Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2015 ; 116-117: 76–86. doi:10.1016/j.prostaglandins.2015.01.002.

Epoxyeicosatrienoic Acid Activation of the Wnt1 Canonical Pathway Mediated by an Increase in Heme Oxygenase 1, Reduces Infarct Size and Cardiac Dysfunction

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Abstract

Myocardial infarction (MI) is complicated by ventricular fibrosis and associated diastolic and systolic failure. Emerging studies implicate Wnt1 signaling in the formation of new blood vessels. Epoxyeicosatrienoic acids (EETs)-mediated up-regulation of heme oxygenase-1 (HO-1) protects against the detrimental consequences of MI in several animal models, however, the mechanism(s) by which this occurs remains unclear. The aim of this study was to examine these mechanisms in the LAD ligation animal model of post infarcted heart failure. Specifically, we sought to clarify the mechanistic basis of the interactions of the Wnt1 canonical pathway, HO-1 and associated angiogenesis. Human microvascular endothelial cells (HMECs) were exposed to anoxia and treated with the EET agonist, NUDSA, in the presence and absence of tin mesoporphyrin (SnMP). Increased capillary density, and Wnt1 and HO-1 expression occurred in cells treated with NUDSA. Anoxic HMECs treated with NUDSA and Wnt1 siRNA, exhibited decreased in the expression of β -catenin and the Wnt1 target gene, PPAR δ ($p < 0.05$ vs. NUDSA). Furthermore, blocking the Wnt 1 antagonist, Dickkopf 1, by siRNA increased β -catenin and PPAR δ expression, and this effect was further enhanced by the concurrent administration of NUDSA. In in vivo experiments, C57B16 mice were divided into 4 groups: sham, mice with MI via LAD ligation and mice with MI treated with NUDSA, with and without SnMP. Increased fractional area change (FAC) and myocardial angiogenesis were observed in mice treated with NUDSA ($p < 0.05$ vs. MI). Increased expression of HO-1, Wnt1, β -catenin, adiponectin, and phospho-endothelial nitric oxide synthetase (p-eNOS), and a decrease in the glycosylated subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, gp91(phox) expression occurred in cardiac tissue of

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mice treated with NUDSA ($p < 0.05$ vs. MI). SnMP reversed these effects. This novel study demonstrates that increasing the canonical Wnt1 signaling cascade with the subsequent increase in HO-1, adiponectin and angiogenesis ameliorates fibrosis and cardiac dysfunction in a mouse model of MI and support the hypothesis that HO-1 is an integral component of the EETs-adiponectin axis and are central for the control of resistance to fibrosis and vascular dysfunction and in part determine how they influence the cellular/vascular homeostasis and provide insight into the mechanisms involved in vascular dysfunction as well as potential targets for the treatment of CVD.

Keywords

epoxyeicosatrienoic acid; heme oxygenase-1; myocardial infarction; angiogenesis; Wnt1

Introduction

The incidence and importance of heart failure complicating myocardial infarction (MI) is well documented [1-3]. MI is a complex and dynamic process which is initiated by coronary occlusion [4, 5]. However, cellular adaptations following MI may dramatically alter the consequences of such coronary occlusions [6]. In particular, enhancing revascularization of ischemic sites in the very acute phase of MI appears to reduce mortality and improve long term outcomes. Inflammation and oxidative stress play a prominent role in the course of MI leading to cardiac remodeling [7-9] and altered cardiac function [10, 11]. Wnts are signaling proteins that are secreted and control diverse biological processes including cell proliferation and angiogenesis [12-14]. Wnt1 is expressed in primary human endothelial cells and Wnt1/b-catenin signaling promotes angiogenesis [15, 16]. Angiogenesis is essential for the restoration of the blood supply to the infarct area as well as the revascularization of the perinecrotic area allowing for recovery of viable myocardium. We therefore speculated that the pharmacological activation of Wnt1 increases angiogenesis and improves cardiac function in post-infarcted heart failure.

Epoxyeicosatrienoic acids (EETs) are formed from arachidonic acid by members of the Cytochrome P450 (CYP) super family [17, 18]. EETs exhibit potent biological effects including vasodilation and inhibition of the inflammatory response [19-21]. Reactive oxidative species (ROS) and inflammation decrease EET synthesis [22]. Emerging studies indicate a role of EETs in improving post-ischemic cardiac function [23-25].

EETs and HO-1 are interdependent; EETs stimulate HO-1 expression, and their vasodilatory action is dependent upon HO activity [26, 27]. Further, targeting vascular endothelial-specific CYP2J2 expression and amplification of EET production led to an increase in HO-1 [28]. The heme-HO system is a key defense mechanism against oxidative stress [29, 30]. Elevated HO-1 levels are critical in the restoration of vascular function through the enhancement of angiogenesis and the decrease of oxidative stress in animal models of myocardial ischemia [31-34]. HO-1 is considered a novel target in the prevention and treatment of cardiovascular disease [35]. HO-1 derived bilirubin is a potent antioxidant [29, 36]. Oxidative stress and hypoxia down regulate the expression and activity of both CYP-

epoxygenases and HO-1 [37-40]. Furthermore, an EET agonist prevented vascular dysfunction in rats fed a high fat diet via a decrease in Bach 1- the transcriptional suppresser of HO-1 resulting in an increase in HO-1 levels, thus, highlighting the integrated role of HO-1 and EET as key to the cellular defence against oxidative stress and increased nitric oxide (NO) bioavalibilty [41]. EETs attenuate metabolic syndrome in HO-2 null mice through the activation of HO-1 dependent pathways [42]. The EET mediated increase in HO-1 provides vascular protection and regulates adiposity [43]. However, the role of EETs on cardiac HO-1 levels and ventricular remodeling in post infarcted heart failure is largely unknown. Therefore, elucidating the role of the EET-HO-1 interplay in affording cardioprotection is critical to the understanding of how cardiac remodeling progresses to oxidative stress and cardiac dysfunction and whether the EET-HO-1 interplay synergistically improves cardiac function.

Although, EETs provide cardioprotection [44], improve vascular integrity [45], have antihypertensive effects on renal function [46-49], and decrease adiposity [41, 50, 51], little is known about the direct effects of EETs on cardiac remodeling and left ventricle function in post infarcted heart failure. This study explores the mechanisms and effectiveness of NUDSA, an EET agonist, administered 5-days after the onset of cardiac remodeling, in ameliorating cardiac dysfunction. The present study demonstrates that NUDSA increases the canonical Wnt1 signaling cascade, and the resultant increase in angiogenesis ameliorates the deleterious effects of MI on cardiac remodeling, and that cross talk exists between EET and HO-1 improving cardiac function in a post MI heart failure animal model. These results offer a portal into a potential therapeutic approach to the treatment of post MI patients.

Materials and Methods

Materials

The EET agonist, 11-(nonyloxy) undec-8(*Z*)-enoic acid (NUDA), is a potent vasorelaxant in mesenteric and renal arteries [41, 52, 53]. For *in vivo* studies, NUDA was conjugated with L-aspartic acid to form (*S*)-2-(11-(nonyloxy) undec-8(*Z*)-enamido) succinic acid (NUDSA) to minimize β -oxidation and improve solubility in an aqueous milieu. NUDSA is a potent EET agonist used in the treatment of cardiovascular disease [54].

Cell culture

HMECs (human microvascular endothelial cells) were purchased from ATTC (Manassas, VA, USA). Cells were maintained in MCDB-131 medium, supplemented with 7% fetal bovine serum, 2 mM glutamic, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.2 μ g/ml hydrocortisone and 10 ng/ml EGF. The cells were fed three times weekly and subcultivated by trypsinization when nearly confluent.

Experimental design for *in vitro* experiment

Anoxia chambers comprised an airtight plastic lunch box with two holes drilled in the lid. A one-way valve was placed in each hole, one allowing gas in and one allowing gas out. Anoxic chambers were used to simulate ischemic conditions in HMECs. Cells were trypsinized and seeded into 75 cm² flasks and when they reached full confluence, they were

placed into the anoxia chambers for 24 hours. Gassing was commenced with pre-warmed, humidified 95% N₂ and 5% CO₂. NUDSA (2 μ M), SnMP (5 μ M), Wnt1 siRNA, Dkk1 siRNA or the scrambled siRNA (Santa Cruz, Dallas, TX, USA) were added to the HMECs before inducing anoxia and the respective treatments were continued for 4 days.

Angiogenesis measurement in HMECs

In vitro capillary tube-like formation was examined in HMECs grown on growth factor-reduced basement membrane Matrigel (BD Biosciences-Discovery Labware, San Jose, CA, USA), as previously described [55]. Formation of tube-like structures was examined 24 hours after addition of NUDSA (2 μ M) with and without SnMP (5 μ M). Cultures were photographed, and the length (micrometer) of the tube-like structures was quantified using Image Pro-Express Software (Cyber Media, India).

Experimental design for *in vivo* experiment

All animal studies were approved by a Chinese PLA Animal Care and Use Committee (SCXK-2007-004) in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals. Thirty two C57 male mice weighing 22-26g at 8 to 10 weeks of age were used. Mice were housed under SPF conditions in a single ventilated cage system, and were fed a diet comprising standard mouse pellets and water ad libitum. C57 mice were divided randomly into four groups: sham operated animals, MI mice and MI mice treated with NUDSA (a gift from John R. Falck, PhD, Dallas TX) and mice treated with NUDSA and the HO activity inhibitor, stannic mesoporphyrin (SnMP). All MI animals underwent left anterior descending artery (LAD) ligation through the transthoracic approach. Commencing 5 days after surgery, NUDSA was administered intraperitoneally every 5 days for four weeks at a concentration of 0.5 mg/kg body weight. SnMP was injected intraperitoneally, 3 times a week at a dose of 20mg/kg body weight, for 2 weeks. All animal groups underwent echocardiography examination 30 days after surgery and were sacrificed. Blood samples were collected in K₃EDTA tubes at sacrifice and the plasma was separated. Samples were flash frozen in liquid nitrogen and maintained at -80°C until assayed.

LAD ligation

Animals were anesthetized using ketamine and xylazine (80 mg/Kg and 10 mg/Kg, respectively), chest shaved, endotracheal intubated and ventilated with 100% oxygen and isoflurane (0.75% to 1.5%) at 180 breaths per minute using a MiniVent Mouse Ventilator (type 845, Harvard Apparatus, Holliston, Ma). A heating pad was used to maintain body temperature at 37° C. Under sterile conditions, a left thoracotomy was performed in the 3th intercostal space, the heart was exposed and the pericardium opened. An 8.0 prolene ligature (Ethicon, Blue Ash, OH, USA) was passed and tied around the proximal left coronary artery, just distal to the left atrial appendage border. Blanching of the anterolateral region of the left ventricle (LV) confirmed infarction. The chest was then closed with a single 5.0 silk suture between the 3th and 4th ribs, the muscle layers were recomposed and the skin closed with a continuous suture. After surgery, all mice were hydrated with saline and given analgesic for two days. Following surgery, mice were housed for 24 hours in an incubator at 37° C and then allowed to recover in single cages and monitored twice a day for 7 days. Sham surgery was performed following the same procedure without coronary ligation.

Echocardiography

Transthoracic echocardiography was performed using a Siemens Acuson Sequoia echo machine with a 15 MHz linear probe. Animals were anesthetized with 3% isoflurane, chests were shaved and temperature controlled anesthesia was maintained with 1.5% isoflurane. Two-dimensional cine loops and M-mode cine loops of a long-axis view and a short-axis view of the Left Ventricle (LV) were recorded. Anterior wall thickness (AWT), End Diastolic Diameter (EDD) and End Systolic Diameter (ESD) of LV, Posterior Wall Thickness (PWT), End Diastolic Area (EDA) and End Systolic Area (ESA) were measured from recorded cine-loop. The M-mode cine loop of the aortic valve was recorded and Doppler analysis of LV flow from the long axis B-mode image was performed by placing the sample volume in the LV, below the mitral annulus. All mice were imaged by a single operator. Fractional Area Change (FAC) was determined with the formula: (EDA-ESA)/EDA. Isovolumic contraction (IVCT), relaxation (IVRT) and ejection times (ET) as well as myocardial perfusion index (MPI) were calculated as described [56].

Histopathological analyses

Hearts were fixed in 4% paraformaldehyde and embedded in paraffin wax according to standard procedures and serial sections were cut using a microtome (7 μ m thickness). Embedded paraffin sections were deparaffinized, rehydrated and finally stained with hematoxylin–eosin and Masson trichrome staining. In Masson trichrome stained slides, collagen fibers stain blue and cytoplasm stains red. A minimum of 20 fields for each slide was examined and evaluated for the presence of necrosis and inflammatory infiltration by two observers blinded to the treatments.

Immunohistochemistry analyses

Embedded sections were deparaffinized, rehydrated and subjected to antigen retrieval in 0.05 M sodium citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide in methanol for 30 minutes. The sections were then incubated (Vector Labs, Burlingame, CA, USA) with rat monoclonal antibody against CD31 (diluted 1:150; BD Pharmingen, USA) for 1 h at room temperature and overnight at 4 °C. After incubation in primary antibody, the sections were sequentially incubated in appropriate biotinylated immunoglobulins, avidin–biotin peroxidase complex (Vector Labs, Burlingame, CA, USA) and in a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich, St. Louis, MO, USA) and 0.33% hydrogen peroxide. All sections were finally counterstained with hematoxylin, dehydrated and mounted. Control reactions were performed by omitting the primary antibody and with isotype-matched irrelevant IgGs as the negative control.

Quantitative analyses—During quantitative analyses, two observers blinded to the treatments computed blue staining for the Masson trichrome staining and immunopositivity for the immunohistochemistry. Digital images of the slices ($\times$ 400 magnifications) were analyzed using an image analyzer (Image Pro Plus, Milan, Italy). The blue staining and the immunopositivity were calculated for arbitrary areas, measuring 20 random fields with the

same area for each sample. The data were pooled to represent a mean value and a statistical analysis was applied to compare the results obtained from the different experimental groups.

RNA extraction and real-time PCR

Total RNA was extracted from mice heart using RNeasy Protect Mini kit (QIAGEN, Maryland, USA) according to manufacturer's instructions. Total RNA (1µg) was transcribed into cDNA using GeneAmp kit (Applied Biosystems, Branchburg, NJ, USA) reverse transcription reagents. Total RNA was analyzed by a quantitative real time polymerase chain reaction (qRT-PCR). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on a 7500 HT Fast Real-Time PCR System (Applied Biosystems). Specific primers for mouse Bach1 were used. For our *in vitro* study, total RNA was also extracted from Human Micro vascular Endothelial Cells (HMECs) to use for qRT-PCR. Specific primers used were human HO-1, Wnt1, B-Catenin, VEGF, PPAR-delta and actin. Each reaction was performed in triplicate. The comparative threshold cycle (Ct) method was used to calculate the fold amplification as specified by the manufacturer. Appropriate positive and negative controls for siRNA were used for the experiments. All experimental samples were normalized using actin as an internal control and normalization was performed in separate reactions.

PRIMER	FORWARD	REVERSE
Mouse Actin	5' TGGCGCTTTTGACTCAGGAT-3'	5' GGGATGTTTGCTCCAACCAA 3'
Mouse Bach1	5'-GGACACTCCTTGCCAAATGCAG-3'	5'-TGACCTGGTCTGGGCTCTCAC-3'
Human HO-1	5'-CGGGCCAGCAACAAAGTG-3'	5'-CGGGCCAGCAACAAAGTG-3'
Human Wnt1	5'-TGTTTGCAAAGACCACCTCCA-3'	5'-TGATTCCAGGAGGCAAACGCAT-3'
β-Catenin	5'-CAAGTGGGTGGTATAGAGG-3'	5'-CAATGGGAGAATAAAGCAG-3'
Human VEGF	5'-CCTGGTGGACATCTTCCAGGAGTACC-3'	5'-GAAGCTCATCTCTCTATGTGCTGGC-3'
Human PPAR γ	5'-GCAGGCTCTAGAATTCCATC-3'	5'-GTGCAGCCTTAGTACATGTC-3'
Human Actin	5'-ACCTGAGAACTCCACTACCCT-3'	5'-GGTCCCACCCATGTTCCAG-3'

Western blot analysis

Frozen heart tissue was pulverized under liquid nitrogen and placed in a homogenization buffer comprising (mmol/l): 10 phosphate buffer, 250 sucrose, 1 EDTA, 0.1 PMSF and 0.1% v/v tertitol, pH 7.5. Homogenates were centrifuged at 27,000×g for 10 minutes at 4°C. The supernatant was isolated and protein levels were assayed (Bradford Method) and immunoblotting was performed in heart tissue as previously described [39, 42]. The supernatant was used for the determination of HO-1, Bach1, Wnt1, β cateninS37A, p-eNOS, adiponectin, gp phox91, p-eNOS, PPARδ and ETS-1. β Actin was used to ensure adequate sample loading for all Western blots.

Statistical Analysis

Data are expressed as means $\pm$ S.E. Significance of difference in mean values was determined using one-way analysis of variance followed by the Newman-Keul's post hoc test. $P < 0.05$ was considered to be significant.

Results

NUDSA increases capillary density and HO-1 and Wnt1 expression in HMECs exposed to anoxia

Our results show that NUDSA is a potent angiogenic stimulant that stimulates capillary-like tube formation in endothelial cells grown on Matrigel compared with cells exposed to anoxia (Figure 1A; $p < 0.05$). SnMP reversed the beneficial effects of NUDSA treatment in endothelial cells (Figure 1A; $p < 0.05$).

Further, we demonstrate that HO-1 expression was increased in HMECs treated with NUDSA following anoxia as compared to HMECs exposed to anoxia alone (Figure 1B; $p < 0.05$). SnMP had no effect on increased levels of HO-1 confirming previous results [57]. In addition, Wnt1 levels decreased in endothelial cells exposed to anoxia and Wnt1 expression increased post NUDSA treatment (Figure 1C; $p < 0.05$). SnMP attenuated the effect of NUDSA on Wnt1 expression.

NUDSA increases β -Catenin and PPAR δ expression in HMECs exposed to anoxia

To elucidate the role of the Wnt1 canonical pathway in the regulation of angiogenesis in HMECs exposed to anoxia, we measured the effect of suppression of Wnt1 using siRNA on β -Catenin and PPAR δ levels. Cells exposed to anoxia had a decreased expression of β -Catenin and PPAR δ as compared to control cells (Figure 2A and 2B, respectively; $p < 0.05$); however anoxic cells treated with NUDSA displayed an increased expression of β -Catenin and PPAR δ compared to cells exposed to anoxia alone (Figure 2A and 2B, respectively; $p < 0.05$). Concurrent treatment of anoxic cells with NUDSA and siRNA Wnt1 decreased the expression of β -Catenin (Figure 2A) and PPAR δ (Figure 2B) as compared to NUDSA alone ($p < 0.05$). To further confirm that Wnt1 is downstream of the EET-HO-1 axis, we treated HMECs exposed to anoxia with Dickkopf 1 (Dkk1) siRNA. NUDSA and siRNA Dkk1 treatment increased β -Catenin and PPAR δ expression as compared to anoxia alone (Figure 2A and 2B, respectively; $p < 0.05$) indicating a mechanistic relationship between the EET-HO-1 axis and the Wnt1 canonical cascade.

NUDSA improves cardiac function in mice 30 days after MI

Groups of animals were comparable with regard to age, body weight and heart rate. No significant differences were found amongst the groups in left ventricle morphology and function. In the MI group 30 days after LAD ligation, the End Diastolic Area (EDA) was increased (Figure 3A and B; $p < 0.05$) and Fractional Area Change (FAC) was reduced when compared to sham operated animals (Figure 3A and C; $p < 0.02$). NUDSA treatment returned both EDA and FAC to normal levels when compared to the MI group ($p < 0.01$). SnMP reversed the beneficial effect of NUDSA on both EDA and FAC in MI mice (Figure 3; A, B

and C). Posterior wall thickness (PWT) was increased ($p<0.05$) in MI mice when compared to sham operated animals. NUDSA reduced PWT to sham operated levels. This was reversed by SnMP. Moreover in the MI group the MPI, a measurement of diastolic function, was increased (Table 1; $p<0.05$) when compared to sham operated animals. NUDSA mitigated this increase. SnMP reversed the beneficial effects of NUDSA on MPI (Table 1).

NUDSA decreases Cardiac fibrosis in mice 30 days after MI

Histopathological examination of cardiac tissue revealed that collagen deposition was higher in hearts of MI mice as compared to the sham operated animals (Figure 4; $p<0.01$). NUDSA decreased fibrosis as compared with MI animals ($p<0.01$). SnMP not only reversed EET mediated attenuation of fibrosis, but increased collagen deposition (Figure 4; $p<0.05$). Additionally, HO activity was measured and the results were in concordance with the HO-1 gene expression in Figure 1 and support the hypothesis that HO-1 is integral components of the EETs-adiponectin circuits and are central for the control of vascular dysfunction and a deficiency in any of these circuits leads to vascular dysfunction in MI.

NUDSA increases Capillary density and Angiogenic markers in mice 30 days after MI

CD31 immunohistochemistry of cardiac tissue revealed a reduction in capillary density in the peri-infarct area of the hearts of MI mice as compared to the sham operated animals (Figure 5A; $p<0.05$). NUDSA increased the capillary density in MI mice as compared to untreated MI mice ($p<0.05$). The increase in capillary density resulted in levels similar to those found in the sham-operated mice. SnMP reversed the beneficial effects of NUDSA treatment in MI mice. Cardiac VEGF and ETS-1 levels were decreased in the MI group when compared to the sham-operated group (Figure 5B and C, respectively; $p<0.02$). NUDSA increased the expression of both VEGF and ETS-1 in MI mice ($p<0.05$) and this effect was reversed by the concurrent administration of SnMP (Figure 5B and C, respectively; $p<0.05$).

Effect of NUDSA on HO-1, Bach1 and the canonical Wnt signaling cascade in mice 30 days after MI

Western blot analysis of cardiac tissue revealed decreased HO-1 expression in the MI group as compared to the sham-operated group ($p<0.05$). NUDSA increased HO-1 expression in MI mice to the levels of the sham-operated group (Figure 6A; $p<0.05$). NUDSA also reduced nuclear levels of bach1 (Figure 6B; $p<0.05$) which was elevated in MI animals compared to the sham operated. Concurrent administration of NUDSA and SnMP restored bach1 levels to those of the MI animals. Further, our results show a decrease in Wnt 1 and β catenin levels in the MI group as compared to the sham-operated group (Figure 6C and D respectively; $p<0.05$). NUDSA increased ($p<0.05$) the expression of both Wnt 1 and β cateninS37A and this effect was reversed by the concurrent administration of SnMP.

Effect of NUDSA on PPAR δ , gp91(phox), adiponectin and p-eNOS expression in mice 30 days after MI

Cardiac PPAR δ expression was decreased in the MI group when compared to the sham-operated group (Figure 7A; $p<0.05$). NUDSA increased the expression of PPAR δ in MI

mice to levels of the sham operated animals and this effect was reversed by the concurrent administration of SnMP (Figure 7A; $p<0.05$). The expression of gp91(phox) was increased in the MI group when compared to the sham-operated group ($p<0.05$). NUDSA reduced cardiac expression of gp91(phox) when compared to the MI group (Figure 7B; $p<0.05$). SnMP increased the expression of gp91(phox) to the levels of the MI group ($p<0.05$). Adiponectin levels were decreased in the MI group when compared to the sham-operated group (Figure 7C; $p<0.01$). NUDSA increased adiponectin levels in mice with MI while SnMP reversed these beneficial effects. The cardiac expression of p-eNOS was reduced in the MI group when compared to sham operated animals ($p<0.05$). NUDSA increased the cardiac expression of p-eNOS (Figure 7D; $p<0.05$); the concurrent administration of SnMP with NUDSA decreased the expression of p-eNOS ($p<0.05$).

Discussion

This is the first study to demonstrate that increased expression of the Wnt1 canonical signaling cascade attenuates cardiac dysfunction and improves angiogenesis via the administration of NUDSA, an EET agonist. We demonstrate, in a mouse model of post-infarcted heart failure, the complete reversal of LV systolic dysfunction 5 days after the onset of cardiac remodeling. The improvements in cardiac function were associated with increased neovascularization and increased adiponectin and p-eNOS levels with a concomitant decrease in Bach1 and oxidative stress. In addition, the reversal of these beneficial effects of NUDSA by SnMP demonstrates the existence of a synergistic relationship between the EET and HO systems. Thus, the EET-HO-1 axis may be considered a target for pharmacological intervention via an increase in the Wnt1 canonical cascade in the treatment of patients with post infarcted cardiac failure.

Angiogenesis is a key process in ischemic heart disease and in the restoration of blood supply to the infarct area after a MI. Our *in vitro* results show that NUDSA induces capillary proliferation in HMECs exposed to anoxia (Figure 1). HMECs exposed to anoxia and treated with NUDSA increased the expression of HO-1, thereby, demonstrating a role of the EET-HO-1 axis in neoangiogenesis. *In vitro* studies have implicated the Wnt1/ β -catenin pathway as a promoter of endothelial cell growth and proliferation [12-14]; however there is a dearth of information regarding the beneficial role of this pathway in improving cardiac function 5 days after the onset of cardiac remodeling due to MI. The canonical Wnt/ β -catenin signaling pathway utilizes β -catenin to transmit a receptor-mediated signal from the cytosol to the nucleus. Expression of Wnt-1 or a stabilized and active form of β -catenin S37A; promotes endothelial cell proliferation [15, 16].

Our results establish a seminal link between the angiogenic effects of the EET-HO-1 axis and stimulation of the Wnt1 canonical pathway. Two lines of evidence characterize this relationship: Firstly, our results demonstrate an increase in the Wnt1/ β -catenin canonical signaling cascade in HMECs exposed to anoxia and treated concurrently with NUDSA. The resultant improvement in capillary proliferation establishes a link between an increase in the levels of EET and Wnt1 signaling. Secondly, siRNA mediated down regulation/ inhibition of Wnt1 prevented the EET-HO-1 axis from increasing the β -catenin and Wnt1 responsive genes; as manifested by PPAR δ expression in HMECs. Further our results show that

inhibition of Dkk1 by siRNA increased β -catenin and PPAR δ expression which was further enhanced by the concurrent administration of NUDSA. These observations indicate that activation of the Wnt1 canonical pathway is beneficial in the induction of angiogenesis in HMECs undergoing EET induction. Thus, these results substantiate our hypothesis that Wnt1 is an EET gene target that, when increased, ultimately results in an increase in angiogenesis and improved cardiac function.

Thereafter, our *in vitro* findings were extended to an *in vivo* study designed to examine the beneficial role of EET-HO-1 axis in attenuating cardiac remodeling via an increase in the Wnt1 canonical cascade. NUDSA treated mice with MI showed increased cardiac HO-1 expression. Consistent with our previous reports, one potential explanation is that epoxides increase HO-1 expression due to suppression of Bach1, a transcriptional regulator of the HO system. Bach1 suppresses HO-1 transcription and inhibition of Bach1 increases HO-1 expression. We demonstrate that NUDSA attenuated Bach1 resulting in increased HO-1 expression in cardiac tissues. Angiogenesis is crucial to the revascularization of the perinecrotic area of the heart leading to improved cardiac function [58, 59]. Our *in vivo* data confirm the neoangiogenic effect of NUDSA in ischemic heart on post-infarcted myocardium via increased levels HO-1. In the present study, pericardial neovascularization was decreased in the MI group when compared to the sham-operated group. NUDSA increased angiogenesis as shown by increased CD31 staining (Figure 5) indicating a substantial role for the EET-HO1 axis in mitigating the clinical deterioration that occurs after a MI. Early neoangiogenesis is responsible for LV functional recovery: the perinecrotic area is regarded as hibernating myocardium and can recover functionally by early reperfusion [60]. HO-1 induction increased cellular metabolic resistance while stimulating angiogenesis and early reperfusion of damaged areas [31, 33, 61]. The present study demonstrates that the reduction in oxidative stress seen in NUDSA treated mice with MI is associated with an increased capillary density manifested by increased CD31 staining and the increased expression of ETS-1 and VEGF which are indicative of increased local neoangiogenesis and endothelium repair. Consistent with these findings, the echocardiographic results demonstrate an improvement in contractile function in MI mice treated with NUDSA as compared to MI mice (Table 1). SnMP reversed the beneficial effects of NUDSA clearly implicating the synergistic relationship of the EET-HO-1-axis and increased HO activity in cardiac protection post MI.

In agreement with our *in vitro* study, our *in vivo* results indicate that the Wnt1/ β -catenin canonical signaling cascade is upregulated in NUDSA treated mice with MI as compared to MI mice. We observed increased densities of capillaries and arterioles in the peri-infarct areas in NUDSA treated mice with MI, suggesting that overexpression of Wnt1 promotes myocardial angiogenesis after MI. Increased Wnt-1 expression promotes capillary proliferation and endothelial cell migration; both processes are critical for angiogenesis and the attenuation of cardiac remodeling, via the transcriptional activation of VEGF [15, 62-64, 64, 65]. VEGF has been linked to increased levels of CYP, and the overexpression of a constitutively active AMPK mutant which increases CYP2C expression. HO-1 acts upstream of the Wnt canonical cascade [16]. EET stimulates canonical Wnt signaling to regulate hematopoietic progenitor cell mobilization and vascular repair [66]. In agreement with these findings, we show that NUDSA treated MI mice exhibit an increased expression of Wnt-1

and β -catenin, and that the concurrent administration of SnMP reversed the beneficial effects of NUDSA. Further, a role for Wnt1/ β -catenin signaling in cardiac remodeling is further supported by the increased levels of Wnt1 target genes, PPAR δ [67] and VEGF in NUDSA treated MI mice. This clearly demonstrates a novel role of EET in attenuating cardiac remodeling via an increase in HO-1 expression and Wnt1/ β -catenin signaling in a mouse model of post-infarcted heart failure.

Oxidative stress plays an integral role in the progression of left ventricular (LV) remodeling and failure that occur after MI [8, 9]. In the present study, the beneficial effects of NUDSA on myocardial function post MI included a reduction in the levels of the oxidative stress marker, gp91(phox) in cardiac tissue. These effects of NUDSA were reversed by SnMP, suggesting that the levels of bilirubin, produced from degradation of heme by HO-1, impact redox balance [33, 61, 68]. Cardiac fibrosis is the consequence of myocardial pathological remodeling, i.e., necrotic and apoptotic myocytes are replaced by collagen fibers. In the present study, cardiac fibrosis was measured in the MI group and found to be increased as compared to the sham operated group. NUDSA treated mice not only had reduced fibrosis, but also increased angiogenesis as shown by CD31 staining. Both EET and HO-1 exhibit anti-ischemic properties [20, 29, 69]. A positive correlation exist between the EET-HO-1 axis and Adiponectin [41, 42]. Adiponectin is critical for endothelial cell survival and increased adiponectin levels improve cardiac function, regulate pAMPK levels, p-eNOS via phosphorylation of LKB1, decrease blood pressure, and attenuate oxidative stress-mediated vascular dysfunction [70-72]. Increased levels of pAKT by EET and HO-1 adiponectin are associated with an increase pAMPK and in p-eNOS levels, and NO bioavailability [70, 73, 74]. In agreement with the above, we demonstrate that the EET-HO-1 axis regulate pAKT and adiponectin regulate pAMKT with a resultant an increase of p-eNOS and improvement in microvascular tone and the relief of paradoxical vasoconstriction. This results in an increase in perfusion in the infarcted area with a resultant decrease in cardiac remodeling.

In conclusion, this study highlights the role of the EET-HO-1 axis in ameliorating cardiac remodeling and improving angiogenesis and cardiac function through an increase in the Wnt1 canonical cascade in an animal model of post infarcted myocardium. This is associated with an increase in the levels of adiponectin, p-eNOS, and VEGF and a decrease in Bach1 levels and oxidative stress summarized in (Figure 8). Additional studies are needed to fully identify the precise mechanism(s) by which Wnt1 improves cardiac function. Much remains to be clarified about the exact role of EET agonist-mediated Wnt1 signaling in MI and heart failure, however, the results described above offer a portal into the crucial role of EET mediated angiogenesis, a result of an increase in HO-1 levels, in cardiac protection and how it might warrant consideration as a target for clinical intervention in patients with MI. The current report support the hypothesis that HO-1 is integral components of the EETs-adiponectin axis and are central for the control of vascular dysfunction and that deficiency in any of these circuits leads to vascular dysfunction CVD.

Acknowledgments

We thank Drs. Paola Pesce, David Sacerdoti and Stephen Peterson for their helpful comments and technical advice. All authors have read and agree with the manuscript as written. This work was supported by National Institutes of Health grants (HL55601, HL34300 to NGA, gifts from the Renfield Foundation to The Rockefeller University

(AK), NIH DK38226 and the Robert A. Welch Foundation (I-0011) to JRF and by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences, Z01 ES025034 to DZ.

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Abbreviations

β-catenin	Beta cadherin associated protein
CYP	Cytochrome P450
Dkk-1	Dickkopf 1
eNOS	Endothelial nitric oxide synthetase
EETs	Epoxyeicosatrienoic acids
ET	Ejection time
FAC	Fractional area change
gp91 (phox)	glycosylated subunit of nicotinamide adenine dinucleotide phosphate(NADPH) oxidase b558
HMECs	Human microvascular endothelial cells
HO	Heme oxygenase
IVCT	Isovolumic contraction time
IVRT	Isovolumic relaxation time
LAD	Left anterior descending coronary artery ligation
MI	Myocardial infarction
MPI	Myocardial Performance Index
p-eNOS	phospho—endothelial nitric oxide synthetase
NUDSA	EET-agonist
PPARδ	Peroxisome proliferator activated receptor delta
ROS	Reactive Oxygen Species
SnMP	Stannic mesoporphyrin
Wnt1b	Wingless-type MMTV integration site family, member 1b

Highlights

- First study to demonstrate that increase in the canonical Wnt1 signaling cascade improves cardiac function in a model of post infarcted heart failure.
- This novel study demonstrates the existence of an epoxide-HO axis, which ameliorates cardiac dysfunction post myocardial infarction.
- Complete reversal of LV systolic dysfunction by the administration of NUSDA five days after the onset of cardiac remodeling.

Figure 1.

(A) Capillary tube-like formation in microvessel endothelial cells. Representative photographs depicting the angiogenic response of NUDSA and Quantitative analysis of capillary length (μm); mean ± SE, n=4. * $p < 0.05$ vs. anoxia; # $p < 0.05$ vs. anoxia + NUDSA, + $p < 0.05$ vs. anoxia + NUDSA + SnMP. RT-PCR analysis of (B) Wnt 1 (C) HO-1 levels in HMECS exposed to anoxia. Results are means ± SE, n=6/group. * $p < 0.05$ vs. anoxia; # $p < 0.05$ vs. anoxia + NUDSA, + $p < 0.05$ vs. anoxia + NUDSA + SnMP.

Figure 2. Effect of NUDSA, siRNA Wnt1 and siRNA Dkk1 on RT-PCR analysis of (A) β cateninS37A (B) PPAR δ expression in HMECS exposed to anoxia. Results are means $\pm$ SE, n=6/group. * $p < 0.05$ vs. anoxia; # $p < 0.05$ vs. anoxia + NUDSA, + $p < 0.05$ vs. anoxia + siRNA Wnt1.

Figure 3.

Effect of NUDSA on fractional area shortening (FAC) and end diastolic area (EDA) measured by echocardiography in C57 mice 30 days post MI; Results are means $\pm$ SE, n=6/group. (A) EDA (Cm²), *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI + NUDSA (B) FAC (%), *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI + NUDSA.

Figure 4.

Effect of NUDSA on cardiac fibrosis in mice 30 days post MI. Results are means $\pm$ SE, $n=6$ /group. (a, b, c, d) Masson trichrome staining for myocardial fibrosis; * $p<0.05$ vs. Sham, # $p<0.05$ vs. MI, + $p<0.05$ vs. MI+NUDSA.

Figure 5.

(A) Immunohistochemistry images of CD31 expression of heart in C57 mice. The graph shows the histomorphometrical analyses of CD31 immunopositivity in heart samples. * $p < 0.05$ vs. sham; # $p < 0.05$ vs. MI, + $p < 0.05$ vs. MI + NUDSA. Effect of NUDSA on Western blot and densitometry analysis of (B) VEGF (C) ET-1 expression in heart tissue in mice 30 days post MI. Data are shown as mean band density normalized to β -actin. Results are means $\pm$ SE, $n = 6$ /group. * $p < 0.05$ vs. Sham, # $p < 0.05$ vs. MI, + $p < 0.05$ vs. MI + NUDSA

Figure 6.

Effect of NUDSA on Western blot and densitometry analysis of (A) HO-1 expression in heart tissue in mice 30 days post MI. Data are shown as mean band density normalized to β -actin. Results are means $\pm$ SE, $n=6$ /group. * $p<0.05$ vs. Sham, # $p<0.05$ vs. MI, + $p<0.05$ vs. MI+NUDSA. (B) RT PCR of Bach1 in heart tissue in mice 30 days post MI. Results are means $\pm$ SE, $n=6$ /group. * $p<0.05$ vs. Sham, # $p<0.05$ vs. MI, + $p<0.05$ vs. MI + NUDSA. Effect of NUDSA on Western blot and densitometry analysis of (C) Wnt 1 (D) β cateninS37A expression in heart tissue in mice 30 days post MI. Data are shown as mean band density normalized to β -actin. Results are means $\pm$ SE, $n=6$ /group. * $p<0.05$ vs. Sham, # $p<0.05$ vs. MI, + $p<0.05$ vs. MI+NUDSA

Figure 7. Effect of NUDSA on Western blot and densitometry analysis of (A) PPARδ (B) gp phox91 (C) adiponectin (D) p-eNOS expression in heart tissue in mice 30 days post MI. Data are shown as mean band density normalized to β-actin. Results are means ± SE, n=6/group. *p<0.05 vs. Sham, #p<0.05 vs. MI, +p<0.05 vs. MI + NUDSA.

Figure 8.

Proposed scheme demonstrate crosstalk between EETs and HO-1 to attenuate post infarcted heart failure. This study highlights the role of the EET-HO-1 axis in ameliorating cardiac remodeling and improving angiogenesis and cardiac energy homeostasis via activation of the Wnt1 canonical cascade in a model of post infarcted myocardium. This is associated with an increase in the levels of adiponectin, p-eNOS along with decreased Bach1 expression and oxidative stress.

Table 1
In-vivo heart measurements in mice 30 days after LAD

	SHAM	MI	MI+NUDSA	MI+NUDSA+ SnMP
ESA (cm)	0.11±0.02	0.15±0.01 ^{**}	0.15±0.02 ^{&&}	0.12±0.02 [#]
EDD (cm)	0.038±0.04	0.37±0.04 ^{**}	0.35±0.02 ^{&&}	0.39±0.04 [#]
ESD (cm)	0.24±0.03	0.31 ±0.03 ^{**}	0.25±0.01 ^{&&}	0.29±0.05
PWT (cm)	0.072±0.009	0.08±0.02 [*]	0.069±0.01 ^{&}	0.08±0.02 ^{*#}
AWT (cm)	0.069±0.009	0.054±0.013 ^{**}	0.046±0.005 ^{**}	0.054±0.005 [*]
EF (%)	0.55±0.05	0.45±0.1 ^{**}	0.56±0.07 ^{&}	0.58±0.03 [*]
ET (msec)	59.4±6.6	59.8±6.4	55±7	66.9±4.6 ^{*&##}
IVCT+IVRT (msec)	17.6±4.2	27.7±12.7 [*]	19.8±4.8	26.6±2.3 ^{*##}
MPI	0.28±0.07	0.46±0.1 [*]	0.36±0.08	0.41 ±0.06 [*]

Values are expressed as mean ± SD; n= number of animals tested;

^{*} P<0.05 and

^{**} P<0.01 vs sham;

[#] P<0.05 and

^{##} P< 0.01 vs. NUDSA-treated group;

[§] P<0.05 and

^{§§} P<0.01 vs. NUDSA + SnMP group;