological replicates N=8 for both young and aged groups and N=15 for middle aged group. P value was determined by one-way ANOVA with Tukey's *post hoc* test. **(C)** Circulating soluble EPCR (sEPCR) levels in young-WT and aged-WT mice under sham or I/R stress (Left). The release ratio of sEPCR/myocardial EPCR (Right). Biological

replicates N=6 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. (**D)** APCs administration reduced I/R stress-induced circulating sEPCR in wild type but not in EPCR$^{R84A/R84A}$ mice. Biological replicates N=9 for young and aged groups, P value was determined by three-way ANOVA with Tukey's *post-hoc* test. Biological replicates N=8 for EPCR$^{WT/WT}$ and EPCR$^{R84A/R84A}$ groups, P value was determined by two-way ANOVA with Tukey's *post-hoc* test. (**E)** Immunoblotting analysis showed that administration of APC leads to increased EPCR levels in wild type but not in EPCR$^{R84A/R84A}$ hearts. Biological replicates N=8 young and aged groups, P value was determined by two-way ANOVA with Tukey's *post-hoc* test. Biological replicates N=6 for EPCR$^{WT/WT}$ and EPCR$^{R84A/R84A}$ groups, P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. (**F)** Immunofluorescence staining showed that APC administration causes myocardium EPCR stabilization in wild type but not EPCR$^{R84A/R84A}$ hearts under sham or I/R conditions. Biological replicates N=8 for each group, P value was determined by two-way ANOVA with Tukey's *post-hoc* test.



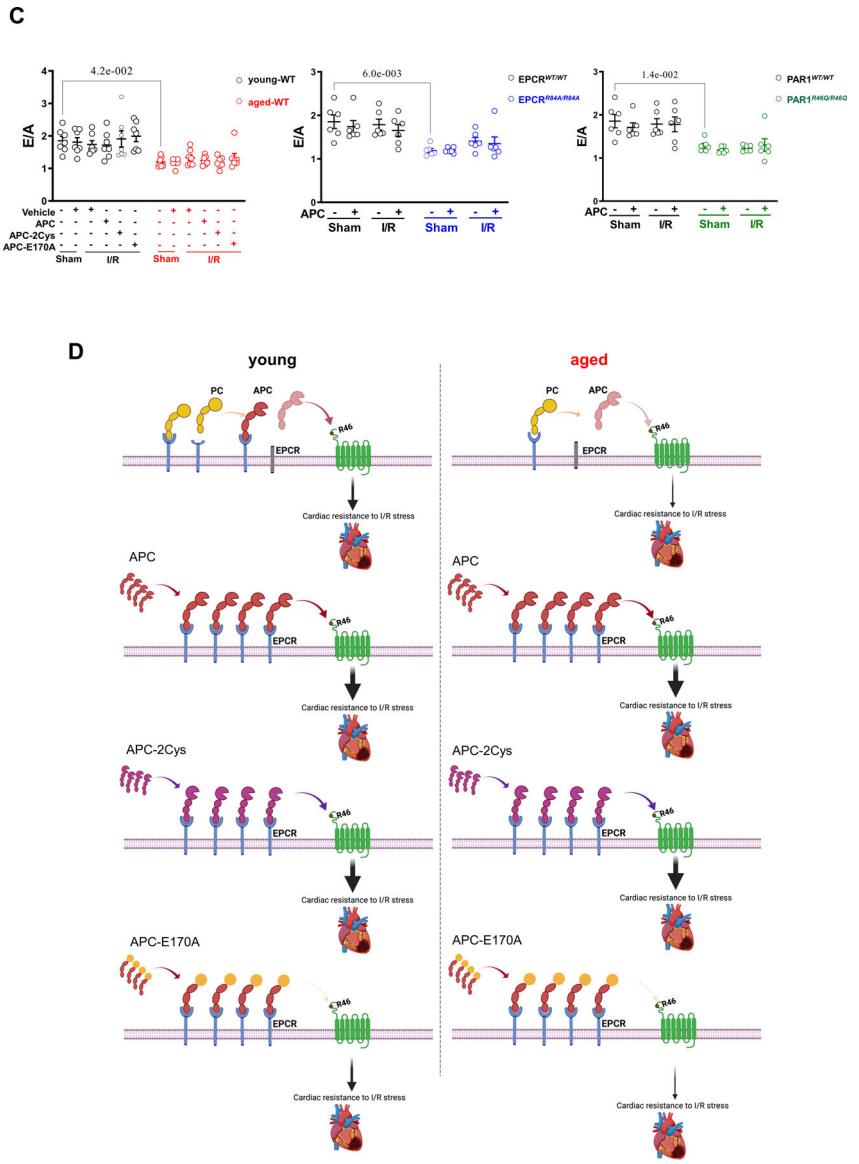
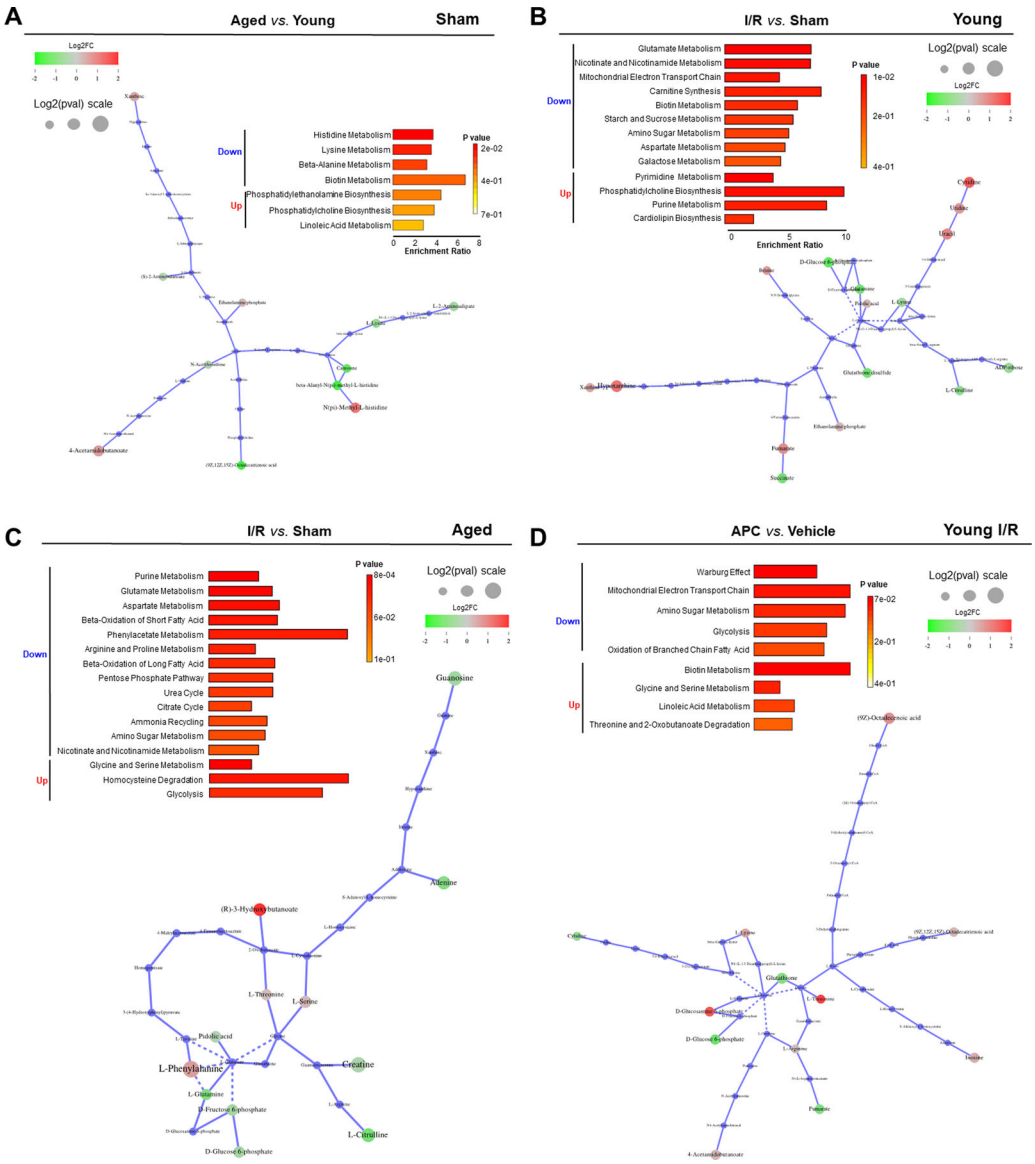


Figure 3. EPCR mediates cardioprotection of the APCs *via* PAR1 biased signaling.

(A) Echocardiography showed that administration of APC and signal-selective APC-2Cys can attenuate the I/R-induced cardiac dysfunction in young/aged-WT as shown by ejection fraction (EF) and fractional shortening (FS). The anticoagulant-selective APC-E170A did not show statistical beneficial effects on I/R hearts (Upper). The cardiac beneficial actions of APC were diminished in EPCR$^{R84A/R84A}$ *vs.* EPCR$^{WT/WT}$ and PAR1$^{R46Q/R46Q}$ *vs.* PAR1$^{WT/WT}$ mice (Middle and Lower). Left: Representative images of M-mode echocardiography. The blue lines were the analysis performed on Vevo Image software to obtain the parameters for systolic function. Right: Quantification analysis of EF and FS with or without APC treatment. Biological replicates N=6 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. (**B)** TTC staining demonstrated that APC and APC-2Cys but not APC-E170A reduced the I/R-induced myocardial infarction in young/aged-WT (Upper). The cardioprotective effects

of APC on I/R-induced myocardial infarction were abolished in EPCR$^{R84A/R84A}$ *vs.* EPCR$^{WT/WT}$ and PAR1$^{R46Q/R46Q}$ *vs.* PAR1$^{WT/WT}$ hearts (Lower). Upper: Representative myocardial infarction images with TTC staining. Lower: Quantification analysis of myocardial infarct size with or without APC. Biological replicates N=7 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. (**C)** Doppler echocardiography showed the diastolic dysfunction was observed in aged-WT, EPCR$^{R84A/R84A}$ and PAR1$^{R46Q/R46Q}$ hearts. Biological replicates N=7 for young and aged groups, N=6 for EPCR and PAR1 mutant and wildtype littermates groups. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. (**D)** The scheme depicts that EPCR and PAR1 mediate APC's cardioprotective effects on ischemic insults caused by I/R.



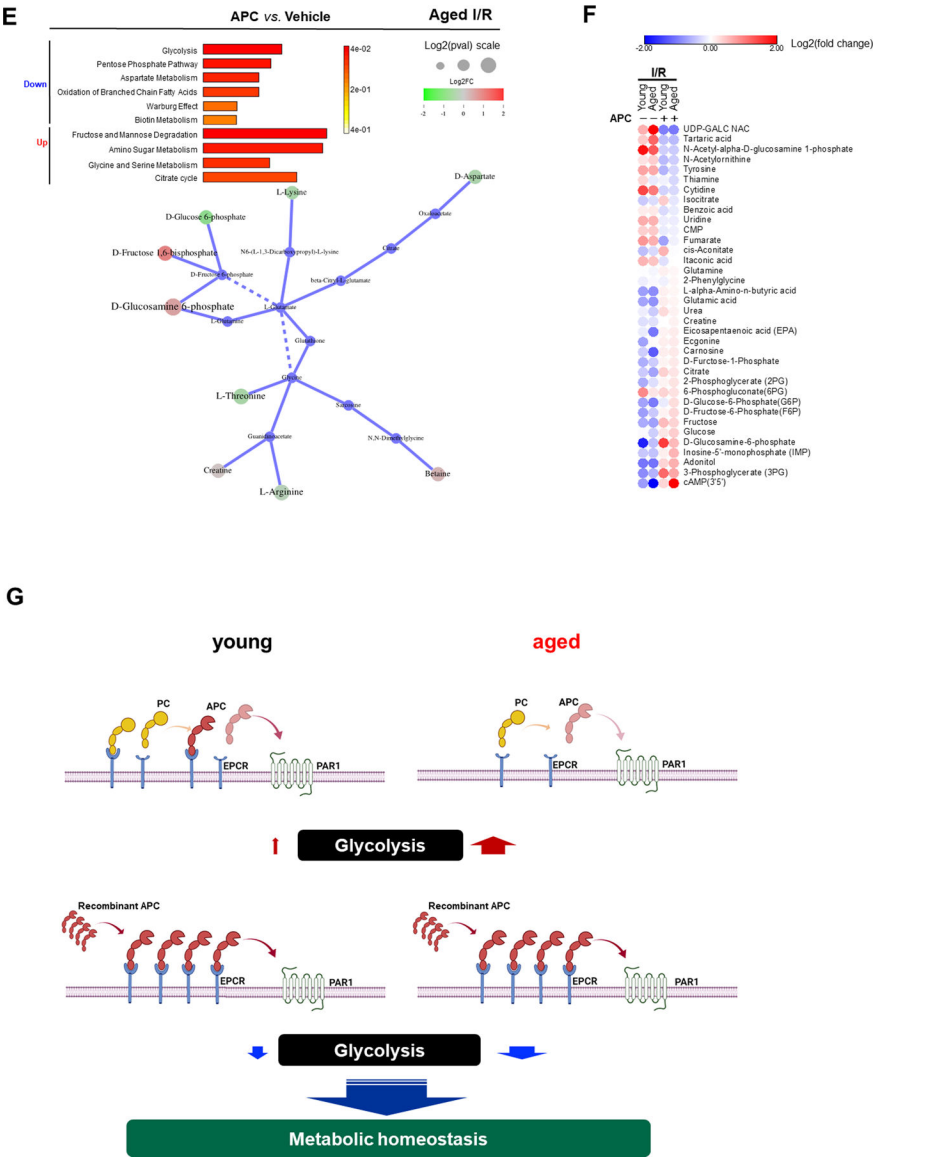
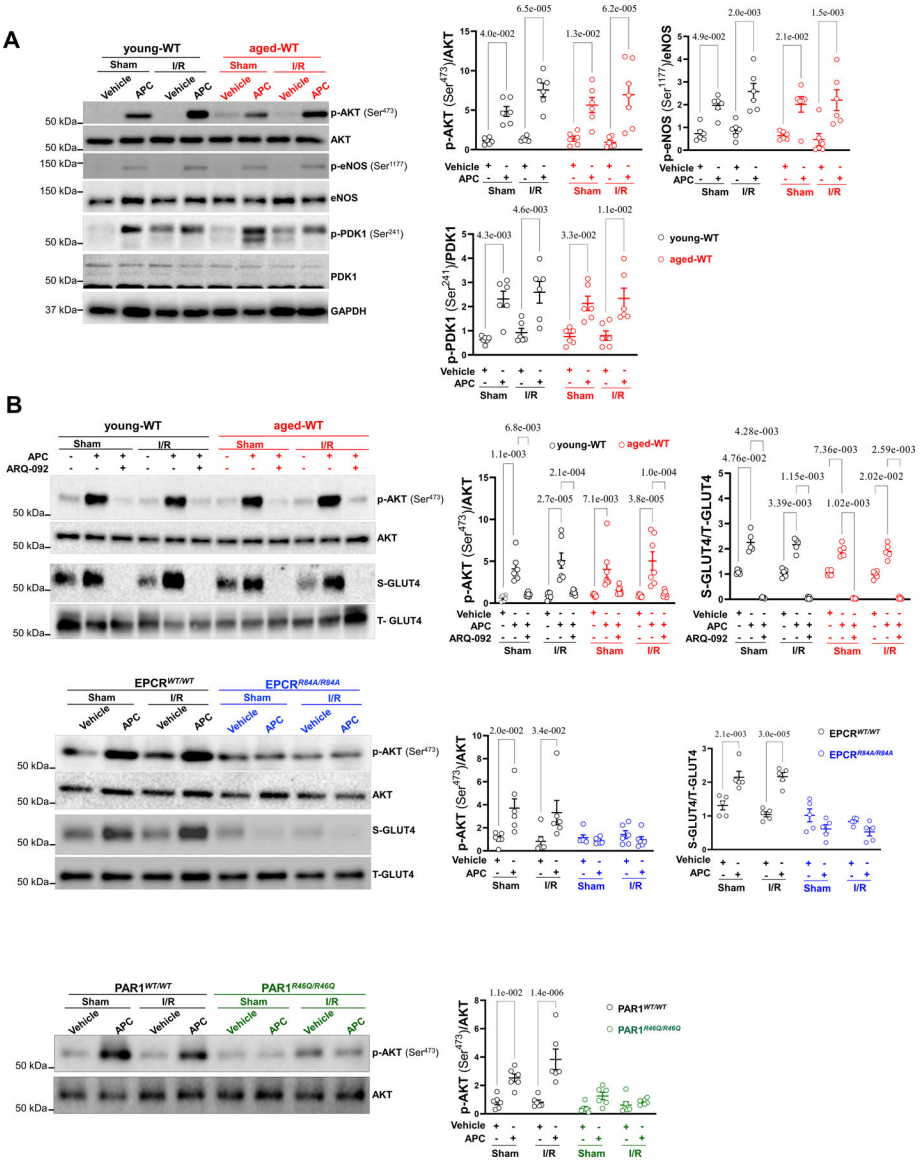


Figure 4. Metabolomics analysis of cardiac substrate metabolism.

The left ventricular tissues were collected from young-WT (3–4 months)/aged-WT (24–26 months) mouse hearts under sham or 45 min of ischemia and 24 h of reperfusion (I/R) with or without APC treatment. Biological replicates N=3 for each group. **(A-E**) Metabolite's alteration network in the comparison listed in the figure. Significant altered metabolites were recognized with p-adjust value <0.05 using Grubbs' test. Color represents the increase (red) and decrease (green) of each gene, fold change ratios are presented in log2 form according to the legend panel. Enrichment score is representing the numbers of metabolites cluster in the same pathway. The gradient red color is representing the p value based on the legend panel. **(F)** Heatmap shows relative abundance of representative significantly modulated metabolites in response to I/R stress in young-WT and aged-WT *vs.* young-WT-sham with or without APC treatment. (**G)** The scheme depicts that administration of APC can modulate cardiac metabolism to rescue the impaired metabolic homeostasis in aging under I/R stress.



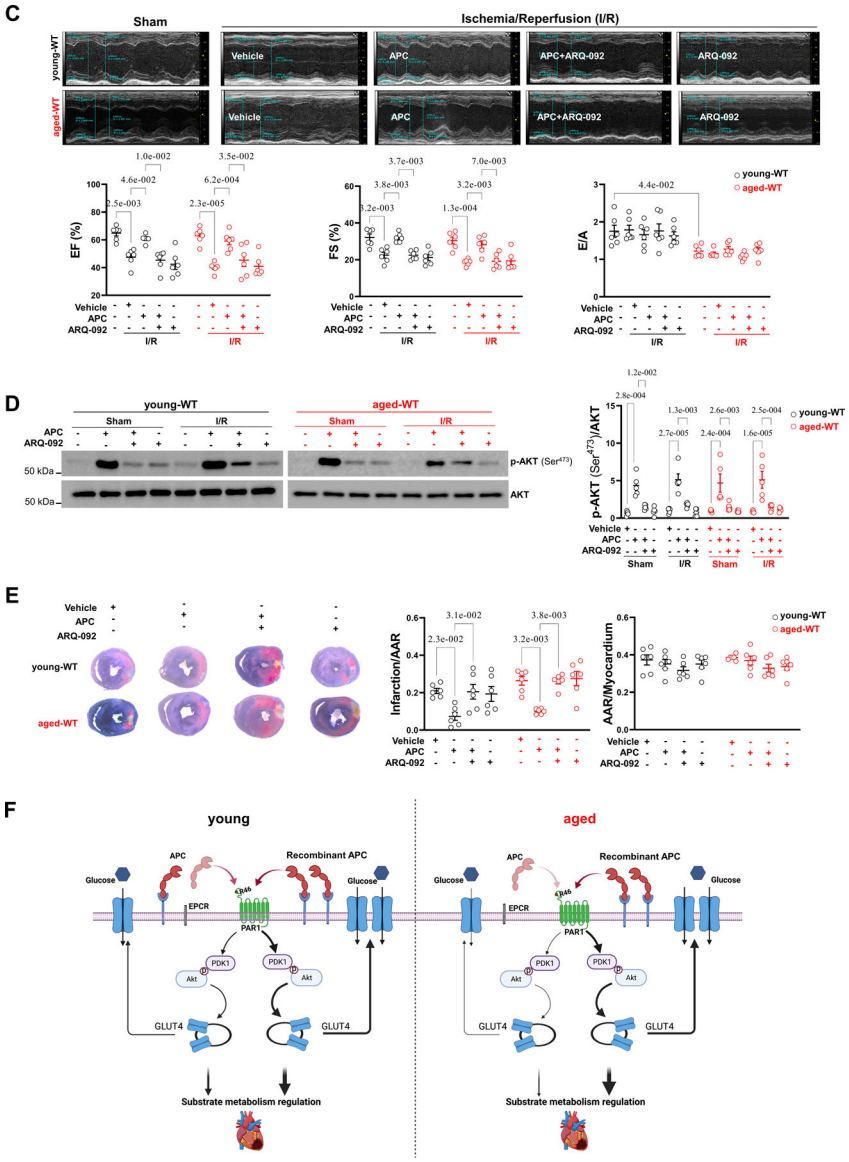
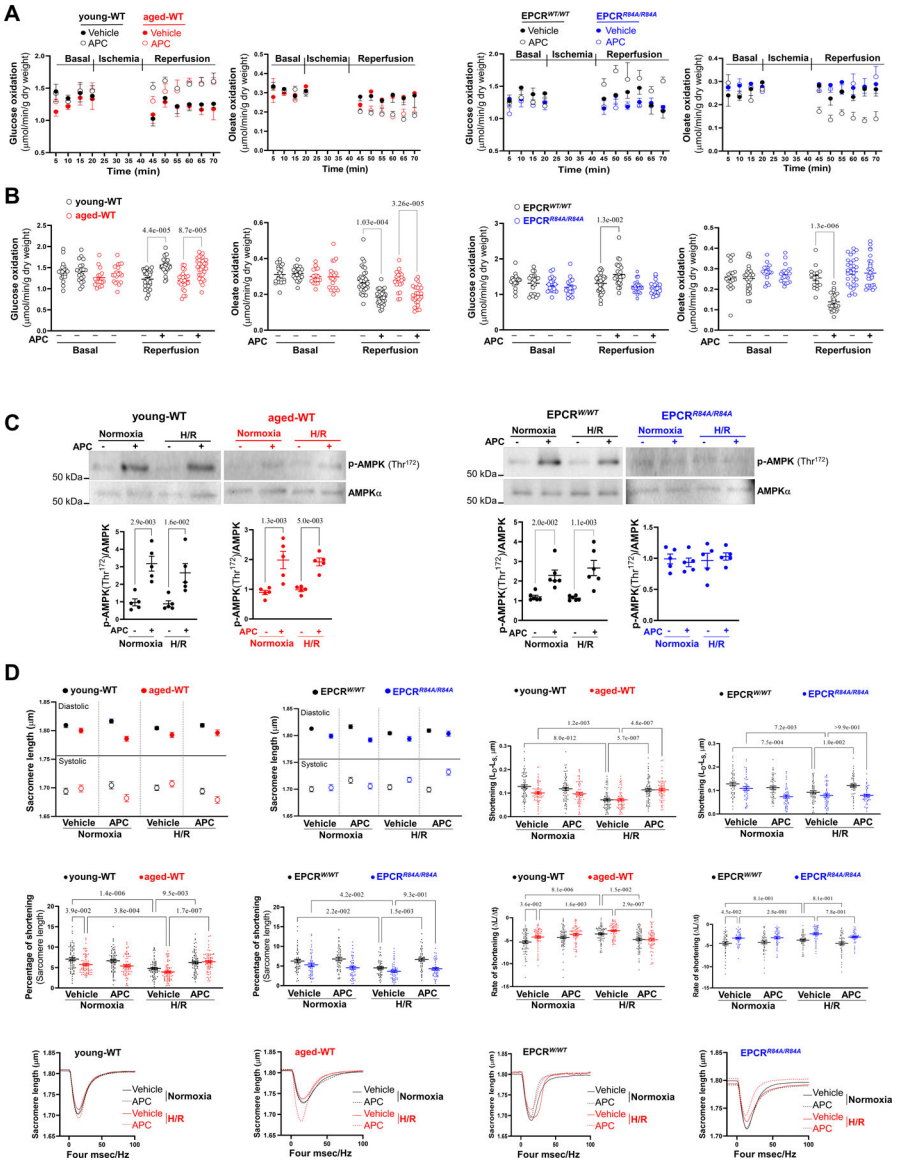


Figure 5. EPCR mediates the metabolic remodeling induced by APC.

(A) Immunoblotting showed the phosphorylation level of upstream kinase PDK1, AKT, and the downstream factor eNOS in young/aged-WT heart under sham or I/R conditions with or without APC treatment. Biological replicates N=6 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. **(B)** Upper, Immunoblotting showed the AKT phosphorylation at Ser^{473} site and total AKT as well as the cell surface labeled GLUT4 (S-GLUT4) and the total GLUT4 (T-GLUT4) in the left ventricles of young-WT/aged-WT hearts under sham or I/R treatment with or without APC and/or AKT inhibitor (ARQ-092). Biological replicates N=7 for each group in detecting AKT phosphorylation, P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. N=5 for each group in detecting GLUT4 trafficking to cell membrane, P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. Middle, Immunoblotting of the phosphorylation of AKT (Ser^{473}) and total AKT, S-GLUT4 and

T-GLUT4 in the left ventricles of EPCR$^{WT/WT}$ and EPCR$^{R84A/R84A}$ heart under sham or I/R with or without APC. Biological replicates N=6 for each group in detecting AKT phosphorylation, P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. N=5 for each group in detecting GLUT4 trafficking to cell membrane, P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. Lower, Immunoblotting of the AKT phosphorylation (Ser^{473}) and total AKT in the left ventricles of PAR1$^{WT/WT}$ and PAR1$^{R46Q/R46Q}$ hearts under sham or I/R with or without APC. Biological replicates N=6 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. **(C)** Echocardiography showed that administration of AKT inhibitor diminished the cardioprotective effects of APC in young/aged-WT as shown by ejection fraction (EF) and fractional shortening (FS). Upper: Representative images of M-mode echocardiography. The blue lines were the analysis performed on Vevo Image software to obtain the parameters for systolic function. Lower: Quantification analysis of EF, FS, and E/A with or without APC and/or AKT inhibitor. Biological replicates N=6 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. **(D)** Immunoblotting of the phosphorylation of AKT in young-WT and aged-WT heart under sham or I/R with APC and/or AKT inhibitor. Biological replicates N=5 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. (**E)** TTC staining demonstrated that administration of AKT inhibitor can diminish the cardioprotective effects of APC in young/aged-WT. Left: Representative myocardial infarction images with TTC staining. Right: Quantification analysis of myocardial infarct size with APC and/or AKT inhibitor (ARQ-092). Biological replicates N=6 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. **(F)** The scheme depicts the administration of APC promotes the activation of PDK1/AKT/GLUT4 signaling pathway to modulate substrate metabolism through EPCR and PAR1 biased signaling during I/R.



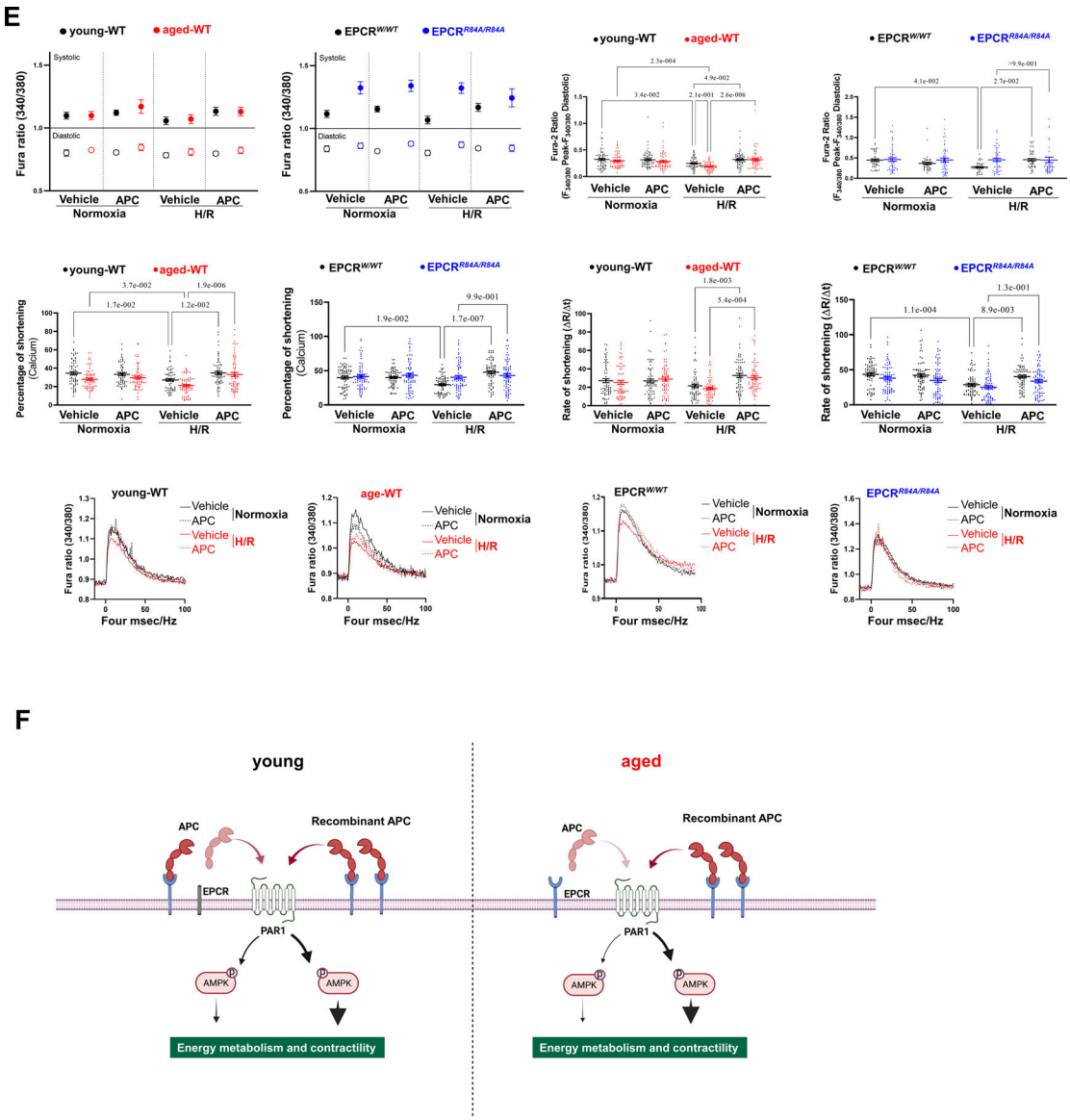


Figure 6. APC modulates energy metabolism and improves the contractile properties of cardiomyocytes.

(A-B) Glucose oxidation and oleate oxidation was analyzed by measuring [^{14}C]-glucose incorporation into $^{14}CO_2$ and incorporation of [9,10-^{3}H] oleate into $^{3}H_2O$, respectively in the *ex vivo* mouse hearts subjected to 20 min ischemia and 30 min reperfusion. The APC administration significantly augmented glucose oxidation in young/aged-WT and EPCR$^{WT/WT}$ but not EPCR$^{R84A/R84A}$ hearts during reperfusion after ischemic stress. (A) Biological replicates N=6 for each group. P value was determined by mixed-effects analysis, with the Geisser-Greenhouse correction. (B) Biological replicates N=6 for each group. P value was determined by mixed-effects analysis, with the Geisser-Greenhouse correction. **(C)** Immunoblotting showed the phosphorylation of AMPK in the isolated cardiomyocytes from young/aged-WT, and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or H/R stress with or without APC treatment. Biological replicates N=5 for each group. P value was determined by Kruskal-Wallis test with Dunn's multiple comparison test. **(D)** The

contractile properties of isolated cardiomyocytes from young/aged-WT, and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or H/R stress with or without APC treatment. Biological replicates N=5, cells number per animal n=16 for each group. P value was determined by mixed-effects analysis, with the Geisser-Greenhouse correction. **(E)** The transient calcium signal response of the isolated cardiomyocytes from young/aged-WT, and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or H/R with or without APC treatment. Biological replicates N=5, cells number per animal n=10 for each group. P value was determined by mixed-effects analysis, with the Geisser-Greenhouse correction. **(F)** The scheme depicts that the administration of APC modulates cardiac energy metabolism and cardiomyocytes' contractility through AMPK signaling.

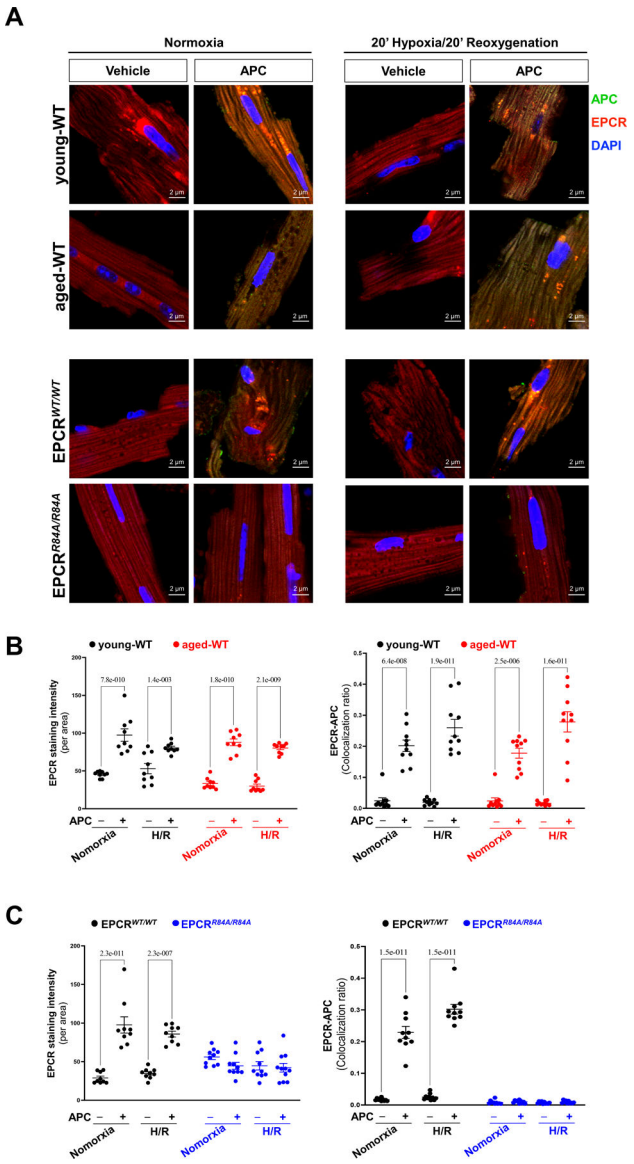


Figure 7. APC administration stabilizes EPCR in cardiomyocytes through binding interactions.

(A) Representative immunofluorescence staining showed the levels of EPCR and APC in cardiomyocytes from young/aged-WT, and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or H/R stress conditions with or without APC treatment. **(B)** The relative levels of EPCR in the isolated cardiomyocytes from young/aged-WT, and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or H/R stress conditions with or without APC treatment. Mice biological replicates N=3, cells number per animal n=3 for each group. P value was determined by mixed-effects analysis, with the Geisser-Greenhouse correction. **(C)** The colocalization ratio between APC and EPCR in the isolated cardiomyocytes from young/aged-WT, and EPCR$^{WT/WT}$/EPCR$^{R84A/R84A}$ hearts under normoxia or H/R stress conditions with or without APC treatment. Mice biological replicates N=3, cells number

per animal n=3 for each group. P value was determined by mixed-effects analysis, with the Geisser-Greenhouse correction.