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A Retinoic Acid β_2-Receptor Agonist Exerts Cardioprotective Effects

Alice Marino, Takuya Sakamoto, Xiao-Han Tang, Lorraine J. Gudas, and Roberto Levi

Department of Pharmacology, Weill Cornell Medicine, New York, New York

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ABSTRACT

We previously discovered that oral treatment with AC261066, a synthetic selective agonist for the retinoic acid β_2-receptor, decreases oxidative stress in the liver, pancreas, and kidney of mice fed a high-fat diet (HFD). Since hyperlipidemic states are causally associated with myocardial ischemia and oxidative stress, we have now investigated the effects of AC261066 in an ex vivo ischemia/reperfusion (I/R) injury model in hearts of two prototypic dysmetabolic mice. We found that a 6-week oral treatment with AC261066 in both genetically hypercholesterolemic (ApoE$^{-/-}$) and obese (HFD-fed) wild-type mice exerts protective effects when their hearts are subsequently subjected to I/R ex vivo in the absence of added drug. In ApoE$^{-/-}$ mice this cardioprotection ensued without hyperlipidemic changes. Cardioprotection consisted of attenuation of infarct size, diminution of norepinephrine (NE) spillover, and alleviation of reperfusion arrhythmias. This cardioprotection was associated with a reduction in oxidative stress and mast cell (MC) degranulation. We suggest that the reduction in myocardial injury and adrenergic activation, and the antiarrhythmic effects, result from decreased formation of oxygen radicals and toxic aldehydes known to elicit the release of MC-derived renin, promoting the activation of the local renin-angiotensin system leading to enhanced NE release and reperfusion arrhythmias. Because these beneficial effects of AC261066 occurred at the ex vivo level following oral drug treatment, our data suggest that AC261066 could be viewed as a therapeutic means to reduce I/R injury of the heart, and potentially also be considered in the treatment of other cardiovascular ailments such as chronic arrhythmias and cardiac failure.

Introduction

Early reperfusion of an occluded coronary artery after myocardial infarction can reduce infarct size, which contributes to preserving left ventricular contraction and preventing the onset of heart failure and other pathologies. However, reperfusion paradoxically can cause further cardiomyocyte death, which is generally known as myocardial ischemia/reperfusion (I/R) injury (Yellon and Hausenloy, 2007; Hausenloy and Yellon, 2013). I/R injury is accompanied by severe arrhythmias, such as ventricular tachycardia (VT) (Bovo et al., 2012) and ventricular fibrillation (VF), which are significant causes of sudden death in patients with ischemic heart disease (Lo et al., 2017).

In the I/R heart, mast cell (MC) activation contributes significantly to arrhythmia generation (Mackins et al., 2006; Reid et al., 2007). Reactive oxygen species (ROS) are a class of highly reactive and unstable molecules, mainly generated in mitochondria, which play important roles in the maintenance of biologic homeostasis (Griendling et al., 2016). During reperfusion, a rise in ROS and toxic aldehyde production (Esterbauer et al., 1991) are responsible for MC degranulation (Yoshimaru et al., 2002; Steiner et al., 2003; Swindle et al., 2004; Heneberg and Dráber, 2005; Swindle and Metcalfe, 2007; Inoue et al., 2008; Koda et al., 2010; Chao et al., 2011; Aldi et al., 2015); this is associated with the release of MC-derived active renin, the first step in the activation of a local renin-angiotensin system (RAS) in the heart, which is responsible for enhanced norepinephrine release and arrhythmias (Mackins et al., 2006; Reid et al., 2007). Monoaminoxidases are mitochondrial enzymes responsible for catecholamine catabolism, which generates hydrogen peroxide, aldehyde, and ammonia as by-products, contributing to oxidative stress and ROS production in the heart (Bianchi et al., 2005; Kaludercic et al., 2010, 2014). Since increased ROS production at the mitochondrial level exacerbates I/R injury, eliciting arrhythmias (Zorov et al., 2000) and left ventricular remodeling (Gaudron et al., 1993), numerous studies have explored therapeutic strategies such as antioxidants to counteract ROS production and oxidative responses, ultimately affording cardioprotection (Kasparova et al., 2015).

Vitamin A (retinol) and its metabolites and derivatives, collectively known as retinoids, are important lipophilic signaling molecules that play critical roles in controlling both vertebrate development and stem cell differentiation in the adult (Gudas et al., 1994; Niederreither and Dollé, 2008). The

Weill Cornell Medicine owns intellectual property (IP) related to AC261066 and has licensed this IP to Sveikatal, Inc. Dr. L.J.G. and Dr. X.-H.T. are founders of Sveikatal, Inc. Dr. A.M., Dr. T.S., and Dr. R.L. report no conflicts of interest.

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ABBREVIATIONS: HDL, high-density lipoprotein; HFD, high-fat diet; I/R, ischemia/reperfusion; LDL, low-density lipoprotein; MC, mast cell; MDA, malondialdehyde; NE, norepinephrine; RA, retinoic acid; RAR, retinoic acid receptor; RARβ_2, retinoic acid β_2-receptor; RAS, renin-angiotensin system; ROS, reactive oxygen species; VF, ventricular fibrillation; VT, ventricular tachycardia; WT, wild type.

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actions of retinoids, such as the potent, biologically active endogenous metabolite of vitamin A, all-*trans* retinoic acid (RA), are primarily mediated by binding to ligand-activated transcription factors, the RA receptors (RARs) α, β, and γ; when RARs bind the pan-agonist RA, they heterodimerize with retinoid X receptors α, β, and γ. The RARs are expressed in the heart during development and in the adult, and RA signaling is active in the post-ischemic heart (Dollé, 2009; Bilbija et al., 2012; Gudas, 2012). The RA signaling pathway can reduce cardiac I/R injury and ROS production (Zhu et al., 2015). RA also displayed protective effects against cardiac arrhythmias (Kang and Leaf, 1995). Moreover, all-*trans* RA protected against doxorubicin-induced cardiotoxicity, in which oxidative stress and I/R-like damage are known to play a major role (Yang et al., 2016; Khafaga and El-Sayed, 2018). Furthermore, supplementation with RA prevented left ventricular dilatation and preserved ventricular function in rats with induced infarction (Paiva et al., 2005). Conversely, in adult rats, vitamin A deficiency caused left ventricular dilatation that led to a major decrease in cardiac function (Azevedo et al., 2010). Loss of RARα specifically in mouse cardiomyocytes resulted in diastolic dysfunction from increased ROS (Zhu et al., 2016).

Accordingly, we investigated possible protective effects of retinoids in an ex vivo I/R injury model in the heart. We chose not to use RA since the liver metabolizes RA rapidly when pharmacological doses of RA are given orally (Muindi et al., 1992a,b), and RA is an agonist for all three RARs, α, β and γ. Instead, we characterized the effects of a retinoic acid β_2-receptor (RARβ_2) selective, synthetic agonist, AC261066 (Lund et al., 2005, 2009) in an ex vivo I/R injury model in the heart in two dysmetabolic murine models because hyperlipidemic states are known to be causally associated with myocardial ischemia and oxidative stress (Stampfer et al., 1991; Yang et al., 2008), and also because we had previously discovered that AC261066 decreases oxidative stress in the liver, pancreas, and kidneys of high-fat diet (HFD)–fed mice (Trasino et al., 2016). We report that AC261066 displays cardio-protective effects in an ex vivo I/R injury model in hyperlipidemic hearts, and our data suggest that AC261066 could be used to reduce I/R injury.

Materials and Methods

I/R in Ex Vivo Mouse Hearts

Wild-type (WT) C57/BL6 and ApoE$^{-/-}$ male mice (C57/BL6 background; Jackson Laboratories, Bar Harbor, ME) were maintained on a regular laboratory chow diet (#5053, Pico Diet, PicoLab Rodent Diet; St. Louis, MO). Six weeks after birth, another group of ApoE$^{-/-}$ mice received, in addition to their chow diet, drinking water containing 3.0 mg AC261066/100 ml in 0.1% dimethylsulfoxide/H_2O for 6 weeks. Twenty minutes after a heparin injection (100 IU, i.p.) to avoid blood clotting, mice were anesthetized with CO_2 vapor and humanely killed by cervical dislocation while under anesthesia (Institutional Animal Care and Use Committee approved). Hearts were quickly excised and cooled in ice-cold Krebs-Henseleit solution (composition: NaCl 120 mM; KCl 4.71 mM; $CaCl_2$ 2.5 mM; $MgSO_4$ 1.2 mM; KH_2PO_4 1.2 mM; $NaHCO_3$ 84.01 mM; Dextrose 11.1 mM; Pyruvic acid 2.0 mM; and EDTA 0.5 mM), and equilibrated with 95% O_2 + 5% CO_2. Hearts were then cannulated via the aorta with a 20-gauge needle and perfused in a Langendorff apparatus (Radnoti, Monrovia, CA) at constant pressure (100 cm H_2O) with Krebs-Henseleit solution at 37°C. Two needle electrodes were attached to the surface of the right atrium and left ventricular apex for ECG recordings. The ECG was recorded online (sample frequency of 1 kHz) and arrhythmia duration was analyzed using Powerlab/8SP (ADInstruments, Colorado Springs, CO). Following 20-minute stabilization, all hearts were subjected to 40-minute normothermic global ischemia, induced by complete cessation of coronary perfusion, followed by 120-minute reoxygenation (reperfusion) with Krebs-Henseleit solution (I/R). I/R-injured areas (i.e., infarct size) were analyzed at the end of reperfusion. Coronary flow was measured by timed collections of the effluent every 2 minutes, and samples were assayed for norepinephrine (NE) by high-performance liquid chromatography with electrochemical detection as previously described (Marino et al., 2017).

Experiments were also performed on WT C57/BL6 mice, maintained on either a standard laboratory chow (Control diet) (#5053; Pico Diet) or a HFD with 45% kcal from fat (#58125; TestDiet; St. Louis, MO) for 3–5 months). Six weeks after the start of the HFD, these mice received either drinking water containing 0.1% dimethylsulfoxide (vehicle) or water/0.1% dimethylsulfoxide containing AC261066 (3.0 mg/100 ml). As powder, AC261066 is stable at room temperature, according to the manufacturer. Our high-performance liquid chromatography results show that AC261066 is stable in drinking water for at least 1 week at room temperature. The half-life in the heart is not known. The scientist performing the ex vivo I/R procedure was blinded to the identities of the groups of mice.

2,3,5-Triphenyltetrazolium Chloride Staining for Ischemic Area in Mouse Hearts

At the end of 120-minute reperfusion, hearts were cut (6 to 7 sections/heart, 2 mm thick), incubated in an aqueous solution of 2,3,5-triphenyltetrazolium chloride (1% w/v) (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 37°C, and transferred to a formaldehyde solution (10% v/v) for overnight fixation. Heart slices were photographed with 16× magnification and analyzed by computerized morphometry using ImageJ software (National Institutes of Health, Bethesda, MD) to measure infarct sizes (expressed as percentage of ischemic vs. total left ventricular area). Infarct size was measured in every slice and then averaged for each single heart. The most representative sections were chosen for illustration.

Malondialdehyde Staining

Cryo-Sectioning. At the end of I/R, mouse hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, and then incubated in 30% sucrose in PBS overnight at 4°C. Tissues were embedded in optimal cutting temperature compound and subjected to cryo-sectioning. The sections were 15 μm thick and stored at −80°C prior to immunostaining.

Immunostaining. Frozen sections were dried at room temperature before staining. Briefly, the sections were washed in PBS, blocked in 10% goat serum plus 0.02% Triton X-100 in PBS for 30 minutes at room temperature, followed by incubation with an anti-malondialdehyde (MDA) antibody (Cat #6463 lot #GR3191333-3, 1:200; Abcam, Cambridge, MA) overnight at 4°C. Sections were then incubated with a goat anti-rabbit IgG secondary antibody at room temperature for 1 hour (ready to use; Thermo Fisher Scientific, Eugene, OR). As a negative control, sections were stained without incubation with the primary antibody. Signals were visualized based on a peroxidase-detection mechanism with 3,3-diaminobenzidine (Thermo Fisher Scientific, Rockford, IL) used as the substrate. Six to eight representative areas of each heart section from three to four mice per group were photographed and analyzed.

Toluidine Blue Staining of Mast Cells

At the end of 120-minute reperfusion, hearts were cut and processed for frozen sections. Heart sections (15 μm thick) were stained with toluidine blue (0.5%) to visualize MCs under transmitted light. MCs were identified with 600× magnification. Intact and

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degranulated MCs were counted in the analyzed sections, and MC degranulation was calculated as a percentage of degranulated MCs over total MCs. Three sections of each heart from 3 to 4 mice per group were analyzed.

Lipid Panel Measurements

Lipid panel measurements were carried out using the CardioChek PA analyzer (PTS Diagnostics, Indianapolis, IN). Briefly, 40 μl of mouse tail blood was applied to test strips to measure the levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides.

Statistical Analysis of Data

Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA). All data are reported as mean ± S.E.M. Statistical analysis was performed only when a minimum of $n = 5$ independent samples was acquired, and was performed with an unpaired t test when comparing two different groups and with one-way analysis of variance followed by Dunnett's post hoc test when comparing more than two groups of data. Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data were considered statistically significant when a value of at least $P < 0.05$ was achieved.

Results

The RARβ_2 Agonist AC261066 Decreases NE Overflows, Reperfusion Arrhythmias, and Infarct Sizes in Murine ApoE$^{-/-}$ Hearts Subjected to Ex Vivo I/R. Since high blood cholesterol levels are a major factor in myocardial ischemia (Stampfer et al., 1991), we first investigated whether the RARβ_2 agonist AC261066 influences relevant parameters of I/R-induced cardiac dysfunction, such as NE release, arrhythmia severity, and infarct size in hypercholesterolemic ApoE$^{-/-}$ mice. For this testing, spontaneously beating Langendorff-perfused mouse hearts were subjected to 40-minute global ischemia followed by 120-minute reperfusion (I/R). In control WT hearts, we found that NE overflows during reperfusion amounted to ~80 pmol/g, ventricular arrhythmias (VT/VF) lasted ~50 seconds, and infarct sizes comprised ~25% of the left ventricle. In ApoE$^{-/-}$ hearts subjected to I/R, NE overflows, VT/VF durations, and infarct sizes were each ~60% greater than in WT hearts. Notably, in hearts from ApoE$^{-/-}$ mice treated orally for 6 weeks with AC261066 and then subjected to I/R ex vivo in the absence of AC261066, NE overflows, VT/VF durations, and infarct sizes were markedly reduced by ~35%–45% compared with those in untreated ApoE$^{-/-}$ hearts subjected to I/R (Fig. 1). Notably, coronary flow in ApoE$^{-/-}$ hearts (3.025 ± 0.46 ml/min) did not differ from that of ApoE$^{-/-}$ hearts treated with AC261066 (2.092 ± 0.25 ml/min). These findings suggest that signaling via RARβ_2 exerts protective effects in a genetic hypercholesterolemic ex vivo I/R heart model.

The RARβ_2 Agonist AC261066 Limits the Increase in Oxidative Stress in Murine ApoE$^{-/-}$ Hearts Subjected to Ex Vivo I/R. Since the hearts from mice treated orally with AC261066 displayed protective features in this ex vivo hypercholesterolemic I/R model in the absence of AC261066 in vitro, we questioned whether these cardioprotective effects resulted from an attenuation of I/R-induced oxidative stress. Accordingly, we investigated whether the RARβ_2 agonist AC261066 influences the production of MDA, a classic marker

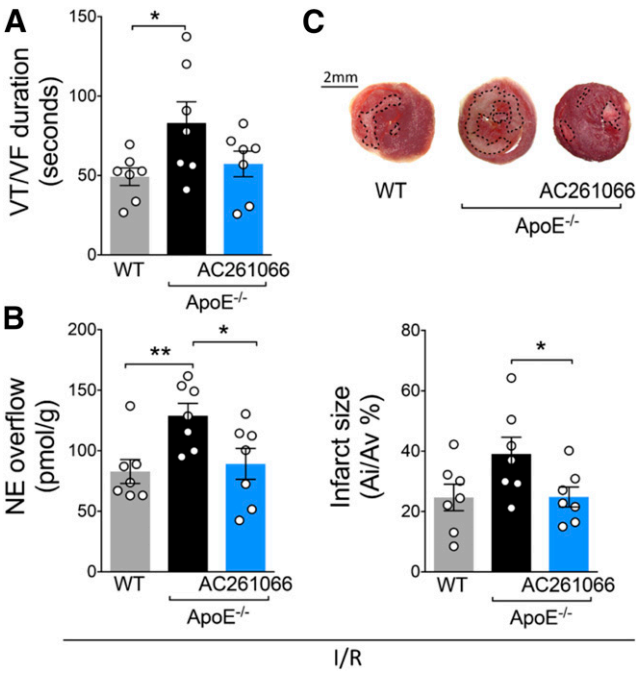

Fig. 1. The RARβ_2 agonist AC261066 reduces NE overflows, alleviates reperfusion arrhythmias, and decreases infarct sizes in hearts isolated from ApoE$^{-/-}$ mice subjected to ex vivo I/R. Mouse hearts were subjected to 40-minute global ischemia followed by 120-minute reperfusion (WT, n = 7; ApoE$^{-/-}$, n = 7; ApoE$^{-/-}$ + AC261066, n = 7). (A) Duration of reperfusion arrhythmias (VT/VF). (B) Overflows of NE in the first 6 minutes from the start of reperfusion. (C) Qualitative and quantitative representation of 2,3,5-triphenyltetrazolium chloride staining in 2-mm-thick left ventricle slices collected at the end of reperfusion. Bars indicate infarcted slice areas as a percentage of total slice areas (Ai/Av %; mean ± S.E.M. of independent experiments). Pale areas indicate I/R-injured tissue, while healthy tissue is colored in red. *$P < 0.05$; **$P < 0.01$, by unpaired t test. Each open circle denotes one mouse.

of oxidative stress (Luo et al., 2014). We found that a 6-week oral treatment with AC261066 markedly reduced the level of MDA in hearts from ApoE$^{-/-}$ mice subjected to I/R compared with their untreated controls; this reduction amounted to ~60% (Fig. 2). These findings suggest that the protective effects provided by the drug AC261066 in ApoE$^{-/-}$ hearts likely result at least in part from a reduction in I/R-induced oxidative stress.

The RARβ_2 Agonist AC261066 Reduces Mast Cell Degranulation in Murine ApoE$^{-/-}$ Hearts Subjected to Ex Vivo I/R. I/R-induced cardiac dysfunction is known to be associated with local MC degranulation and consequent release of noxious mediators elicited by toxic aldehydes produced in the setting of oxidative stress (Koda et al., 2010). Thus, we determined whether the cardioprotection afforded by AC261066 in I/R is accompanied by a reduction in MC degranulation. Frozen sections of WT, ApoE$^{-/-}$, and AC261066-treated ApoE$^{-/-}$ murine hearts subjected to I/R were stained with toluidine blue to identify MCs and assess their degranulation. We found that in hearts from WT mice, I/R elicited MC degranulation, which amounted to ~30% of the total MC number. In hearts from ApoE$^{-/-}$ mice, I/R-induced MC degranulation increased to ~50%, but in AC261066-treated ApoE$^{-/-}$ hearts I/R-induced MC degranulation was reduced by ~30% compared with that in untreated ApoE$^{-/-}$ hearts (Fig. 3). These data indicate that the protective effects provided by AC261066 in ApoE$^{-/-}$ hearts subjected to ex vivo

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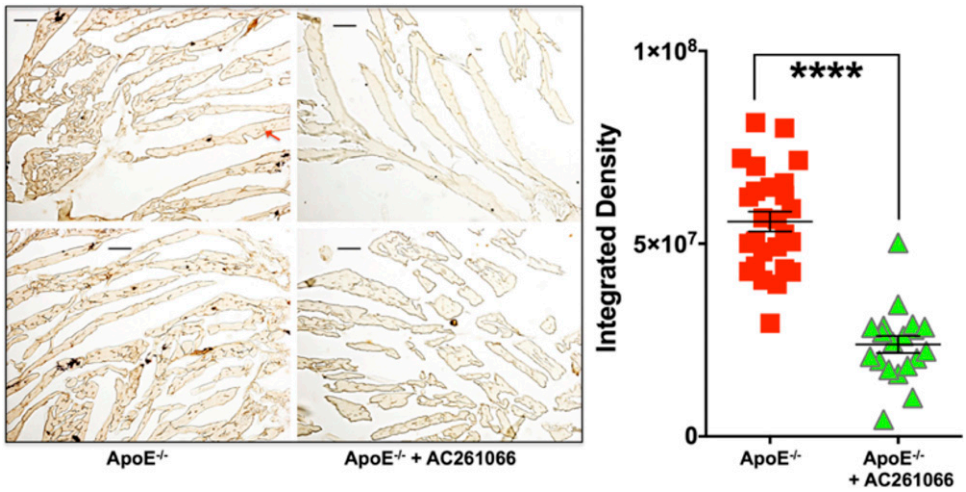

Fig. 2. MDA levels in heart sections of ApoE$^{-/-}$ mice either untreated or treated with AC261066 and subjected to ex vivo I/R. Following I/R, hearts were fixed, embedded in optimal cutting temperature compound, and sectioned. Sections were stained with a MDA antibody. (Left-hand side) Representative images of oxidative stress staining in ApoE$^{-/-}$ and ApoE$^{-/-}$ + AC261066. Anti-MDA stain in mouse heart frozen sections, the red arrow points to brown spots denoting MDA. Original magnification, 200×; Scale bar, 50 μm. Two samples per group are shown. (Right-hand side) Quantification of MDA levels in all fields. Six to eight representative areas of each heart section from three to four mice per group were photographed and analyzed. The quantification was carried out using ImageJ (National Institutes of Health). Student's t test was used for statistical analysis (****$P < 0.0001$).

I/R may result from a decrease in MC degranulation elicited by products of oxidative stress.

The RARβ_2 Agonist AC261066 Decreases NE Overflows, Reperfusion Arrhythmias, and Infarct Sizes in HFD-Fed Murine Hearts Subjected to Ex Vivo I/R. Our studies with ApoE$^{-/-}$ mice indicated that AC261066 exerts cardioprotective anti-I/R effects. Therefore, we next investigated whether cardioprotection also occurs in hearts from WT C57BL6 mice fed a HFD, which is known to enhance oxidative stress and cause cardiac dysfunction (Zeng et al., 2015). For this testing, spontaneously beating, Langendorff-perfused hearts from chow-, HFD-, and HFD + AC261066-fed mice were subjected to 40-minute global ischemia followed by 120-minute reperfusion (I/R). In hearts from chow-fed mice, NE overflows during reperfusion amounted to ~60 pmol/g, ventricular arrhythmias (VT/VF) lasted ~60 seconds, and infarct sizes comprised ~25% of left ventricles (Fig. 4). In hearts from HFD-fed mice subjected to I/R, NE overflows were as large as in hearts from chow-fed WT, and VT/VF durations and infarct sizes were ~4- and 2-fold greater than in chow-fed WT hearts, resopectively. In hearts from HFD-fed mice treated orally with AC261066 prior to ex vivo I/R, NE overflows, VT/VF durations, and infarct sizes were markedly reduced by ~45%–65% compared with those in untreated HFD hearts (Fig. 4). Notably, coronary flow in HFD hearts (3.02 ± 0.19 ml/min) did not differ from that of HFD hearts treated with AC261066 (3.015 ± 0.27 ml/min). Thus, the RARβ_2 agonist AC261066 also displays protective effects in a non-genetic, obese mouse ex vivo I/R heart model.

Oral AC261066 Treatment Does Not Reduce Total Cholesterol, Triglyceride, HDL, and LDL Blood Levels in ApoE$^{-/-}$ Mice. There were no significant differences in body weight among WT, ApoE$^{-/-}$, and ApoE$^{-/-}$ mice treated orally with AC261066 (Fig. 5A). Total cholesterol, triglycerides, HDL, and LDL blood levels were markedly increased in ApoE$^{-/-}$ mice compared with WT controls, whereas HDL levels were slightly reduced in ApoE$^{-/-}$ and AC261066-treated ApoE$^{-/-}$ mice when compared with WT. Most importantly, oral treatment of ApoE$^{-/-}$ mice with AC261066 did not modify total cholesterol, triglycerides, HDL, and LDL blood levels (Fig. 5B), indicating that the blood lipid profile does not play a major role in the cardioprotective effects of this RARβ_2-selective agonist.

Discussion

The purpose of our investigation was to characterize the effects of a RARβ_2-selective, synthetic agonist, AC261066, in an ex vivo I/R injury model in the heart. We chose to test the

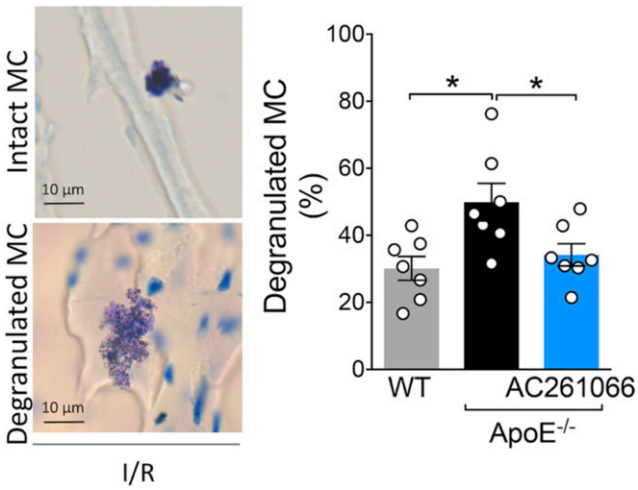

Fig. 3. The RARβ_2 agonist AC261066 reduces MC degranulation in ApoE$^{-/-}$ mouse hearts subjected to ex vivo I/R. Frozen heart sections of WT, ApoE$^{-/-}$, and ApoE$^{-/-}$ + AC2610166 mouse hearts subjected to I/R (n = 7, 7, and 7) were stained with toluidine blue. (Left-hand side) Representative images of intact and degranulated cardiac MCs in WT hearts subjected to ex vivo I/R. (Right-hand side) Quantification of MC degranulation, calculated as a percentage of degranulated MC over total MC. *$P < 0.05$ by one-way analysis of variance, followed by Tukey's post-hoc test.

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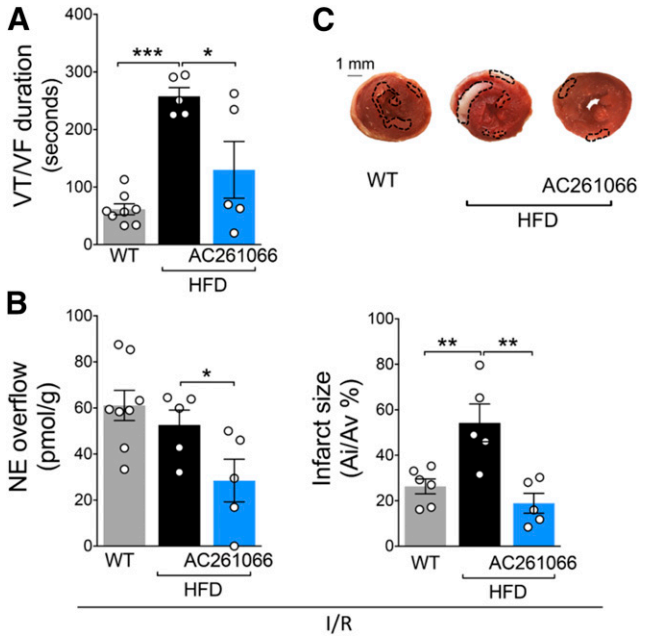

Fig. 4. The RARβ_2 agonist AC261066 reduces NE overflows, alleviates reperfusion arrhythmias, and decreases infarct sizes in hearts from HFD-fed mice subjected to ex vivo I/R. Mouse hearts were subjected to 40-minute global ischemia followed by 120-minute reperfusion (WT control, n = 8; HFD, n = 5; HFD + AC261066, n = 5). (A) Duration of reperfusion arrhythmias (VT/VF). (B) Overflows of NE collected during 6 minutes from the start of reperfusion. (C) Qualitative and quantitative representations of 2,3,5-triphenyltetrazolium chloride staining in 2-mm-thick left ventricle slices. Scale bar, 1 mm. Bars indicate mean ± S.E.M. of independent experiments. Pale areas indicate I/R-injured tissue, while healthy tissue is colored in red. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, by unpaired Student's t test.

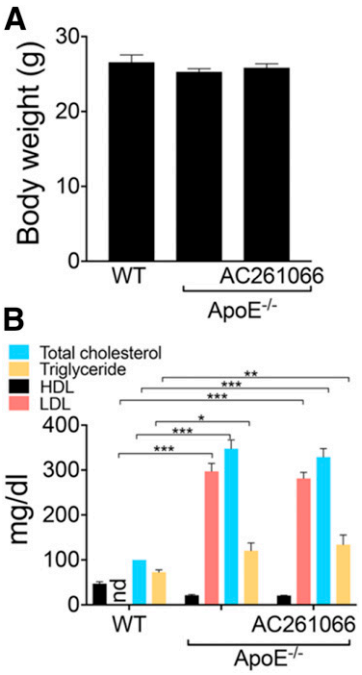

Fig. 5. Body weight and total cholesterol levels of ApoE$^{-/-}$ mice are not affected by the RARβ_2 agonist AC261066. (A) Body weight of WT, ApoE$^{-/-}$, and AC261066-treated ApoE$^{-/-}$ mice at the time of the experiment (WT, n = 7; ApoE$^{-/-}$, n = 7; ApoE$^{-/-}$ + AC261066, n = 7). (B) Blood levels of total cholesterol (light blue), triglycerides (yellow), HDL (black), and LDL (red; nd = not detectable in WT) in ApoE$^{-/-}$ and AC261066-treated ApoE$^{-/-}$ mice. Bars indicate mean ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by two-way analysis of variance followed by Tukey's post-hoc test.

effects of AC261066 in two dysmetabolic murine models because hyperlipidemic states are known to be causally associated with myocardial ischemia and oxidative stress (Stampfer et al., 1991; Yang et al., 2008), and also because we had previously discovered that AC261066 decreases oxidative stress in the liver, pancreas, and kidneys of HFD-fed mice (Trasino et al., 2016). We found that a 6-week oral treatment with AC261066 in both genetically hypercholesterolemic (ApoE$^{-/-}$) and obese (HFD-fed) mice exerts protective effects when their hearts are subsequently subjected to I/R ex vivo in the absence of added drug. Most importantly, this cardioprotection ensued without any major changes in the hyperlipidemic state, indicating that the cardioprotective effects of this RARβ_2-selective agonist do not derive from hypothetical modification of the blood lipid profile (see Fig. 5). Furthermore, although RAR and retinoid X receptor activation has been shown to relax resistance vessels via the endothelium-dependent NO-cGMP pathway (Wang et al., 2013), AC261066-induced cardioprotection was not associated with an increase in coronary flow, since treatment with AC261066 did not modify coronary flow in hearts from ApoE$^{-/-}$ and HFD-fed mice.

Langendorff-perfused mouse hearts subjected to I/R undergo a sizeable infarct of the left ventricle (Marino et al., 2017). A prominent characteristic of AC261066-afforded protection in our ex vivo heart model was the reduction in I/R-induced infarct size in the hearts of both the ApoE$^{-/-}$ and HFD-fed WT mice. In as much as the extent of myocardial injury associated with I/R results at least in part from oxidative stress and formation of toxic aldehydes (Chen et al., 2008, 2014a), we measured the MDA levels, a known marker of oxidative stress (Luo et al., 2014), in post-I/R hearts, and found them to be significantly reduced in sections from AC261066-treated ApoE$^{-/-}$ mice compared with untreated ApoE$^{-/-}$ control mice. Accordingly, this RARβ_2-selective agonist most likely reduces I/R-induced oxidative stress, and thus infarct size. Since the production of oxygen radicals and toxic aldehydes occurs primarily at the cardiomyocyte mitochondrial level (Tsutsui et al., 2011; Cadenas, 2018), we surmise that intranuclear RARβ_2 activation recruits a yet to be uncovered signaling pathway, which ultimately results in oxidative stress reduction. It is also conceivable that the AC261066-induced reduction in infarct size results from a decrease in apoptosis, favored by the reduction in oxidative stress, or involves other protective mechanisms.

Cardiac I/R is accompanied by systemic and local sympathetic neural activation, which characteristically results in abundant NE release in the heart (Koyama et al., 2013; Chen et al., 2014b; Schwartz, 2014); I/R-induced activation of a local RAS is a major contributor to this enhancement of NE release (Mackins et al., 2006). As expected, we found that NE coronary spillover was significantly increased during reperfusion in chow- and HFD-fed hearts, as well as in WT and ApoE$^{-/-}$ hearts. A novel finding was that oral treatment with AC261066 markedly curtailed I/R-induced NE overflow in the hearts from both the ApoE$^{-/-}$ and HFD-fed mice. Although it has not been previously demonstrated, it is

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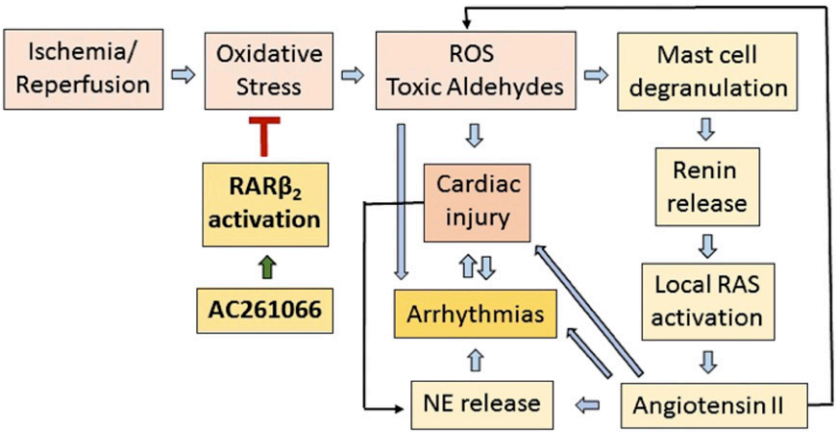

Fig. 6. Proposed mechanism for the cardioprotective effect of AC261066.

plausible that RARβ_2 activation directly interferes with NE exocytosis from sympathetic nerve endings. However, a major mechanism of this protective effect probably derives from diminished activation of local cardiac RAS, likely a result of the decreased MC degranulation also elicited by AC261066 treatment. Indeed, enzymatically active renin is present in cardiac MCs, and renin release during I/R constitutes the first step of local RAS activation that culminates in angiotensin-induced cardiac dysfunction, including excessive NE release (Mackins et al., 2006; Reid et al., 2007).

We think that the reduction in NE release that occurred in the hearts of AC261066-treated mice also contributed to the decrease in infarct size. Indeed, I/R-induced cardiac injury is known to be enhanced by the vasoconstriction and increased oxygen demand associated with hyperadrenergic states (Mann, 1998). Concomitantly, RAS activation and increased angiotensin production likely contributed to the enhancement of cardiac injury, given the recognized capacity of angiotensin to promote the formation of oxygen radicals (Wolf, 2000).

Whether AC261066 treatment decreased the I/R-induced degranulation of cardiac MCs by one or more mechanisms directly involving MC exocytotic pathways remains to be determined. Nonetheless, given the well-known capability of oxygen radicals and toxic aldehydes to degranulate MCs (Aldi et al., 2014) we propose that the AC261066-induced reduction in MC degranulation results from the attenuation of oxidative stress and toxic aldehyde formation by this selective RARβ_2 agonist.

Preeminent in the cardioprotective effects of AC261066 in our ex vivo murine I/R model was alleviation of reperfusion arrhythmias, characterized by abbreviation of VT and VF. Given the notorious arrhythmogenic effects of catecholamines (Podrid et al., 1990; Schömig et al., 1991, 1995), it is probable that the attenuation of NE release from I/R hearts of the AC261066-treated mice played a major role in the antiarrhythmic effects of AC261066. At the same time, since oxygen radicals are known to elicit cardiac arrhythmias by multiple mechanisms (Beresewicz and Horackova, 1991; Thomas et al., 1998; Song et al., 2006; Yang et al., 2010), it is likely that the AC261066-induced reduction in oxidative stress contributed to the alleviation of reperfusion arrhythmias. In addition, since angiotensin is highly arrhythmogenic, both directly and via oxygen radical production (Fleetwood et al., 1991; Curtis et al., 1993), the AC261066-induced reduction of MC degranulation, and thus of renin release and RAS activation, is likely to have contributed to its antiarrhythmic effects.

In conclusion, we found that oral treatment of both genetically hypercholesterolemic ApoE$^{-/-}$ and HFD-fed obese WT mice with the selective RARβ_2 agonist AC261066 afforded cardioprotection in their ex vivo hearts subjected to I/R. Cardioprotection consisted of attenuation of infarct size, diminution of NE spillover, and alleviation of reperfusion arrhythmias. This cardioprotection was associated with a reduction in oxidative stress and MC degranulation. We suggest that the reduction in myocardial injury and adrenergic activation, as well as the antiarrhythmic effects, result at least in part from decreased formation of oxygen radicals and toxic aldehydes known to elicit the release of MC-derived renin, promoting the activation of local RAS leading to enhanced NE release and reperfusion arrhythmias (Fig. 6). In as much as these beneficial effects of AC261066 occurred at the ex vivo level following oral drug treatment, our data suggest that AC261066 could be considered not only as a therapeutic means to reduce I/R injury of the heart, but also as a drug for patients affected by other cardiovascular ailments, such as chronic arrhythmias and cardiac failure.

Future perspectives will include studies in ex vivo hearts from conditional RAR$\beta_2^{-/-}$ mice to ascertain whether cardiac RARβ_2 is the sole or primary site of the cardioprotective actions of AC261066. To verify the cardioprotective action of AC261066 at a systemic preclinical level, additional studies will be conducted in vivo.

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Authorship Contributions

Participated in research design: Marino, Sakamoto, Tang, Gudas, Levi.

Conducted experiments: Marino, Sakamoto, Tang.

Performed data analysis: Marino, Sakamoto, Tang, Gudas, Levi.

Wrote or contributed to the writing of the manuscript: Marino, Tang, Gudas, Levi.

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Address correspondence to: Dr. Roberto Levi, Department of Pharmacology, Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065. E-mail: rlevi@med.cornell.edu

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