Alternative Therapeutic Modalities in Treatment of Ischemic Heart Disease

Experimental studies on retinoic acid signaling in myocardial remodelling and remote gene therapy using heme oxygenase-1

by

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ABBREVIATIONS

ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2
AOSMC	Human aortic smooth muscle cells
AtRA	All-trans retinoic acid
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CF	Cardiofibroblasts
CM	Cardiomyocytes
CMP	Non-ischemic dilated cardiomyopathy
CO	Carbon monoxide
CRABP 2	Cellular retinoic acid binding protein 2
Fe	Free iron
HIF-1á	Hypoxia-inducible factor 1 alpha
HMOX-1	Heme oxygenase-1
HUVEC	Human umbilical vein endothelial
NO	Nitric oxide
NYHA	New York Heart Association Classification of the stages of heart failure
PcDNA	Empty vector
PCI	Percutaneous coronary intervention
PTA	Percutaneous transluminal angioplasty
RA	Retinoic acid
RARE	Retinoic acid response elements
RARE-Luc	Retinoic acid response element luciferase reporter
RARs	Retinoic acid receptors

RBP1	Retinol binding protein 1
RBP4	Retinol binding protein 4
STRA6	Stimulated by retinoic acid gene
SMC	Smooth muscle cells

LIST OF PAPERS

The thesis is based on the following papers, which will be referred in the text by their Roman numerals:

Paper I **Bilbija Dusan**, Haugen Fred, Sagave Julia, Dahl Christen Peder, Gullestad Lars, Elmabsout A. Ali, Sirsjö Allan, Valen Guro
Retinoic acid target gene expression in ischemic heart disease

Submitted

Paper II **Bilbija Dusan**, Haugen Fred, Baysa Anton, Sagave Julia, Bastani Nasser, Levy F. Olav, Sirsjö Allan, Blomhoff Rune, Valen Guro

Retinoic Acid signalling is activated in the postischemic heart and may influence remodelling

PLoS One; 2012 Sep 28;

Paper III **Bilbija Dusan**, Gravning Jørgen, Attramadal Håvard, Valen Guro

Protecting the heart through delivering DNA encoding for heme oxygenase-1 into skeletal muscle

Life Sci; 2012 Aug 17;

INTRODUCTION

Ischemic heart disease

Ischemic heart disease is the leading cause of death worldwide [1]. The hallmark of disease is formation of atherosclerotic plaques in coronary arteries. The process is initiated by endothelial injury and further promoted by inflammation, lipid accumulation, proliferation of smooth muscle cells (SMC) and matrix deposition. It starts at an early age, where fatty streaks are seen in autopsies of children as young as five years of age [2-4]. Plaques usually consist of a gel-like, lipid-rich core covered with a fibrous cap consisting of a layer of sclerotic vessel tissue. It is the lipid core which is the more dangerous component because it can increase in size and destabilize the plaque [5]. Plaques with increased lipid content are more prone to rupture, leading to the intraluminal thrombosis of coronary arteries and consequently acute myocardial infarction or sudden death [6, 7]. Approximately 75% of acute coronary syndromes is caused by underlying plaque rupture [5]. Well-known risk factors such as high blood pressure, high cholesterol and glucose levels may contribute to the process. It is thought infection or inflammation may enhance plaque rupture [5, 6]. Currently composition and vulnerability of plaques cannot be revealed by any available diagnostic method. However not all plaques are prone to rupture and transformation from an asymptomatic fibroatheromatous plaque to a lesion at high risk for rupture, are not fully understood and it is a therapeutic window. Patients suffering from coronary artery atherosclerosis can get symptoms such as effort angina which is stable, unstable coronary syndrome, which is transitory ischemia and at risk for developing infarction, and finally, myocardial infarction with thrombus formation [1-7].

Myocardial remodelling

Initaly, remodelling begins as adaptive response to myocardial injury, which lead to alterations in myocardial cell phenotype, myocardial morphology and progressive functional deterioration [8]. It is caused by conditions such as myocardial infarction [9], hypertension, aortic stenosis (pressure overload) [10], mitral insufficiency (volume overload) [11], myocarditis (inflammatory cardiac muscle disease) [12], idiopathic [13] and others. In this overview the focus is on remodelling after myocardial infarction since this was the subject of our investigation. Occlusion of a coronary artery, if not timely revascularized by percutaneous coronary intervention (PCI), thrombolysis or coronary artery bypass grafting**,** results in regional ischemia and ultimately death of ischemic cardiomyocytes [14, 15]. An increased hemodynamic load challenges the remaining cardiomyocytes, which first lengthen and then adapt by increasing cell size and number of contractile units, with lack of a concomitant effect on muscle cell proliferation [16-21]. Reactivation of embryonic gene programs may be important for this process. Increased cell size comes with the high price of greater oxygen demand in the already ischemic myocardium, thus further promoting loss of remaining cardiomyocytes [16, 22-24]. Remodelling may be further enhanced by inappropriate responses of damaged myocardium to neurohormonal stimulation [25, 26]. The left ventricular wall thins and the chamber dilates resulting in gradually increasing residual volume and decline of ejection fraction [15, 23, 24]. Myocardial integrity is maintained by cardiofibroblasts, which proliferate excessively, first in the infarct zone and then beyond, leading to with accumulation of collagen and cardiac fibrosis [27-29]. Myocardial infarction induces a sterile inflammation and reabsorption of necrotic tissue, also contribute to remodelling [30, 31]. For patients that survive the acute phase of myocardial infarction, the rate of remodelling is directly proportional to the infarct size [32]. The greater the infarct the more rapid is postinfarction left ventricular dilatation and reduction of ejection fraction [33]. An increasing amount of patients suffer from heart failure, which is probably due to improved treatment of acute infarction combined with an aging population. However, as mortality and morbidity of the heart failure remains unacceptably high, it argues for inadequacy of our current therapy.

Paradigm shift in treatment of heart failure

Some 30 years ago, the cardiorenal pathophysiological theory prevailed, and heart failure patients were treated with diuretics, vasodilatory and inotropic drugs. In the 1980 the neurohumoral understanding took over, leading to use of angiotensin-converting-enzyme inhibitors, beta-adrenergic antagonists and aldosterone antagonists. Obviously these treatments are inadequate. Recent studies are revealing an inflammatory component in progression of heart failure, launching the cardioinflammatory model of heart failure [34, 35]. Patients with heart failure have progressively elevated levels of inflammatory cytokines, both in the circulation and in the failing heart, suggesting activation of the innate immune system secondary to cardiac stress [36-39]. Initially it was observed that in contrast to the healthy heart that does not express tumor necrosis factor-alpha (TNFα), failing heart generates large quantities of TNFα. The level of TNFα expression is directly proportional to the severity of the disease [40, 41]. Experimental studies with transgenic mice show that cardiac-specific overexpression of TNFα causes gradual left ventricular dilatation and decline in myocardial function [42-44]. Proinflammatory hypothesis of heart failure was supported with experimental studies, which show that inhibition of TNFα prevents myocardial remodeling [45-47]. However, the recent clinical trials designed to suppress TNFα resulted in worsening of heart failure [48-50] and the inflammatory theory of myocardial remodelling did not result in any novel treatments

Current treatments of ischemic heart disease

The objective in current treatment of ischemic heart disease is to slow down the progression of the disease, relieve symptoms, improve quality of life, reduce the need for hospitalization and ultimately prolong life. Different therapeutic approaches or combinations are used for targeting different components of this complex disease.

Reduction of risk factors

The relationship between atherosclerosis and high blood glucose and cholesterol levels is now well established. Perhaps the best therapeutically strategy in treatment of the heart remodeling is preventing coronary disease from developing by adopting a healthy lifestyle. Healthy lifestyle habits such as low-fat diet [51, 52], limiting alcohol and quitting smoking [53, 54], regular physical activity [55, 56] and maintaining a healthy weight [57] have favorable effects and should be practiced from the earliest days [58, 59]. However, for most of the patients this is not enough and they need pharmacotherapy, with oral antidiabetic agents and cholesterol lowering drugs Reducing risk factors with cholesterol and glucose lowering drugs slow down the progression of atherosclerosis [60-63]. Statins not only slow down progression, but can also reverse the process of atherosclerosis [63-66].

Surgical alternatives

For some patients conventional pharmacological therapy is insufficient in preventing atherosclerosis of coronary arteries. Revascularization can be performed either by percutaneous coronary interventions (PCI) [67, 68] or more invasive open heart coronary artery bypass grafting (CABG) [69, 70]. Those procedures reestablish lumen of occluded vessel, for a short while [71, 72]. Initially the occurrence of restenosis after PCI was as high as 20-50% [73], while after introduction of stents the occurrence of restenosis was dramatically reduced to 5-10% [74]. It still remains a significant clinical problem [75]. Patients undergoing CABG compared with PCI of a native vessel generally have worse long-term clinical outcome [76].

Treating the failing heart

Numerous strategies that intervene with inappropriate neurohormonal stimulation are used in clinical practice with various degrees of success in the treatment of heart failure. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers [77] lower blood pressure, improve blood flow and decrease cardiac workload [78]. Beta adrenergic blockers slow the rate of contraction, reduce heart rate, lower blood pressure and reduce the risk of heart arrhythmias [79], while diuretics prevent accumulation of fluids and maintain blood pressure [80]. When all pharmacological strategies fail, the only option is heart transplantation. Even though the survival of transplanted patients has greatly improved, the donor is almost never perfect and numerous challenges remain: rejection of donor heart, graft infections, unspecific graft failure and sclerosis, and long term immunosuppression issues [81-84]. The largest limitation of cardiac transplantation is donor availability.

For patients who are out of options we have been exploring alternative therapeutically approaches in treatment of ischemic heart disease.

Potential of retinoic acid in treatment of cardiovascular remodelling

Retinoic acid metabolic pathway and mechanism of action

Retinoic acid metabolites exert a wide range of effects on fetal development and cardiac morphogenesis, and furthermore on tissue homeostasis, cell proliferation, differentiation and apoptosis in postnatal life [85-87]. Mammalian cells cannot synthetize vitamin A (retinoic acid), which is obtained from diet in the form of retinyl esters or from β-caroten, which are converted to retinol and stored in the liver. The homeostasis of vitamin A is strictly regulated, since excessive free vitamin A can intervene with cell function and survival. It is delivered to the targeted cells as retinol bound to retinol binding proteins (RBPs). RBPs protect both vitamin A from enzymatic damage and cells from unregulated diffusion of this potent signaling molecule. RBPs are found

both extracellularly and intracellularly. Vitamin A becomes toxic in vivo when it surpasses the binding capabilities of RBPs. Furthermore, the vitamin A - RBP complex is recognized by cell surface receptors (STRA6), which binds exclusively to RBP and induces release of vitamin A into the cell. The RBP-STRA6 system functions as a physiological mechanism of vitamin A uptake [88-90]. The STRA6 expression level is suggested to be a good predictor of retinol status in the cells [91]. Intracellular retinol is in a metabolic cascade converted to the biologically active derivatives all-trans RA (atRA), 13-cis RA or 9-cis RA by metabolic enzymes (i.e. oxidation enzymes of the ALDH family) [92], which are delivered to the nuclear retinoic acid receptors by cellular binding proteins (CRBPs and CRABPs) [93, 94]. Excessive vitamin A is deactivated by conversion into the inactive metabolites 4-oxo-RA, 4-OH-RA, 18-OH-RA and 5,8-epoxy-RA by cytochrome p450 enzymes (CYP26A1 and CYP26B1) [95]. AtRA signals through binding to the nuclear receptor RAR (α, β, or γ), while its enantiomer 9-cisRA and 13-cisRA binds to RAR or RXR (α, β, or γ) [96]. The receptors behave as ligand-activated transcription factors that form heterodimers and bind to DNA sequences called retinoic acid respond elements (RAREs), located within the promoter of target genes [94, 97]. The heterodimers have two functions; modulate the frequency of transcription of target genes after binding to RARE, and cross-talk with other signalling pathways, such as thyroid hormone receptors, vitamin D receptors, peroxisomal proliferator-activated receptors, and several orphan receptors [94].

Retinoic acid and vascular remodelling

Percutaneous transluminal angioplasty (PTA) is established as routine procedure for treatment of patients suffering from peripheral arterial occlusive disease [98-100]. In principle, a catheter with small folded balloon is advanced over the wire to the narrow segment of the vessel that needs to be treated. Inflation of the balloon compresses the plaque and dilates the artery, improving the blood flow. Restenosis is quite common in PTA and PCI of the coronary arteries. At the cellular level, restenosis is characterized by migration and proliferation of local smooth muscle cells (SMC)

at the site of endothelial injury, resulting in formation of hyperplastic neointima [101]. Intact endothelium plays an important role in vascular homeostasis. Endothelium-derived nitric oxide mediates endothelium-dependent vasodilation, inhibits leukocyte adhesion, SMC migration, platelet adhesion and aggregation, and the expression of plasminogen activator inhibitor-1, a known prothrombotic protein [102]. Reendothelisation after vessel injury and control of SMC proliferation were early recognized as phenomenons of importance to reduce the occurrence of restenosis.

Retinoic acid was found to induce proliferation of human umbilical vein endothelial cells (HUVEC) *in vitro* [103], and to accelerate reendothelisation after vessel injury *in vivo* [104]. It also restricts proliferation of SMC *in vitro* [105-107] and *in vivo* [105-110]. Control of proliferation of those to cells seems to be mediated by signaling through RAR (α, β, or γ). Anti-proliferative effects of RA on SMC and reduction of the occurrence of restenosis was documented across various species [105-111] including humans [112]. When administrated orally [110, 113], injected intramuscularly [114], injected at the place of vessel injury [115], or endogenously generated [116], RA prevents neointimal hyperplasia resulting in a marked reduction of the vessel cross-sectional area and reduction of the intimal/medial thickening ratio.

Probably the most underinvestigated cell which contributes to vascular remodelling is fibroblasts. Their migration from adventitia into neointima was observed in several studies [117-119]. One study showed that myofibroblasts characterized by the expression of SM α-actin also coexpress CRBP-1, a retinoic acid–responsive gene [120]. However, effect of RA on vascular fibroblast proliferation and migration was not investigated.

Retinoic acid and myocardial remodelling

The RA signal transduction pathway is recognized as a master regulator of embryonic cardiomyocyte proliferation and differentiation [121-123]. Disturbances in RA homeostasis, both

lack of and excess of RA, during heart formation may result in congenital cardiovascular malformations [124]. Myocardial remodelling is associated with reactivation of embryonic gene programs [20, 21, 125]. Postnatal RA signal transduction pathway appears to have role in preserving the normally differentiated phenotype of cardiomyocytes and restricting cardiofibroblast proliferation and fibrosis. *In vitro* studies on rat neonatal cardiomyocytes and cardiofibroblasts demonstrate that RA can antagonize decrementing hypertrophic stimuli of some major promoters of remodelling such as endothelin, angiotensin II and phenylephrine [126-131]. It was observed that RA inhibits hypertrophic responses of cardiomyocytes evoked by cyclic stretching [127]. Role of RA in maintainance of cardiac structure and function was demonstrated *in vivo*. RA prevented myocardial fibrosis during the development of hypertension in spontaneously hypertensive rats [114]. Dietary depletion of RA from tissues resulted in ventricular dilatation and decline of systolic function in rats [132]. Tissue insufficiency of RA enhanced remodelling after myocardial infarction [133], while supplementation with RA after myocardial infarction attenuated ventricular remodelling [134]. The above studies demonstrate a favourable effect of RA on myocardial remodelling. However, if RA signaling is activated in the process of cardiac remodelling remains to be investigated.

Potential of gene therapy in treatment of myocardial remodelling

For patients where conventional pharmacological therapy is not sufficient and distribution of coronary artery disease disallows revascularization, gene therapy may offer an alternative. The potential of gene therapy is virtually unlimited. In theory targeting vascular cells could restrict proliferation and reoccurrence of stenosis, or promote angiogenesis. Gene delivery to cardiomyocytes could antagonize hypertrophic stimuli and promote survival after i.e. myocardial infarction. Targeting cardiofibroblasts could perhaps limit fibrosis. Gene therapy has already

reached clinical trials [135]. The selection of the gene and method of transfer is determined by the therapeutic objective. No matter what the purpose is, the ultimate challenge is safe, sufficient and lasting transfer of the selected gene to the targeted cells [136, 137]. DNA encoding for protective factors can be injected directly into the myocardium, delivered through the coronary arteries or veins, or into the pericardium [137]. However, achieving sufficient nuclear uptake of delivered DNA by targeted cells proves to be challenging. Two main gene delivery strategies have emerged over the years: non-viral delivery systems and viral vectors [136, 137].

Non-viral gene delivery in treatment of myocardial remodelling

Non-viral delivery systems are usually plasmid DNA, DNA – liposome complex or different polymers used both as protection of DNA from enzymatic damage, and enhanced cellular uptake of DNA [138-140]. Those DNA complexes are successfully used *in vitro*, but with moderate success *in vivo.* Applying those strategies in order to transfer genes with intracellular effects to the myocardium appears to be difficult, since successful transfection of a greater number of cells is needed [141]. Naked DNA cannot sufficiently penetrate the cells and usually a very local transfection is achieved [137, 142, 143], while 99% of DNA which does not enter the cell is degraded by endonucleases within minutes [144-147]. The highest transfection efficiency of naked DNA *in vivo* was achieved with direct myocardial injection and it was estimated to reach barely a few hundred cells [148]. Despite low transfection efficiency, resulting in a very local expression, this approach has been used experimentally and clinically with considerable success [137, 149]. In experimental animal models direct myocardial injection of naked DNA into ischemic myocardium encoding for transient stromal cell-derived factor-1 alpha [150] or hepatocyte growth factor prevented the progression of heart failure [149]. Clinical trials show that injection of DNA encoding for vascular endothelial growth factor gene 2 to patients with ischemic heart disease increased their tolerance to physical activity [141]. However, injecting DNA into the heart muscle is an invasive procedure associated with tissue damage, and due to the hearts' protective placement in

the thorax, is unlikely to represent a large-scale clinical treatment. Use of non-viral delivery systems could perhaps be a method of choice for transfection of vascular cells *in vivo,* since naked DNA transfer is not associated with systemic dissemination and inflammation, which are considerable risks if vector enters arterial blood [151]. It was demonstrated that adequate gene transfer of therapeutic genes into the vascular wall can be achieved using endovascular stents coated with different polymers containing DNA [152-154].

Viral gene delivery in treatment of myocardial remodelling

Gene delivery by viral vectors is another approach with high likelihood of success. The principle is relying on the natural ability of viruses to recognize, penetrate and deliver their genetic material to cells [155]. Viral vectors achieve successful transfection of greater number of the cells within the targeted organ than the approaches mentioned in the previous chapter, but with considerable risks [156]. The selectivity of viral vectors remains an issue, since viruses usually infect numerous cell types [151, 155]. Delivered DNA can practically incorporate into any cell and in any place in the human genome resulting with dysfunction or mutation, particularly in cells with high mitotic index. Malignant mutations resulting in cancer have been already reported in clinical trials using gene therapy. Correction of hematopoietic stem cells, carrying X-linked severe combined immunodeficiency, with retrovirus, caused T cell leukemia in 4 of 20 patients [157, 158]. Another issue to consider is the possibility of systemic dissemination and inappropriate inflammatory responses, which could be life-threatening. Using an adenoviral vector in the treatment of ornithine transcarbamylase deficiency at the University of Pennsylvania resulted with the death of a patient. This was attributed to the dissemination of the viral vector, massive inflammatory response, disseminated intravascular coagulation, acute respiratory distress and multiorgan failure [151, 159]. Gene therapy of myocardial remodelling is gaining momentum. Many different viral vectors have been tried with various degrees of success and unwanted side effects [160]. Evidence suggests

that adenoassociated viruses (AVV) are currently the vectors of choice when targeting cardiomyocytes [161-163]. Serotypes AAV 1, 6, 8 and 9 have been recognized to have higher tropism for cardiomyocytes than all previous viral vectors used in cardiac gene therapy [163, 164]. They are not associated with any human disease and they can be administered systemically. AVV 9 is very cardioselective with an amazing transfection efficiency of almost 100%, resulting in long lasting myocardial expression and virtually no unwanted complications [161, 163, 165, 166]. Even though some other organs are transfected, hundreds of patients have been treated without observing unwanted effects [135]. Currently under investigation is clinical trial of sarcoplasmic reticulum calcium ATPase 2a transfer to the failing ischemic heart, using AVV1 as vector [167].

Remote myocardial protection by injecting naked DNA encoding for HMOX-1 into the skeletal muscle

Remote myocardial gene therapy is a new approach [137, 156, 168]. It is based on the principle of remote ischemic preconditioning [137]. The principle refers to the phenomenon by which exposure of non-cardiac tissue to episodes of ischemia and reperfusion prior to the cardiac ischemia induces myocardial protection. The protection appears universal [169-178]. Remote ischemic preconditioning is likely to convey protection through the secretion of the protective factors into the blood stream. Direct injection of DNA to transfect skeletal muscle was used efficiently before and it is currently considered as the method of choice for DNA based vaccines [168, 179]. If the gene transfer is followed by in vivo electroporation, transfection is far more efficient, lasts longer, and proves to be potential approach for delivery of genes encoding for secretory factors [141, 168, 180, 181]. Unlike the genes with intracellular effects, secretory factors can exert their protective effect even with much lower transfection efficiency [137, 141]. Gene delivery of hypoxia-inducible factor 1 alpha (HIF-1α) into the skeletal muscle protects against acute and chronic ischemic injury of the heart *in vivo*, *ex vivo* and *in vitro* [137, 156, 168]. Injection of DNA encoding for HIF-1α followed by electroporation achieved stable, lasting and local expression up to 8 weeks later, with no leakage

to any other organ [168]. Others have confirmed that this approach can achieve sustainable overexpression up to one year [182]. HIF-1α gene delivery resulted in increased expression of heme oxygenase 1 (HMOX-1) in the treated skeletal muscle, and increased circulating levels of bilirubin, a downstream product of HMOX-1 [168]. Hypothesizing that HMOX-1 was an important factor for the beneficial effects evoked by HIF-1α, the HMOX-1 blocker zinc deuteroporphyrin 2,4-bis-glycol was given together with HIF-1α [168]. This abolished the protective effects of HIF-1α when hearts were isolated and perfused with global ischemia and reperfusion, suggesting that HIF-1α mediates protection through HMOX-1 [168]. HIF-1α delivery also caused a general angiogenesis, which may be procarcinogenic in patients. The next step was to investigate if remote gene delivery of HMOX-1 could prevent cardiac remodelling and preserve function, without causing general angiogenesis.

HMOX-1 is stimulated by both hypoxia and oxidative stress in various cell types including cultured endothelial, cells vascular smooth muscle cells and cardiomyocytes [183-186]. It is a stress-induced protein which plays a major role in endogenous cellular protection against injury caused by adverse stimuli [187-190]. HMOX-1 is an ubiquitous enzyme which metabolizes free heme into carbon monoxide (CO), free iron (Fe) and biliverdin [190], which is further rapidly convertetd into bilirubin by biliverdin reductase [191]. Anti-oxidative, anti-inflammatory and anti-apoptotic properties of HMOX-1 seems to be mediated by those metabolites [192]. Activation of HMOX-1 in hypoxic conditions in animal tissues *in vivo* and cell cultures *in vitro* is mediated by hypoxia-inducible factor-1 (HIF-1) [193]. Over the years it was observed that HMOX-1 can be activated also by alternative mechanisms, which are different from those activated by hypoxia such as atrial natriuretic peptide and angiotensin II [194-197]. Taken together with the findings of Czibik [156, 168], HMOX-1 was selected as an interesting candidate for remote gene delivery.

METHODOLOGICAL CONSIDERATIONS

Human material

We get exclusive but limited insight into RA signaling in human ischemic and dilated cardiomyopathy through obtaining the left ventricular free wall biopsies in different stages of disease. Biopsies were collected from patients undergoing coronary artery bypass grafting (CABG, n=11). Thru-cut biopsies were taken after cannulation for extracorporeal circulation was completed, but prior to cardioplegic arrest. The elective patients had coronary artery disease associated with mild cardiac dysfunction graded as NYHA classes I-II with left ventricular ejection fraction >60 %. Moreover, biopsies of the left ventricular free wall were also collected from still-beating explanted human hearts from patients with end-stage heart failure undergoing cardiac transplantation due to postinfarct heart failure (CAD, n=9) or non-ischemic dilated cardiomyopathy (CMP, n=15). The patients were in NYHA classes III-IV with ejection fraction <35%. Gene expression was compared to donor hearts, which were obtained from sex- and age-matched healthy individuals with no history of heart disease, dying suddenly of non-cardiac reasons (DONOR, n=7). The patients are heterogenic. Many variables may influence results such as gender, age, past medical history, sampling procedure, stage of heart failure. We have control from where we obtain the biopsies, but we have no control on the distribution of coronary plaques with in each biopsy. We were not able to measure RA metabolite content in the hearts of the patients that were undergoing coronary artery bypass grafting, since this would require a fairly large biopsy of left ventricular tissue that would undoubtedly harm a patient. In order to investigate gene expression of RA target genes in atherosclerotic plaque samples were obtained from 9 patients scheduled for endarterectomy due to >80% carotid artery stenosis. Unfortunately, we could not obtain atherosclerotic lesions from the coronary arteries. As a control to this, a bit of the renal artery was collected from patients without a history of cardiovascular disease scheduled for nephrectomy (n=5). We did not have access to normal carotid arteries for comparisons in the same vascular bed. Even though those arteries have histologically the same structure as coronary arteries, the different origin of the samples

could be a source of variation. In addition, gene expression in the non-sclerotic renal arteries could be influenced by the underlying disease.

Animals

Mice are small, easy to maintain, reproduce quickly and they do not need much space, which makes them attractive for research. Genetically they are not more different from humans than any other larger animal [198]. The mouse genome has been sequenced revealing that 99% of mouse genes have matching human homologs [199, 200]. Until now various transgenic and knockout models have been designed to investigate pathophysiology of different human diseases [199, 201, 202]. Moreover, most mice used in research are inbred, matched in age and gender, which reduces genetic variability and make them suitable for lab studies of gene expression. We have some examples of experimental studies with mice in preclinical trials which resulted with development of successful therapeutic modalities for humans. Often mentioned is the pioneer work of Professor Pier Paolo Pandolfi of Harvard Medical School, USA. He used genetically engineered mice in order to simulate different types of acute promyelocytic leukaemia. This resulted in development of different target therapies which were successfully transferred to humans [203].

However, there are considerable physiological differences between mice and humans such as basal heart rate of 400-600 beats per minute, different responses to some drugs and differences in immune cell composition [204]. For our study only male transgenic retinoic acid response element luciferase reporter mice and regular C57BL/6 wild type mice were used. We took into consideration that female sex hormones could influence results. Estradiol induces retinoic acid receptors [205] and interaction between estrogen receptors and RARs has been described [206, 207]. Gender aspects of RA signaling are scientifically exiting and may be persued in the future.

Male transgenic retinoic acid response element luciferase reporter mice (RARE-Luc; www.cgene.no) of the same weight were used for experiments. RARE-luc mice on C57BL/6

background have an artificial construct containing the luciferase gene under the control of three copies of RARE derived from the RAR-β2. The advantage of this model is that it allows observation of RARE mediated transcription in *in vivo* in real time. Luciferase was used as a reporter to assess the transcriptional activity of RARs. Transcribed luciferase reacts with luciferin resulting in photon emission measured in a CCD camera, which is somewhat proportional to the degree of RARs activation. Thus, if we would treat the cells with a known RAR agonist such as RA we would observe an increase in luciferase readings. Animal models give high control of experimental conditions however translation of results to humans cannot be performed. Interspecies differences must be considered.

However, the luciferase model has also some disadvantages. Firstly RARE can be activate by retinoic acid, but also by other ligands or post-translational modifications that are present in infarct conditions. RARs are known to be a polyvalent cooperator for various nuclear receptors such as thyroid hormone receptors, vitamin D receptors, peroxisomal proliferator-activated receptors, and several orphan receptors [94]. Moreover, there are at least three RARs isotypes (RARα, RARβ and RARγ). Theoretically if we would like to identify ligand responsible for RARs activation we would most likely have to use transgenic animals in which each of the three RAR isotypes have been previously knocked down. Furthermore, finding anti-luciferase antibody has shown to be almost impossible, preventing us from using immunohistochemistry or bioluminescence microscopy.

Mouse model of *in vivo* permanent ligation of the left coronary artery

There are several models of remodelling in relation to coronary artery disease which could be used: acute myocardial infarction by surgical ligation of left anterior descending coronary artery or embolization, myocardial infarction followed by reperfusion and the model of chronic myocardial ischemia by permanently ligating the left coronary artery. [208, 209]. A model of chronic myocardial ischemia was used to investigate in vivo activation of RARs after myocardial infarction and to investigate cardioprotective properties of HMOX-1 (Paper II and paper III). Advantages of

this model are low cost and simple procedure. The major limitation of the procedure is that it is surgically demanding.

Mice are anesthetised with isoflurane and intubated. Intubation with mechanical ventilation is important step. The ligation procedure requires opening of the chest, which result in disruption of negative intrathoracic pressure. Mechanical ventilation is necessary to maintain respiration, and it protects the animal from depressive effects of isoflurane on respiratory function. Left-sided thoracotomy is performed. The pericardium is cut open, and permanent ligation of the descending branch of the left coronary artery is made 1,5 mm under the tip of the left auricle. The challenge is to ligate in one quick move so that the heart is not preconditioned and to make even ligations in the same place so that infarct size or effect of infarct can be compared between animals. Unfortunately, a certain degree of variability exists partly due to individual variations in coronary anatomy and partly due to manual factors. In acute models of ischemia this can be corrected thorough measuring area at risk (Evans blue staining) [210]. In the chronically infarcted heart this cannot be done due to tissue adherances. Injury to lung parenchyma will result in lethal injury which is almost impossible to repair. Avoidance of this requires a well-trained operator and a fairly large number of animals. In our hands mortality was around 45%. Subsequently the intercostal space, muscles of the external thoracic wall and skin must be sutured leaving no perforations of the thoracic wall, so that negative pressure can re-establish, which is essential for recovery of spontaneous breathing. Extubation is performed upon spontaneous breathing. Animals were placed in a “mini intensive care unit” postoperatively, maintaining an environment of 30°C over night. All animals received 0,5 ml saline intraperitonealy prior to surgery to compensate for fluid loss and 0,1 mg/kg of Temgesic subcutaneously for analgesia. Sham operated controls were included. In sham operation, the surgical procedure is identical, with a suture perforating the heart, but no ligation is placed and no infarct is induced. A model of chronic myocardial ischemia was used as it allows investigation of not only cardiac protection by assessing area of fibrosis as surrogate to infarction area, but it also enable investigation of cellular responses to stress and

tissue damage repair. A model of myocardial infarction followed by reperfusion was not used. This model is associated with higher mortality rate.

Investigation of retinoic acid target gene expression and proliferation of cardiac cells

The heart consists of several cell types, where the most abundant are cardiomyocytes and cardiofibroblasts followed by endothelial cells and smooth muscle cells. An *in vitro* approach is a good model for dissecting and analysing the response to atRA in various regimes. However, *in vitro* studies are in many ways artificial models, with biological conditions very different from those of *in vivo*. Cells in cultures are not under control of homeostatic mechanisms and control pathways which are found in living organisms.

In paper II we isolated murine cardiofibroblasts and cardiomyocytes using the method established by O'Connell [211]. In principle, cells are released from matrix by digestion with collagenase and cardiomyocytes are separated from cardiofibroblasts by serial centrifugations [211]. Cardiofibroblasts are collected from the first centrifugation, transferred to a separate tube, resuspended, and plated. The method takes advantage of cardiomyocyte size, which is considerably larger than cardiofibroblast. The advantages of the method is that it gives opportunity to investigate gene expression and RA signaling in different cardiac cells, isolated from the healthy region of the heart, peri-infarcted zone or the infarct itself. The isolation procedure weakness is that the method is highly non-physiological and could affect gene expression, which may no longer be representative to the conditions observed in vivo. Further, maintaining cells in culture may alter their phenotype. HUVEC and SMC proliferation was evaluated by counting trypsinized cells with a hemocytometer (Burker) after 24h and 72h. Cells were counted in triplicates of 5 independent experiments. Cardiofibroblasts proliferation in matched time points was evaluated by measuring EdU incorporation as a marker of cell division, using flow cytometer. AOSMC and HUVEC were purchased as primary cells and proliferation was investigated by manual counting. The major drawback for primary cardiomyocytes, which are cells with low stem cell potentiall, is that they gradually die off. They are therefore not possible to maintain for more then 24-28 hours.

Gene expression

Through the studies gene expression was evaluated at the mRNA level using real time qPCR. A key assumption in studying mRNA expression is that it may predict protein expression. This hypothesis was confirmed in mouse experiments by finding increased expression of RBP1 and ALDH1A2 at protein level concomitantly with increase of expression of corresponding mRNA. The assumption was made on the fact that in eukaryotes export of mRNA from the nucleus is mandatory before translation is possible. However, in eukaryotes post-transcriptional modifications and RNA degradation play important role in regulation of protein expression in the cell, and protein expression should be investigated as the most important end point. In paper I we were not able to investigate protein expression in the hearts of the patients undergoing coronary artery bypass grafting, as the biopsies obtained with a thru-cut needle were too small. Gene expression was examined instead. In paper II as surrogate to protein expression luciferase activity was measured. In paper III gene expression of HMOX-1 was confirmed only in treated muscle, and the corresponding protein remains to be elucidated. One reason for doing qPCR only in that study is that so few cells were transfected and finding representative parts of the muscle for protein determination by western blot is cumbersome. Another challenge when investigating relative gene expression is choice of proper endogenous control. The mRNA levels of target gene are related to endogenous control according to the standard formula 2-ΔΔCT, where ΔΔCT= (CTTarget_sample - CTendogenous control _sample)-(CTTarget_calibrator –CTendogenous control_calibrator). The endogenous control must be stably expressed and not affected by infarction. Ideally the expression levels should be comparable in different cardiac cells. Evaluation of gene expression in the mouse material was done using 18S ribosomal RNA. The stability of 18S was tested in our lab by comparing gene expression before and after myocardial infarction, which was stable. It is considered stable also by others [216-218]. However, 18S is not stable in humans [216] and other endogenous controls had to be used. Beta actin was used as endogenous control for HUVEC and

AOSMC, while in human heart material hypoxanthine phosphoribosyl transferase was used with reference to the literature [216].

AIMS OF THE STUDY

1. To evaluate the content of retinoic acid metabolites in postinfarct remodelling hearts

2. To elucidate influence of retinoic acid on proliferation of cardiovascular cells

3. To investigate a possible activation of retinoic acid receptors in acute myocardial ischemia and postinfarction remodelling

4. To evaluate gene expression of the retinoic acid metabolic pathway in acute myocardial ischemia and postinfarction remodelling

5. To evaluate if remote gene delivery of HMOX-1 prior to myocardial infarction can prevent cardiac remodelling and preserve function

6. To evaluate if delivery of DNA encoding for HMOX-1 into the skeletal muscle will induce generalised angiogenesis

SUMMARY OF RESULTS

Paper I

Left ventricular biopsies were collected from still-beating explanted human hearts due to dilated cardiomyopathy (CMP) or postinfarction heart failure (CAD), and compared with donor hearts (Donor). Additional heart biopsies were taken from patients undergoing coronary artery bypass grafting (CABG). Atherosclerotic plaques were collected from patients during endarterectomy due to >80% carotid artery stenosis and compared to the non-sclerotic renal arteries that were collected during nephrectomy from patients without a history of cardiovascular disease. Gene expression of key enzymes in RA metabolism was analysed using qPCR. Determination of endogenous retinoids in hearts was performed using triple-stage liquid chromatography/tandem mass spectrometry. We observed that RA target genes involved in delivery of retinol into the cell (RBP1, RBP4 and STRA6) together with genes involved in transformation of intracellular retinol into the active forms of retinoic acid (ALDH1A1 and ALDH1A2) were dramatically upregulated in hearts of patients with coronary artery disease with stable angina undergoing coronary revascularistion, as well as in atherosclerotic lesions. All-trans retinoic acid accumulated in the postinfarcted failing human hearts of patients with coronary artery disease. Stimulation of cardiomyocytes, cardiofibroblasts, smooth muscle cells, and endothelial cells with atRA increased gene expression of the key enzymes governing RA metabolism. Cardiofibroblast and smooth muscle cell proliferation was reduced by atRA, which promoted endothelial cell proliferation. Coronary artery disease leads to increased expression of key enzymes governing retinoic acid metabolism. AtRA accumulated in the failing human heart. All investigated cell types present in the heart had induced expression of retinoic acid target genes when stimulated with atRA, which influenced cell proliferation.

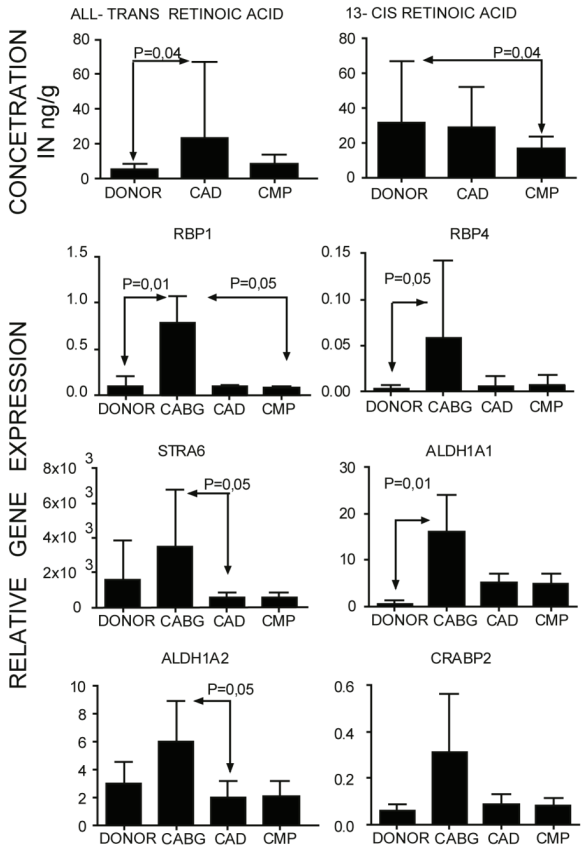

Figure 1. Expression of RA targeted genes and concentration of endogenous RA metabolites

Left ventricular biopsies were collected from still-beating explanted human hearts from patients with end-stage heart failure undergoing cardiac transplantation due to dilated cardiomyopathy (CMP, n=15) or postinfarction heart failure (CAD, n=9). Explanted healthy donor hearts (DONOR, n=7) were controls. Endogenous retinoid concentrations were measured by triple-stage liquid chromatography/tandem mass spectrometry. Gene expression of retinoic acid target genes was investigated with addition of biopsies from patients undergoing coronary artery bypass grafting (CABG, n=11). RNA was extracted and amplified with real time PCR.

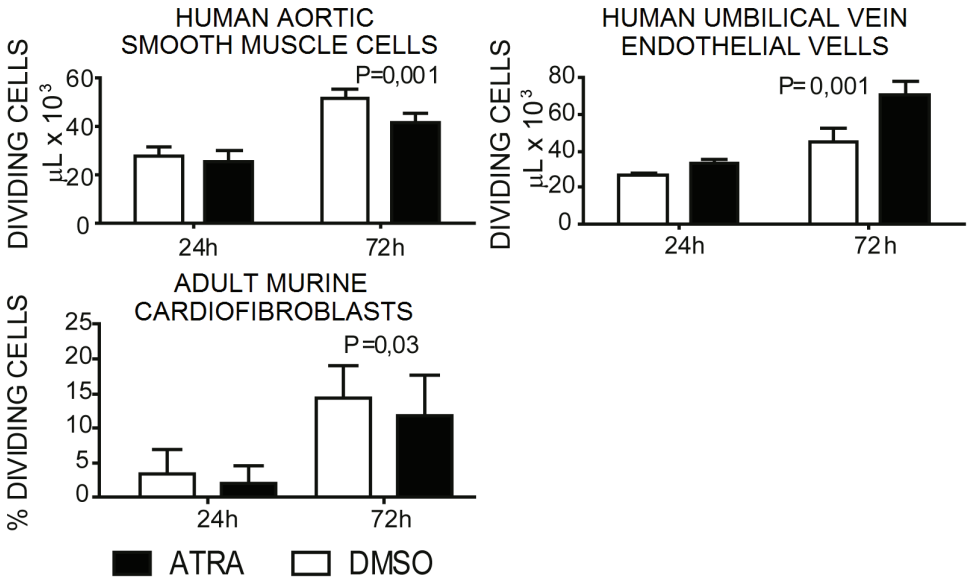

Figure 2. Effect of ATRA on proliferation of cardiac cells.

Human aortic smooth muscle cells and human umbilical vein endothelial cells were cultured in medium supplemented with 1 µM atRA or the dissolvent DMSO. Proliferation of those cells was evaluated by counting cells with a hemocytometer. Adult murine cardiofibroblasts were isolated from C57BL6 mouse hearts and cultured in medium supplemented with 1 µM atRA or the dissolvent ethanol. Cell proliferation was evaluated by adding EdU and evaluating incorporation with flow cytometry. Data are mean±SD of 4 independent isolations in duplicates

Paper II

Myocardial infarction was induced through ligating the left coronary artery in mice. In vivo cardiac activation of the retinoic acid receptors (RARs) was measured by imaging RARE-luciferase reporter mice, and analysing RAR target genes by real time qPCR up to six weeks afterwards. Endogenous retinoids in postinfarcted hearts were analysed by triple-stage liquid chromatography/tandem mass spectrometry. Cardiomyocytes (CM) and cardiofibroblasts (CF) were isolated from infarcted and sham operated RARE luciferase reporter hearts and monitored for RAR activity and expression of target genes. The effect of atRA on CF proliferation was evaluated by EdU incorporation.

The main finding of this paper was that endogenous RA signaling was activated in the mouse heart with permanent coronary artery ligation. A dramatic increase of luciferase signal was found *in vivo* in the thoracic region of RARE-luciferase reporter mice with induced infarction, peaking the first postoperative week **.** The signal was verified to originate exclusively from the heart by *ex vivo* organ imaging, with maximal signal emission in the infarcted zone**.** This was accompanied by increased cardiac gene and protein expression of the RAR target genes retinol binding protein 1 and aldehyde dehydrogenase 1A2, while gene expression of cytochrome P450 26B1 was downregulated. Increased expression of retinol transporting genes coincided with accumulation of retinol in infarcted hearts. Cardiofibroblasts and cardiomyocytes isolated from infarcted hearts had most RARE-Luc activation, evident as higher luminescence, than those from sham operated hearts. This was accompanied by increased fibroblast expression of the RA target genes RBP1, ALDH1A2, CYP26B1, RARα and RARγ, while RARβ was more highly expressed in cardiomyocytes. AtRA inhibited proliferation of murine cardiac fibroblasts *in vitro*. The RA signalling pathway is activated in postischemic hearts and may play a role in regulation of damage and repair during remodelling.

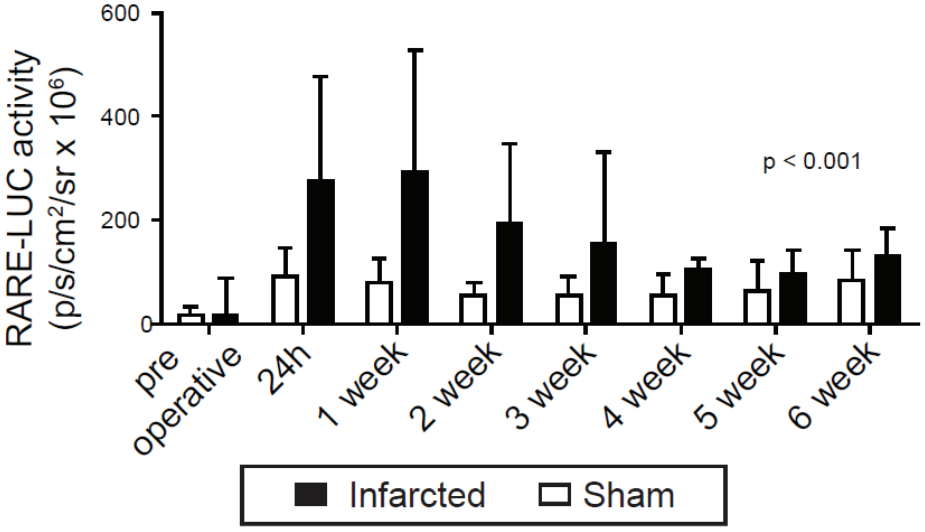

Figure 1: *In vivo* imaging of cardiac retinoic acid signalling after myocardial infarction
To evaluate *in vivo* cardiac activation of the retinoic acid receptor RAR, RARE-luciferase reporter mice were subjected to myocardial infarction through permanent coronary artery ligation or sham operation (n=7-10 of each). *In vivo* luciferase activity was measured in a CCD camera after injection of luciferin preoperatively and serially after infarction. The digitized luciferase signal is quantified and shown as bar graphs (mean±SD).

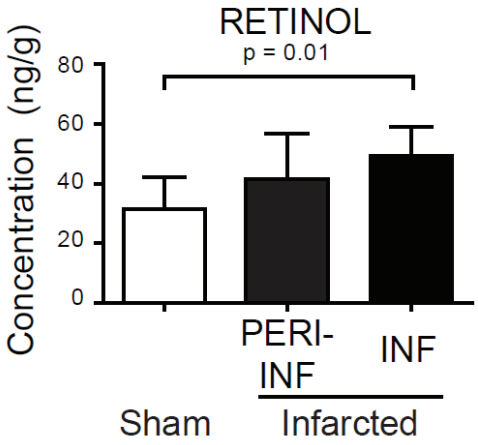

Figure 2: Concentration of endogenous RA metabolites Wild type C57Bl6 mice were subjected to *in vivo* ligation of the left coronary artery or sham surgery, and hearts were sampled serially. Endogenous retinoic acid metabolite concentrations were evaluated by triple-stage liquid chromatography/tandem mass spectrometry one week after induction of myocardial infarction. The infarcted zone of the left ventricle (INF), the periinfarcted zone (PERIINF) and left ventricles from sham operated hearts (SHAM) were investigated (n=6). Data are shown as mean±SD.

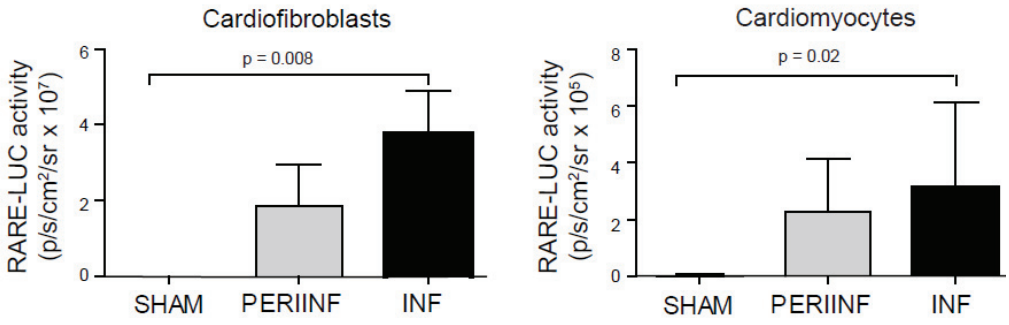

Figure 3: *In vitro* imaging of retinoic acid signaling after myocardial infarction
Cardiomyocytes and cardiofibroblasts were isolated from left ventricular tissue from RARE-luciferase reporter hearts one week after myocardial infarction or sham operation (SHAM). Cells were isolated from the infarct (INF) and periinfarct zone (PERIINF). After plating for three hours, non-viable cells were removed and luciferin was added for imaging. The upper panel shows a representative image of one experiment. The lower panel shows mean±SD of n=5 experiments in each group. Note that the Y-axis labelling is different.

Paper III

HMOX-1 was cloned from murine cDNA into pcDNA3.1. C57BL6 mice sedated with isoflurane were injected with 15 µg of pcDNA3.1/HMOX-1, empty vector, or saline alone into the right quadriceps in a total volume of 50 µL saline (n=5-8 in each group). Bipolar pulses were used to enhance nuclear uptake. The transfection efficacy was evaluated by real time PCR and in situ hybridization of the transfected muscle, contralateral muscle, and heart. One week later echocardiography was performed using the Vevo 770 System. Mice were subjected to myocardial infarction by permanent ligation of the descending left coronary artery, and kept for 6 weeks before functional evaluation by echocardiography and tissue harvesting. Cardiac output and cardiac index were calculated. The electroporated muscle, the contralateral muscle and the hearts were harvested, embedded in OCT and frozen in nitrogen. Concurrently, LV tissue was collected for real time PCR analysis of the markers of hypertrophy α-skeletal actin, atrial natriuretic peptide and brain natriuretic peptide. OCT embedded hearts were sectioned and stained with hematoxylin-eosin and Masson's trichrome. Infarct size was measured and calculated. Furthermore sections of treated quadriceps muscle, contralateral muscle, and hearts were evaluated for angiogenesis through staining with the endotelial marker CD31. Quantification was performed using Adobe Photoshop CS3.

Gene delivery of HMOX-1 lead to a local expression of HMOX-1 in the treated muscle, but not in any other organ. HMOX-1 treated mice had reduced infarct size and improved function evident as higher ejection farction, improved fractional shortening and higher stroke volume. HMOX-1 did not cause angiogenesis in the heart or skeletal muscle.

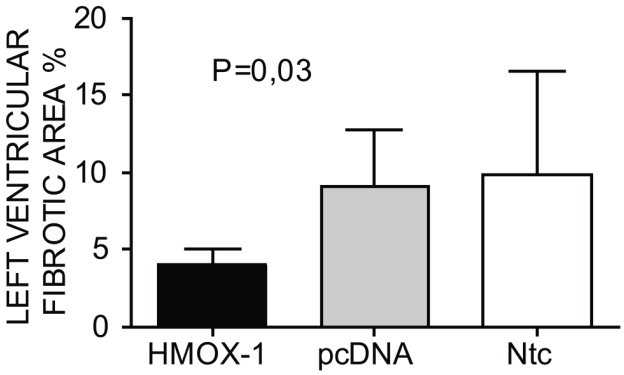

Fig 1. Evaluation of cardiac morphology
Right quadriceps muscles of mice were treated with DNA encoding for HMOX-1, empty vector (PcDNA) or saline (Ntc). One week later permanant coronary artery ligation was induced. Six weeks later hearts were excised and cross sectioned for histological evaluation of infarct size by Masson trichrome staining. When infarct size was quantified, HMOX-1 treated mice had smaller infarcts. Values are mean ± SD of n= 7-10 per group.

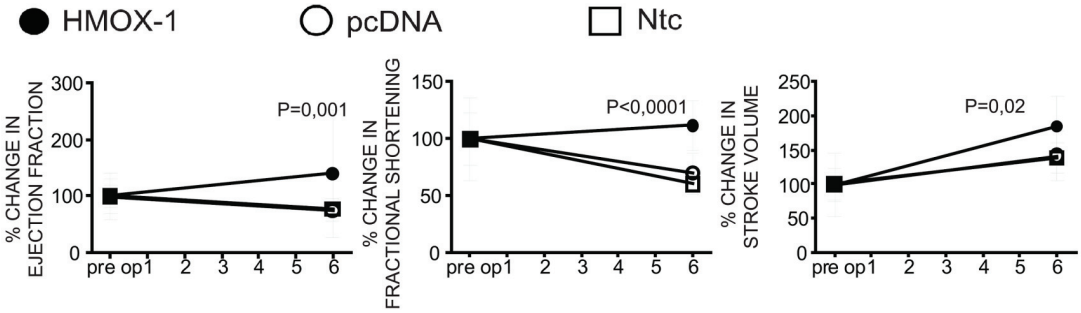

Fig 2. Cardiac performance

Evaluation of cardiac performance in mice after the right quadriceps muscles were treated with DNA encoding for HMOX-1, empty vector (PcDNA) or saline (Ntc). Seven days later preoperative echocardiography was performed followed by induced myocardial infarction. Six weeks later cardiac performance was reassessed by echocardiography. Two-dimensional guided M-mode recordings of the left ventricle in the short and long axis viewed at the level of the papillary muscle were obtained. Changes in ejection fraction, fractional shortening, stroke volume were calculated. All data are presented as mean±SD of n=7-10.

GENERAL DISCCUSION

Retinoic acid in ischemic heart disease

The first two papers in this thesis investigate possible endogenous activation of retinoic acid signalling pathway in conjunction with myocardial ischemia and remodelling. We used biopsies from human ischemic hearts in different stages of coronary disease, as well as failing hearts due to cardiomyopathy, samples of atherosclerotic plaques, a rodent model of in vivo myocardial infarction using RARE-luciferase reporter mice and *in vitro* investigations of RA effects on RA target gene expression and proliferation of cardiovascular cells.

Retinoic acid in myocardial remodelling

AtRA in human remodelling

Gene expression of RA target genes was dramatically upregulated in hearts of patients with coronary artery disease with stable angina undergoing coronary revascularistion, as well as in atherosclerotic lesions. The gene expression pattern suggests increased retinoic acid delivery in ischemic hearts, possibly in an attempt to accumulate retinoic acid metabolites. Upregulation of mRNA for RBP1, RBP4 and STRA6 in ischemic hearts could indicate increased activity of the RBP-STRA6 system, which are the enzymes involved in physiological vitamin A uptake [88]. STRA6, which is induced by retinoids, is suggested as a good predictor of retinol status in cells [91]. The latter hypothesis was supported by the finding that postinfarcted failing human heart accumulated all-trans retinoic acid. Hearts failing due to dilated cardiomyopathy of non-ischemic origin had reduced levels of 13-cis retinoic acid, a finding which is difficult to interprete. To our knowledge no previous studies addressed the expression pattern of RA targeted genes or myocardial content of retinoic acid metabolites in human ischemic heart disease or failing hearts of other etiologies. The differences in accumulation atRA versus 13-cisRA may reflect differences in the pathophysiology underlying the disease states. RA plays an important role in cardiac

morphogenesis during fetal development, and increased RA signalling in failing hearts could be due to reactivation of embryonic gene programs, which are reactivated in decrementing pathophysiological conditions such as hypoxia, ischemia and hypertrophy. Reactivation of embryonic gene programs is associated with cell survival under stress [20, 21].

AtRA in murine remodelling

In order to investigate *in vivo* endogenous activation of the RA signaling pathway in a more controlled manner, we subjected RARE-luciferase reporter mice to myocardial infarction. This approach revealed for the first time activation of endogenous RA signalling pathway in postischemic mouse hearts, which was concomitant with increased expression and translation of RA target genes, resulting in accumulation of retinol. It seems to be independent of hypoxia-inducible factor 1 alpha, as administration of HIF-1α inhibitor in conjunction with infarct induction had no effect on the signal. Different metabolites were accumulated in human and mouse hearts, which could be attributed to the interspecies differences or different time points of tissue collection. Furthermore, the postinfarcted mouse heart was remodelling, but not failing. Thus, the human and muse models are not directly comparable.

Our finding that endogenous RA signalling was activated in the mouse heart with permanent coronary artery ligation resulting in retinol accumulation is in a concurrence with a previous study. Exogenous administration of radiolabelled vitamin A in conjunction with myocardial infarction increased RA in hearts and plasma of rats, while labeled kidney and liver retinol was lower in those animals [219]. The authors speculated that it would be beneficial for an ischemic heart to increase antioxidant content. However, in that study RA was exogenously administrated, which is not physiological and does not reflect endogenous activation of RA signalling in the postinfarcted heart. We did not address effects of RA on myocardial remodelling in vivo in the present study. Experiments performed by others are arguing for beneficial role of RA in cardiac remodelling [132, 219].

Role of RARs activation in ischemic hearts

Activation of RARs in ischemic and postinfarcted hearts may play a role in regulation of damage and repair. Studies on rats with tissue deficiency of vitamine A showed that this promotes cardiac remodelling and ventricular dysfunction per se [132, 219]. When myocardial infarction was induced in rats with tissue vitamin A deficiency, adverse left ventricular remodelling was intensified [133]. Conversely, supplementing rats with retinoic acid in a model of tobacco smoke- induced left ventricular remodelling prevented remodelling [220]. Retinoic acid supplementation also prevented remodelling induced by left coronary artery ligation [134]. The lack of data on human subjects makes it difficult to interpret the importance of these data for human pathology. One study demonstrates that oral by administered isotretinoin, a first-generation synthetic 13-cis-RA which acts systemically and non-selectively can influence cardiac morphology and function in investigated patients, evident as increase of ventricular mass and a decrease in the size of the chambers [221]. The main weakness of that study is that they lack adequate non treated controls.

Investigation of RAR response in cardiomyocytes and cardiofibroblasts after myocardial ischemia showed that cells from the infarcted RAR reporter hearts had increased luminescence compared to cells from sham operated hearts. Expression of RA target genes increased concomitantly with luminescence, showing that RARE-luc activation led to transcription of RARE target genes. This activation was most abundant in cardiofibroblasts.

This observation led us to believe that RA signalling may influence proliferation of different cardiovascular cells. In support of this hypothesis is the finding of increased expression of RA target genes in atherosclerotic plaques, which indicates that the cells within the plaque have been stimulated by retinoic acid. This has been suggested before by finding increased transcript levels of the retinoic acid receptor responder-1 gene in unstable carotid artery atherosclerotic plaque [222]. To explore this possibility the response of RA target genes and cell proliferation was investigated in different mouse and human cardiovascular cell types.

Effect of atRA on cardiovascular cell types

AtRA effects on cardiofibroblasts

All-trans retinoic acid stimulation of mouse cardiofibroblast induced expression RBP1, STRA6, CYP26B1, CRABP 1 and RARβ, and inhibited cell proliferation, supporting a potential beneficial role of atRA during remodelling. Others have studied the effect of atRA on proliferation of neonatal cardiac cells. It was observed that hypertrophic responses evoked by cyclic stretching of cardiomyocytes was inhibited by atRA [127]. In neonatal cardiofibroblasts, Wang and co-workers found that atRA dose dependently reduced angiotensin-induced hyperplasia, and reduced the total cell protein content [223]. Wu and collaborators (1996) used atRA to counteract hypertrophic responses to endothelin in neonatal cardiomyocytes [129]. We confirm that this applies also to adult murine cardiofibroblasts *in vitro.* The effect of RA on fibroblast proliferation is assumed to be benefial, as it would potentially reduce in vivo scar formation in postinfarcted tissue.

AtRA effect on smooth muscle cells

All-trans retinoic acid stimulation of human AOSMC induced expression of the same genes and as expected restricted proliferation. These findings are in agreement those of others. Control of SMC proliferation was early recognized as a phenomenon of importance for restenosis and the effect of RA on SMC has been investigated. Numerous studies show that RA restricts proliferation of SMC *in vitro* [105-107]. Early *in vitro* studies performed by Miano in 1996 confirm the presence of retinoic acid receptors on SMC, and observed that RA induced activation of RARs, and prevented proliferation of SMC induced by platelet-derived growth factor-BB serum [105]. Observations *in vitro* showed that RA can both prevent and promote proliferation of SMC. Interestingly, RA alone promoted proliferation of SMC isolated from normal vessels, while it prevented proliferation of SMC originating from hyperplastic neointima, evoked by mitogen stimuli [107]. Different response of SMC originating from healthy vessel and thickened neointima indicates possible differences in SMC phenotype. Further investigations *in vitro* showed that RA prevents proliferation of SMC

induced by some growth factors such as angiotensin II and endothelin [224, 225]. It was later documented that RA prevents proliferation signaling through RAR (α, β, or γ). Neuville showed that RAR and RAR-alpha agonists, but not RXR agonists, inhibited SMC proliferation [106]. Anti-proliferative effects of RA was concomitant with upregulated expression of differentiation marker genes SM- myosin heavy chain and SM α-actin, proposing that RA promotes differentiation of phenotypically altered SMC from neointima [226]. Studies performed on the RA inactivating gene CYP26B1, showed that silencing the CYP26B1 gene using siRNA or reducing CYP26B1 enzymatic activity using an inhibitor (R115866) enhanced RA-mediated signalling, resulting in reduced proliferation of SMC [116].

More importantly RA reduces the occurrence of restenosis *in vivo* [105-110]. Oral administration of RA in the rabbit iliac artery atherosclerotic model in conjunction with balloon angioplasty, resulted in a marked reduction of the vessel cross-sectional area and reduced the ratio of intimal/medial thickening [113]. Chronic RA treatment was found to prevent hypertension-induced thickening of intramyocardial and intrarenal arteries in rats [114]. Moreover, local administration of RA immediately after vessel injury in a model of experimental atherosclerosis of the rabbit carotid artery prevents early neointimal hyperplasia and preserves overall vessel diameter [115]. Similar effects on SMC proliferation and neointimal hyperplasia were also observed in a rabbit model of vein bypass grafting, where oral administration of RA decreased cell proliferation and increased apoptosis in the intima of healing vein bypass grafts [110].

AtRA effect on endothelial cells

RA stimulation of HUVEC had similar effects on gene expression, but with different biological results on proliferation. The effect of RA on endothelial cell proliferation is controversial. Some older studies reported both stimulatory effects [227, 228] and inhibitory effects [229].

More recent studies demonstrate both that RA and the RAR agonist Am80 induce proliferation of human umbilical vein endothelial cells *in vitro* [103]. *In vivo* studies indicate that RA treatment accelerates reendothelisation after vessel injury in rat balloon-injured aorta [104]. However, the

underlying mechanism by which RA reestablishes normal endothelial function remains unknown. Intact endothelium plays an important role in vascular homeostasis. Two studies reported that RA increases nitric oxide production by endothelial cells [230, 231]. In the first study it was observed that both RA and RAR agonist Am580 induced production of nitric oxide in human dermal microvascular endothelial cells, while the effect was abolished by the RAR antagonist LE540 [230]. In the second study the authors attributed this effect to the upregulation of the dimethylarginine dimethylaminohydrolase [231].

Unlike cardiofibroblasts, smooth muscle cells, and human endothelial cells, which are actively dividing cells, cardiomyocytes do not divide. They could be only studied in a short time frame. We observed that RA stimulation induced expression of RA target genes. However we were not able to attribute functional effect to this. RA was used before to counteract hypertrophic responses to endothelin in neonatal cardiomyocytes [129].

Future perspectives

The current therapy of ischemic heart disease is based on reduction of risk factors by lowering triglycerides, increasing HDL, decreasing LDL, decreasing blood pressure or controlling hyperglycaemia, but not on inhibiting proliferative responses of cells involved in cardiovascular remodelling. The antiproliferative properties of atRA are already used in the clinics against acute promyelocytic leukemia and some forms of acute myeloid leukemia. Perhaps the antiproliferative properties of atRA could be used to slow remodeling and preserve function of the cardiovascular system.

Protecting the heart through delivering DNA encoding for heme oxygenase-1 into skeletal muscle

Remote delivery of HMOX-1 is cardioprotective

Remote delivery of DNA encoding for HMOX-1 was cardioprotective, evident as preserved cardiac structure and function 6 weeks after coronary artery ligation, while angiogenesis was not induced by HMOX-1 treatment. In our study overexpression of HMOX-1 was found exclusively in the transfected right quadriceps muscle, which is in accordance with previous findings with this model of gene delivery [168, 232]. Observed cardioprotective properties of HMOX-1 are supported by numerous earlier studies. Transgenic animals overexpressing HMOX-1 have remarkably increased tolerance to myocardial ischemia, evident as reduced infarct size, preserved left ventricular function and improved recovery after ischemia compared to wild type animals [233-235]. Gene deletion of HMOX-1 decreases hypoxic tolerance leading to larger infarctions and more left ventricular dilatation when exposed to prolonged hypoxia than wild types [233, 236]. Delivery of HMOX-1 using adenoassociated vector prior to induction of ischemia dramatically reduces ischemic damage of heart, liver, and skeletal muscle [236]. Modification of mesenchymal stem cells with HMOX-1 prior to transplantation into the ischemic heart enhances their survival, attenuates left ventricular remodelling, and improves the functional recovery of hearts two weeks after infarction as compared with transplantation of naive stem cells [237]. However, this is the first report of beneficial effects of remotely delivery of HMOX-1 against myocardial infarction. We were not able to find to the direct mechanisms underlying cardioprotection in present study.

Proposed mechanism of action of HMOX-1

The cardioprotective effects of HMOX-1 may be due to its downstream metabolites. We have previously shown that treating HL-1 cells with HIF-1α, HMOX-1, carbon monoxide donors, or bilirubin protects against induced injury [168]. Evidence suggests that biliverdin is potent

antioxidant, which enhances resistance to oxidative injury [239, 240]. Cellular depletion of bilirubin by RNA interference with HMOX-1 markedly increases tissue levels of reactive oxygen species resulting in apoptotic death of HeLa cells [241]. Furthermore, HMOX-1 appears to be the only endogenous source of carbon monoxide in mammalian cells, which is recognised as an intracellular signalling molecule involved in vasorelaxation and inhibition of inflammation, apoptosis, lipid peroxidation and proliferation of smooth muscle cells [240, 242-245]. Accordingly, we and others have found that use of water-soluble carbon monoxide releasing molecules promotes cardioprotection *in vitro* and *in vivo*. Cardiomyocytes exposed to CORM-3 exhibited greater tolerance to hypoxia-reoxygenation and oxidative stress, while isolated mouse hearts perfused with buffer containing CORM-3 had improved performance and reduced infarct size [246]. A recent study indicates that CO strongly activates c-kit+ stem/progenitor cells after myocardial infarction, and promotes the differentiation of c-kit+ cells into vascular smooth muscle cells and cardiomyocytes [247]. Iron is known to generate ROS, which could damage various cellular components [248, 249]. However, iron generated by HMOX-1 also induces ferritin which sequestrates iron from the cell [250250]. Ferritin was shown to be a cytoprotective antioxidant [251-253]. The intriguing mechanism of cardioprotection after remote delivery of HMOX-1 into the skeletal muscle remains to be elucidated, but it is likely to involve secretion of one or more of the discussed molecules into the circulation.

Remote delivery of HMOX-1 did not cause angiogenesis

We did not find evidence of increased vascularisation after HMOX-1 treatment in our study. HMOX-1 is considered to promote angiogenesis [254]. Overexpression of HMOX-1 in ischemic mouse hearts or in skeletal muscle after hind limb ischemia promotes neovascularization through the induction of vascular endothelial growth factor, stromal cell derived factor-1 and the recruitment of circulating progenitor cells [255, 256]. However, in these studies, either transgenic animals overexpressing HMOX-1 or viral gene delivery to cardiac muscle was used. In our study

we had overexpression of HMOX-1 only in the transfected muscle, where very few muscle fibers were transfected. Possibly the amount of HMOX-1 overexpression achieved locally in the transfected muscle was insufficient to induce angiogenesis.

Future perspectives

Gene delivery of HMOX-1 into the skeletal muscle profoundly reduced the adverse effects of induced myocardial ischemia. Timely delivery of HMOX-1 with a single injection into the skeletal muscle might protect inoperable patients with coronary artery disease from the detrimental effects of prolonged or repeated ischemia. The mechanism of protection in this experimental setting remains to be elucidated.

CONCLUSIONS

1) Coronary artery disease leads to increased expression of key enzymes governing retinoic acid metabolism.

2) AtRA is accumulated in the postinfarcted failing human heart, while 13-cis RA was decreased in hearts with dilated cardiomyopathy compared to non failing donor hearts.

3) When cardiovascular cells were stimulated with AtRA, expression of RA target genes was induced

4) AtRA reduced proliferation of mouse cardiofibroblasts and human smooth muscle cells, while it promoted proliferation of endothelial cells.

5) In a model of *in vivo* myocardial infarction in RARE-LUC reporter mice, increased luciferase signal was found in the infarcted hearts.

6) Expression of RA target genes increased concomitantly with luminescence, showing that RARE-luc activation led to transcription and translation of RARE target genes.

7) Increased expression of RA target genes coincided with accumulation of retinol in infarcted mouse hearts.

8) Cardiofibroblasts had higher RAR activation postinfarct than cardiomyocytes.

9) Gene delivery of HMOX-1 lead to a local expression of HMOX-1 in the treated muscle, but not in any other organ.

10) Remote delivery of DNA encoding for HMOX-1 was cardioprotective, evident as preserved cardiac structure and function.

11) HMOX-1 did not cause angiogenesis in the heart or skeletal muscle.

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Retinoic Acid Signalling Is Activated in the Postischemic Heart and May Influence Remodelling

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Abstract

Background: All-trans retinoic acid (atRA), an active derivative of vitamin A, regulates cell differentiation, proliferation and cardiac morphogenesis via transcriptional activation of retinoic acid receptors (RARs) acting on retinoic acid response elements (RARE).We hypothesized that the retinoic acid (RA) signalling pathway is activated in myocardial ischemia and postischemic remodelling.

Methods and Findings: Myocardial infarction was induced through ligating the left coronary artery in mice. *In vivo* cardiac activation of the RARs was measured by imaging RARE-luciferase reporter mice, and analysing expression of RAR target genes and proteins by real time RT-PCR and western blot. Endogenous retinoids in postinfarcted hearts were analysed by triple-stage liquid chromatography/tandem mass spectrometry. Cardiomyocytes (CM) and cardiofibroblasts (CF) were isolated from infarcted and sham operated RARE luciferase reporter hearts and monitored for RAR activity and expression of target genes. The effect of atRA on CF proliferation was evaluated by EdU incorporation. Myocardial infarction increased thoracic RAR activity *in vivo* ($p<0.001$), which was ascribed to the heart through *ex vivo* imaging ($p = 0.002$) with the largest signal 1 week postinfarct. This was accompanied by increased cardiac gene and protein expression of the RAR target genes retinol binding protein 1 ($p = 0.01$ for RNA, $p = 0{,}006$ for protein) and aldehyde dehydrogenase 1A2 ($p = 0.04$ for RNA, $p = 0{,}014$ for protein), while gene expression of cytochrome P450 26B1 was downregulated ($p = 0.007$). Concomitantly, retinol accumulated in the infarcted zone ($p = 0.02$). CM and CF isolated from infarcted hearts had higher luminescence than those from sham operated hearts ($p = 0.02$ and $p = 0.008$). AtRA inhibited CF proliferation *in vitro* ($p = 0.02$).

Conclusion: The RA signalling pathway is activated in postischemic hearts and may play a role in regulation of damage and repair during remodelling.

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Introduction

Retinoic acid metabolites, the active derivatives of vitamin A, are involved in tissue homeostasis in health and disease [1]. Retinoic acid (RA) orchestrates signal transduction pathways regulating embryonic development and cellular differentiation and proliferation [2]. Vitamin A is obtained from the diet as retinyl esters, or from provitamin A carotenoids as β-carotene, which are converted to retinol. Delivery of retinol to cells and its transformation into active retinoic acid metabolites is complex. It requires delivery of retinol by retinol binding proteins (RBPs), transport across the cell membrane by membrane receptor for plasma RBP (STRA6), synthesis of biologically active forms by metabolic enzymes (i.e. oxidation enzymes of the ALDH family) and delivery of metabolites to nuclear retinoic acid receptors by cellular binding proteins (CRBPs and CRABPs). Endogenous levels of RA are self-regulated by cytochrome P450 superfamily of enzymes (CYP26A1, CYP26B1 and CYP26C1), which convert all-trans RA (atRA) to hydroxylated inactive forms [3].

Heart failure is an increasing clinical challenge due to improved treatment of myocardial infarction and a steadily aging population. The process of remodelling may be initiated by myocardial injury such as infarction or pressure- or volume overload [4]. It is at first an adaptive response to maintain normal function, but when detrimental stimuli overpower adaptive capacity progressive decompensation follows. Remodelling is often associated with activation of fetal gene programs [4]. Our current understanding of the processes of remodelling and heart failure development is incomplete, and treatment regimens remain to be improved.

Vitamine A may play a role in remodelling of the heart. During early embryogenesis RA orchestrates organogenesis and formation of the heart across various species [5]. Dietary intake of alpha- and beta-carotene reduced the risk of acute myocardial infarction in a case-control study of coronary artery disease patients [6]. In adult rats, vitamin A deficiency causes left ventricular dilatation resulting in a decline of cardiac function [7]. Evidence suggests that supplementation with atRA may prevent left ventricular dilatation and preserve ventricular function in rats with induced infarction [8]. RA may oppose various hypertrophic stimuli *in vitro* and preserve a normal phenotype of cardiomyocytes [9]. Thus, atRA may be a therapeutic candidate for the prevention and therapy of cardiac hypertrophy and remodelling in postnatal life. However, the endogenous expression pattern of RA target genes in the acute phase of infarction and in long term remodelling is not well characterized.

RA exists as the derivatives atRA, 13-cis RA or 9-cis RA. AtRA exerts its actions mainly through binding to the nuclear receptor RAR (α, β, or γ), while its enantiomer 9-cis RA binds to RAR or RXR (α, β, or γ), [10]. The receptors act as ligand-dependent transcription factors, and form heterodimers binding to promoter RAR elements (RARE) [11]. The heterodimers have two functions; modulate the frequency of transcription of target genes after binding to RARE, and cross-talk with other signalling pathways. RXR is a polyvalent cooperator for various nuclear receptors such as thyroid hormone receptors, vitamin D receptors, peroxisomal proliferator-activated receptors, and several orphan receptors [5]. RAR/RXR knockout mice develop different forms of heart defects dependent on the isoform knocked out, including heart malformations, defects in the conduction system, and heart failure [12]. Overexpression of RAR or RXR induces dilated cardiomyopathy, depresses cardiac function, and causes congestive heart failure [13].

We hypothesized that myocardial infarction evokes RA signaling and that RA regulated genes may have effects on postinfarction remodelling of the heart. A mouse model of in vivo infarction, RARE-luciferase reporter mice, as well as analysis of cardiac cells and tissue were used to test the hypothesis.

Materials and Methods

Animals

All experiments were performed according to the Declaration of Helsinki, and the experiments were approved by the Norwegian Animal Research Authority. Male C57BL/6 mice (25–30 g) or male retinoic acid response element luciferase reporter mice (RARE-Luc) (www.cgene.no) of the same weight were used for experiments as detailed below. Briefly, fertilized zygotes were obtained from superovulated C57BL/6 females mated to C57BL/6 males. Linearized DNA, containing three copies of RARE derived from the RAR-β2 attached to the luciferase firefly gene was injected into pronuclei of the zygotes. Zygotes were then transferred into the oviducts of pseudopregnant mice. All the mice used in these experiments were heterozygous for the transgene and back–crossed for more than six generations from founder mice to C57BL/6 [14].

Induction of *in vivo* Myocardial Infarction

Mice were anesthetised with isoflurane and intubated, and surgery was performed as previously described [15]. Mechanical ventilation was used to maintain a respiratory rate of 135/min with pure oxygen mixed with 1.5–2.0% isoflurane, and a left-sided thoracotomy performed. The pericardium was cut open, and permanent ligation of the descending branch of the left coronary artery was made 1.5 mm under the tip of the left auricle using 8–0 silk suture. Sham operated mice underwent exactly the same procedures, except that the silk suture around the coronary artery was not tightened but rapidly withdrawn. Subsequently the intercostal space, muscles of the external thoracic wall and skin were sutured with 6/0 polyester. Extubation was performed upon spontaneous breathing. Animals were placed in a "mini intensive care unit" postoperatively, maintaining an environment of 30°C over night. All animals received 0.5 ml saline intraperitoneally prior to surgery to compensate for fluid loss and 0.1 mg/kg of buprenorphine hydrochloride (Temgesic, Schering-Plough AS) subcutaneously for analgesia. Postoperatively animals were inspected daily and Temgesic was administrated on the first postoperative day and later when animal behaviour suggested pain.

In vivo Imaging of Cardiac Retinoic Acid Signalling

To evaluate in vivo cardiac activation of the retinoic acid receptors (RAR), luciferase reporter mice were subjected to myocardial infarction as described above or sham operated ($n = 7–10$ in each group). After shaving the ventral thoracoabdominal wall, mice were anesthetized with isoflurane (1.5–2.0%) and luciferase activity was measured in vivo serially after infarct induction using luciferin (Biosynth, Basel, Switzerland) and an IVIS 100 CCD camera (Xenogen, CA, USA). Luciferin (200 µl of 20 mg/mL; Biosynth, Basel, Switzerland) was injected intraperitoneally, and the thoracic region was imaged 12 minutes later (time span decided after pilot studies). Mice were imaged prior to infarct induction or sham operation, and followed up to six weeks after infarction. Data acquisition and quantification were done with the software Living Image (Xenogen). Light emission in the thoracic region was quantified as photons/s/cm^2/sr. After the last *in vivo* imaging six weeks postinfarction, organs were harvested and imaged *ex vivo* to confirm that the signal was from the heart.

Effect of Hypoxia Factor 1α (HIF-1α) Inhibition on Retinoic Acid Signalling *in vivo*

In order to investigate if retinoic acid signalling during ischemia was dependent on HIF-1α, RARE-luc reporter mice ($n = 4$ in each group) were injected i.p.with 25 mg/kg of the HIF-1α inhibitor PX-478 (conc. 250 ug/mL in 0.9% NaCl) or vehicle alone 2 hours prior to ligation of the coronary artery. The concentration of PX-478 was chosen based on experiments performed in similar in vivo models [16,17]. PX-478 was injected daily up to 7 days postinfarction, and the RARE-luc activity was imaged daily 2 hours after administration of HIF-1α-inhibitor. Imaging of thoracic RARE luc was performed as described previously, and 7 days postinfarct hearts were extracted for ex vivo imaging. Infarct size was determined by TTC staining, and the bioluminescent signal was related to infarct size estimated in Photoshop.

Ex vivo Imaging of Cardiac Retinoic Acid Signalling

Additional RARE-luc mice were subjected to myocardial infarction or sham operation ($n = 7–8$/group) for organ imaging one week postinfarction, when the *in vivo* signal was at its strongest. Mice were anesthetized with isoflurane, injected with luciferin, and hearts, lungs, thymus, liver, spleen, pancreas and epididymal white adipose tissue were surgically removed. Organs were placed in a petridish and the luciferase signal measured and quantified in the CCD camera.

Heart Sampling for Gene Expression

In another series of experiments, C57BL/6 wild type mice were subjected to *in vivo* induced infarction or sham operation as described above, and hearts were sampled serially postinfarction (after 24 hours, 1 week, 4 weeks, and 6 weeks) for RNA extraction and amplification of RA-regulated target genes. A control group without infarction was added (n = 6 to 8 in each group at each time point).

Western Blot

Protein expression was investigated using western blot technic, as described in more detail in online supplement (Methods S3). Tissue samples from infarcted and sham operated hearts one week after induction of infarction (n = 7 of each) were homogenized in RIPA lysis buffer (Millipore, Temecula, CA, USA, supplemented with Halt^{TM} Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific), and 40 µg protein of each was separated on SDS-polyacrylamide gels (Criteron, BIoRad) and transferred onto nitrocellulose membranes (Amersham Biosciences Europe, Germany). Membranes were incubated in TBS +0.1% Tween-20, first with 5% skimmed milk powder, then over-night at 4°C with goat polyclonal anti-RBP-1 (1:2500; Cat # PAB6754, Abnova) or rabbit polyclonal anti-ALDH1A2 (1:1000; Cat # 13951-1-AP, Proteintech, Manchester, UK). HRP-conjugated secondary antibodies were used to vizualize protein bands on photographic film by chemiluminescence (ECLplus; Pierce, Rockford, IL, USA). Scanned images of exposed films and membranes stained with Ponceau solution for protein loading evaluation, were analyzed using the Image Quant software, and signal intensity of target protein bands were related to the Ponceau staining to account for protein loading and blotting efficiency.

Measurement of Retinoic Acid Metabolites

Infarcted or sham operated RARE luciferase reporter hearts used for *ex vivo* luciferase activity were also used for measuring RA metabolites 1 week postinfarction. The infarcted zone of the left ventricle was dissected from periinfarcted tissue and samples were separately snap frozen in liquid nitrogen. The samples from sham operated hearts were uses for comparison. Heart samples were homogenized in ice-cold phosphate-buffered saline with a motorized homogenizer (Pro Scientific, Inc., Oxford, CT), and retinoids were extracted with ice-cold acetonitrile containing ^{13}C-labeled atRA as an internal standard (IS). The concentration of the IS was 10 ng/mL. An aliquot was injected into a 4000 Q TRAP LC-MS/MS instrument with APCI ionization (Applied Biosystems, California). The liquid chromatography-mass spectrometry conditions were as described previously [18], except that the separating column was an ABZ Plus (75 by 3 mm [inner diameter], 3-µm particles; Supelco, Pennsylvania). The entire procedure was performed under red light. Calibration graphs were constructed by linear least-squares regression analysis, plotting peak area ratios of the analyte concentration and the IS against the corresponding concentrations. Quantification was carried out by interpolation and linear least-squares regression [18].

Isolation and Culture of Adult Mouse Cardiomyocytes and Cardiofibroblasts

Adult mouse cardiomyocytes and cardiofibroblasts were isolated from hearts from 5 infarcted and 5 sham operated RARE-luc reporter mice using the method described by [19]. Cells were isolated for evaluation of RARE reporter gene activity on the 7th postoperative day as described in more detail in online supplement (Methods S1). The infarcted and periinfarcted zone of the left ventricle were dissected and used for cell isolation in parallel with extracting cells from sham operated hearts. Hearts were perfused with digestion buffer containing Collagenase II (Worthington Biochemical, Lakewood, NJ), and mechanically disrupted. Cardiomyocytes were separated from cardiofibroblasts by serial centrifugations [19]. Cardiofibroblasts were collected from the first centrifugation, transferred to a separate tube, resuspended, and plated [20]. Cardiomyocytes were resuspended laminin (BD- biosciences) coated six-well plates (see online supplement). The cells were incubated for 3 hours at 37°C in an atmosphere supplied with 2 or 5% CO_2, before RARE reporter gene activity was measured using the same CCD camera as for *in vivo/ex vivo* imaging after adding luciferin (100 µL of 20 mg/mL). Finally, cells were harvested for mRNA isolation and stored at –80°C.

RNA Extraction and Real-time q-PCR

RNA was extracted using RNeasy Mini Kit (QIAGEN inc.) with an additional phenol-chloroform extraction step and in-column DNase treatment (QIAGEN). Random hexamers for priming (3 min at 70°C) were used for reverse transcription of 1 µg of RNA from whole heart extracts and 200 ng of RNA from isolated cardiomyocytes and cardiac fibroblasts. Reverse transcription was followed by a modified First Strand cDNA Synthesis Protocol with Superscript III (Invitrogen) and RNasin (Promega) enzymes and cDNAs were amplified using real-time PCR. Primers for RAR target genes and endogenous control rpl32 were designed with Primer Express 3.0 software and are shown in online supplement (Table S1). SYBR green (Applied Biosystems, Foster City, CA, USA) was used for detection. A predesigned Real-Time PCR primer pair and probe (TaqMan Gene Expression Assays, Applied Biosystems) for detection of 18S rRNA was used as endogenous control for whole heart extracts, whereas rpl32 was used as endogenous control for cardiomyocytes and cardiofibroblasts after performing pilot studies on endogenous control suitability. For details of PCR reaction, please see supplementary material online (Methods S2).

In vitro Evaluation of atRA Effects on Cardiofibroblast Proliferation

Cardiofibroblasts were isolated from 4 healthy C57BL/6 mouse hearts. Cells were incubated up to 96 h in medium supplemented with 1 µM atRA dissolved in ethanol (Sigma Aldrich; St. Louis, USA) and 10 µM EdU (5-ethynyl-2′-deoxyuridine; nucleoside analogue to thymidine which is incorporated into DNA during active DNA synthesis). atRA and EdU supplemented medium was changed every 24 h. Control experiments were performed using ethanol only. Cells were harvested for proliferation evaluation by EdU incorporation by flow cytometry as described in more detail in the online supplement (Methods S4).

Statistics

Repeated measurements of luminescence activity postoperatively in the thoracic region were evaluated using repeated measures ANOVA. The non-parametric Mann–Whitney *U* test was used to compare gene expression data, luminescence between explanted organs and isolated cells, where a non-Gaussian distribution was assumed. Data are presented as mean ± SD. Differences were considered significant when $P<0.05$ and a tendency was regarded when P was 0.05–0.08.

Results

Thoracic Retinoic Acid Signalling is Activated Early after Myocardial Infarction

RAR luciferase reporter mice were used to determine if RA signalling measured as luminescence was activated in the heart after induced infarction through *in vivo* imaging. The RAR reporter activity was increased in the thoracic region of infarcted animals, indicating activation in the heart. Infarction-induced thoracic RAR activity reached the highest level at the end of the first postoperative week and gradually declined, but remained higher than sham throughout the six weeks observation period (Fig. 1).

Retinoic Acid Signalling is Independent of Hypoxia-inducible Factor 1α

RARE-luc activity was measured and quantified in RARE luc reporter mice treated daily with the HIF-1α inhibitor PX-478 or saline in conjunction with infarct induction. No differences were found between groups during 1 week observation (Figure S1).

Retinoic Acid Signalling was Increased Specifically in the Infarcted Heart

Since *in vivo* signalling in the thoracic region could be of non-cardiac origin, selected organs were harvested and imaged in the CCD camera to verify the source of signal. One week postinfarction, when the *in vivo* signalling peaked, the RAR reporter activity was higher in infarcted hearts than in sham operated hearts (Fig. 2). There were no differences in luminescence between groups in any of the other imaged organs (Fig. 2). RAR reporter activity remained higher in infarcted hearts than in sham operated hearts six weeks postinfarct (results not shown).

Increased *in vivo* Expression of RA Target Genes

Other mice were subjected to in vivo infarction or sham operation for sampling of hearts for RNA extraction and amplification with real time PCR 24 hours, 1, 4 and 6 weeks postinfarct. Expression of RBP1 mRNA was increased one week postinfarction (Fig. 3a). ALDH1A2 was increased compared with sham 24 hours and 1 week postinfarction (Fig. 3b). CYP26B1 tended to be downregulated early post infarction and 4 weeks later (Fig. 3c). STRA6, CRABP1, CRABP2 and RAR α,β,γ, were unchanged (data not shown).

Increased Expression of RA Transporting and Metabolising Proteins

To investigate whether retinoic acid transporting and metabolizing proteins are induced after myocardial infarction, we extracted proteins from infarcted and sham operated hearts one week after coronary artery ligation for western blotting with antibodies against RBP1 and ALDH1A2. Protein expression of RBP1 and ALDH1A2 were significantly upregulated in infarcted hearts one week after infarct, but not in sham operated hearts (Fig. 3d).

The Infarcted Myocardium Accumulates Retinol

Investigation of endogenous RA metabolite concentrations in infarcted and sham operated RARE-luc hearts one week later by triple-stage liquid chromatography/tandem mass spectrometry showed accumulation of retinol in infarcted hearts. Increased levels of retinol were found in the infarcted tissue itself and not in the periinfarct zone or in sham operated hearts (Fig. 3e). Retinal, 13-cis RA or atRA were unchanged (data not shown).

Cardiofibroblasts are the Major Source of Luminescence in the Infarcted Heart

Cardiomyocytes and cardiofibroblasts were isolated from the infarcted area and the periinfarct zone of infarcted hearts, or from sham operated RARE-luc reporter hearts. Luciferase activity was measured after plating of cells. Fibroblasts isolated from the infarcted zone of infarcted hearts had increased luminescence compared with fibroblasts from sham operated hearts (Fig. 4). Luminescence was similar in cells harvested from the periinfarcted zone and the infarct zone. Hardly any luciferase activity was detected in cardiomyocytes of sham

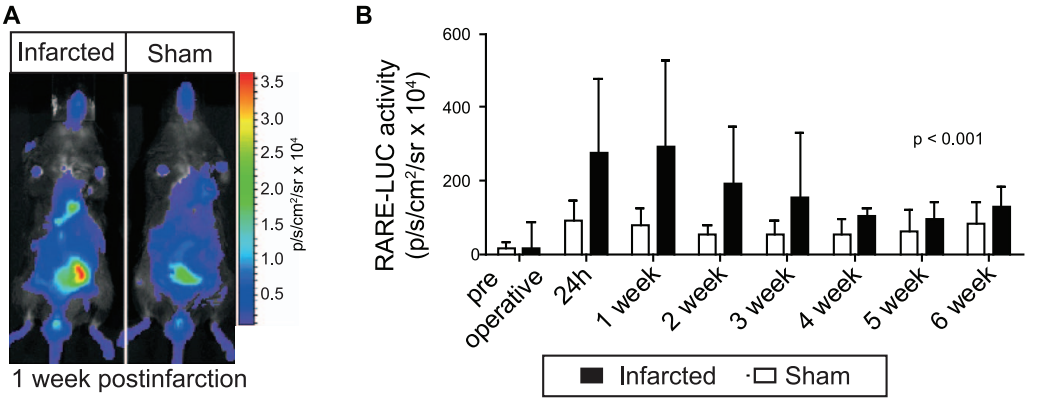

Figure 1. *In vivo* imaging of cardiac retinoic acid signalling after myocardial infarction. To evaluate *in vivo* cardiac activation of the retinoic acid receptor RAR, RARE-luciferase reporter mice were subjected to myocardial infarction through permanent coronary artery ligation or sham operation (n = 7–10 of each). *In vivo* luciferase activity was measured in a CCD camera after injection of luciferin preoperatively and serially after infarction. A representative image of an infarcted and a sham-operated mouse is shown on the left (**A**). On the right, the digitized luciferase signal is quantified and shown as bar graphs (**B**; mean±SD).
doi:10.1371/journal.pone.0044740.g001

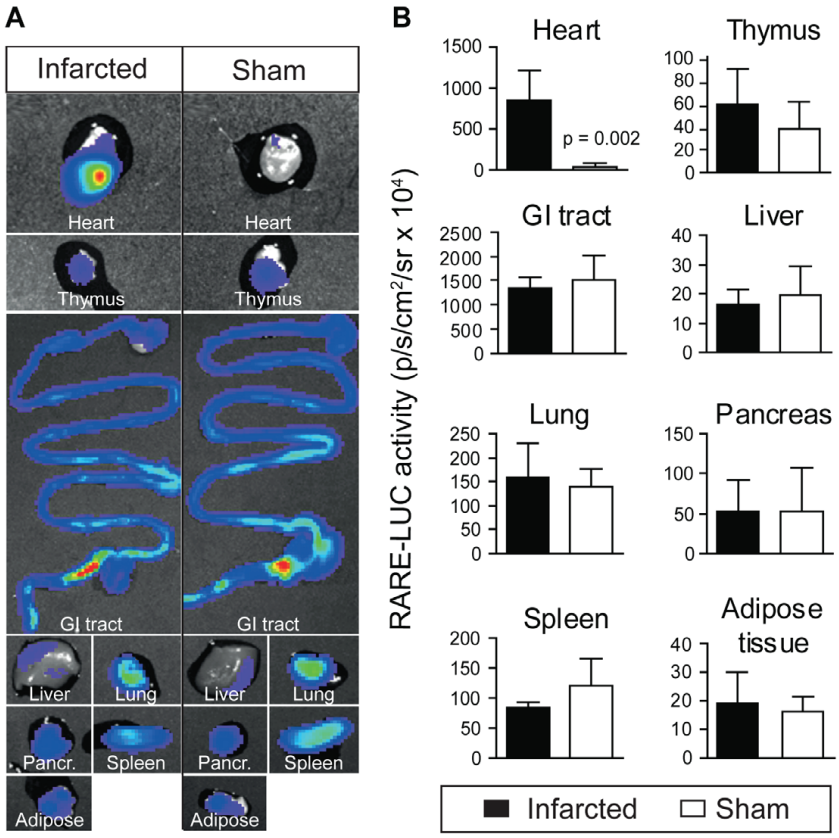

Figure 2. ***Ex vivo*** **imaging of cardiac retinoic acid signalling after myocardial infarction.** To verify that the signal in the thoracic region detected *in vivo* originated from hearts, organs were harvested one week after infarction or sham operation following in vivo luciferin injection in RARE-luciferase reporter mice. Organs were placed in a petridish and the luminescence signal quantified. A representative image of one single experiment is shown on the left (**A**), where hearts, thymus, gastrointestinal tract (GI-tract), liver, lungs, pancreas, spleen and epididymal white adipose tissue from one infarcted and one sham operated animal are shown. When the signal was quantified from 6 independent experiments, the luminescence was exclusively increased in infarcted hearts, and not in sham operated hearts or any other organs (**B**). Data are shown as mean±SD.
doi:10.1371/journal.pone.0044740.g002

operated mice, while the signal intensity increased after infarction with no differences between infarcted and periinfarcted cells (Fig. 4).

Gene Expression of RA Target Genes in Infarct Zone is Higher in Cardiofibroblasts than in Cardiomyocytes

The cells isolated from the infarct zone of hearts used for RARE-luc activation were used for RNA extraction and amplification of RA target genes through real time PCR. The CT value of the endogenous control rpl32 was similar in both cell types. In accordance with increased luciferase activity in cardiofibroblasts, gene expression of RBP1, CYP26B1, ALDH1A2, RARα and RARγ was higher in cardiofibroblasts than in cardiomyocytes. RARβ was the only examined gene which was more highly expressed in cardiomyocytes (Fig. 5a).

In vitro Evaluation of atRA Effects on Cardiofibroblast Proliferation

Incubation of cardiofibroblasts with 1 μM atRA inhibited cell proliferation in vitro when EdU incorporation was evaluated 96 hours later by flow cytometry (Fig. 5b).

Discussion

The main finding of the present study was that the RA signalling pathway was activated in the mouse heart with permanent coronary artery ligation. Using a reporter mouse with firefly luciferase coupled to RA response element, a dramatic increase of luciferase signal was found *in vivo* in the thoracic region of mice with induced infarction, peaking the first postoperative week. The signal was verified to originate exclusively from the heart by *ex vivo* organ imaging, with maximal signal emission in the infarcted zone. This was accompanied by increased cardiac expression of genes and proteins involved in regulation of RA

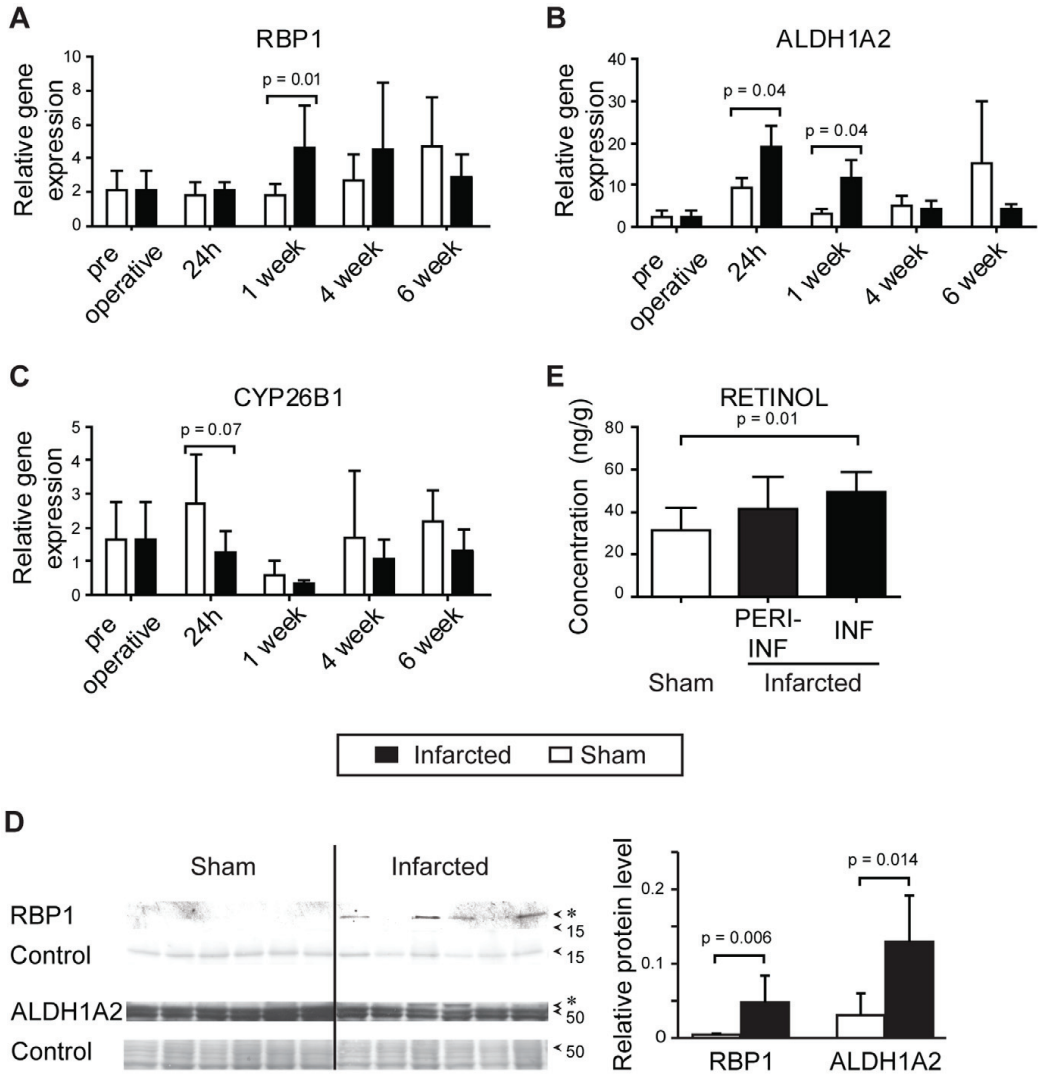

Figure 3. Expression of retinoic acid target genes and endogenous metabolites after infarction. Wild type C57Bl6 mice were subjected to *in vivo* ligation of the left coronary artery or sham surgery, and hearts were sampled serially for RNA extraction and amplification of retinoic acid target genes with real time PCR, or proteins were extracted for western blotting. Hearts were also sampled from mice without any surgery (preoperative). Gene expression of retinol binding protein 1 (RBP1) (**A**), aldehyde dehydrogenase 1A2 (ALDH1A2) (**B**), and cytochrome p45026B1 (CYP26B1) are shown (**C**). (n = 6–8 in each group at each time point). **D)** Representative western blot analysis of retinoic acid transporting and metabolizing proteins one week after induction of infarction or sham operation. Ponceau solution was used as protein loading (control). Histograms show the relative density of RBP1 at 16 kDa and ALDH1A2 at 53–57 kDa in infarcted and sham operated hearts. **E)** Endogenous retinoic acid metabolite concentrations were evaluated by triple-stage liquid chromatography/tandem mass spectrometry one week after induction of myocardial infarction. The infarcted zone of the left ventricle (INF), the periinfarcted zone (PERIINF) and left ventricles from sham operated hearts (SHAM) were investigated (n = 6). Data are shown as mean±SD.
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metabolism RBP1 and ALDH1A2, while CYP26B1 mRNA, an endogenous enzyme responsible for atRA degradation, was downregulated. Increased gene and protein expression of retinol transporting proteins coincided with accumulation of retinol in infarcted hearts. Cardiofibroblasts and cardiomyocytes had RARE-Luc activation postinfarct, and increased fibroblast expres-

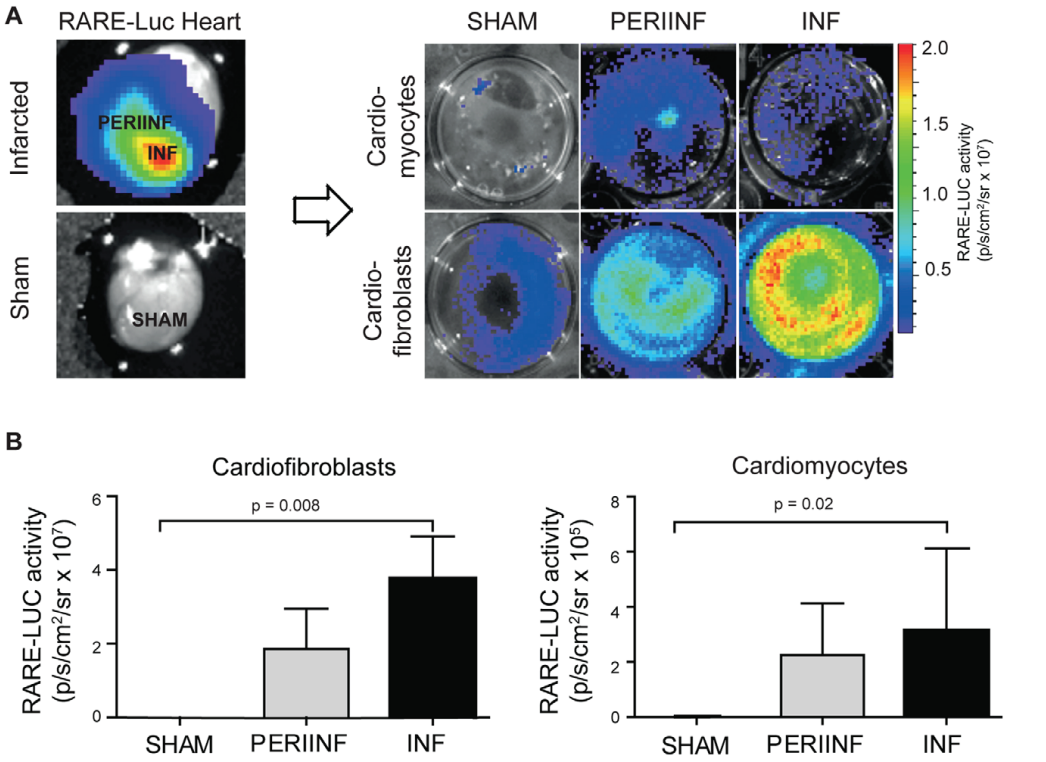

Figure 4. ***In vitro*** **imaging of retinoic acid signaling after myocardial infarction.** Cardiomyocytes and cardiofibroblasts were isolated from left ventricular tissue from RARE-luciferase reporter hearts one week after myocardial infarction or sham operation (SHAM). Cells were isolated from the infarct (INF) and periinfarct zone (PERIINF). After plating for three hours, non-viable cells were removed and luciferin was added for imaging. The upper panel shows a representative image of one experiment (**A**). The lower panel shows mean ± SD of n = 5 experiments in each group (**B**). Note that the Y-axis labelling is different.
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sion of the RA target genes RBP1, ALDH1A2, CYP26B1, RARα and RARγ, while RARβ was more highly expressed in cardiomyocytes. AtRA inhibited proliferation of cardiofibroblasts *in vitro*. Anti-proliferative effects of atRA in cardiac fibroblasts is clinically appealing, as dietary RA supplementation is easy to perform in humans with myocardial infarction. The antiproliferative properties of atRA are already used in the clinics as treatment of different leukemias.

To our knowledge, no previous studies have reported activation of retinoic acid signalling in the heart in conjunction with myocardial ischemia and remodelling. It seems to be independent of hypoxia-inducible factor 1 alpha (Fig. S1). The increased RARE-luc activity as well as increased expression of retinol transporting genes and proteins could lead to cardiac accumulation of retinoic acid postinfarction. This hypothesis was supported by the finding of altered content of retinol in postinfarcted hearts in the current study. Our findings concur with one previous study investigating RA content in postischemic hearts. Palace and coworkers injected postinfarcted rats with radiolabelled vitamin A. Radiolabelled vitamin A was increased in hearts and plasma of rats with myocardial infarction, while labelled kidney and liver retinol was lower in those animals. The authors speculated that it would be beneficial for an ischemic heart to increase antioxidant content. However, accumulation of exogenously administrated atRA does not necessarily reflect the role of endogenous atRA postinfarction.

Activation of RARs in the post ischemic heart may play a role in regulation of damage and repair. We did not address the role of retinoic acid *in vivo* in the present study. This has previously been done by others: Rats with tissue insufficiency of vitamin A had spontaneous cardiac remodelling and ventricular dysfunction [7]. When myocardial infarction was induced in rats with tissue vitamin A deficiency, adverse left ventricular remodelling was intensified [21]. Supplementing rats with retinoic acid in a model of tobacco smoke-induced left ventricular remodelling could prevent remodelling [22]. Retinoic acid supplementation could also prevent remodelling induced by left coronary artery ligation [8].

The heart consists of several cell types, where cardiofibroblasts and cardiomyocytes are the most abundant. To explore the relation between cell types and RAR response, cardiomyocytes and cardiofibroblasts were isolated from infarcted hearts. Interestingly, cardiofibroblasts isolated from the infarcted or periinfarcted zones had increased luminescence compared to fibroblasts from sham

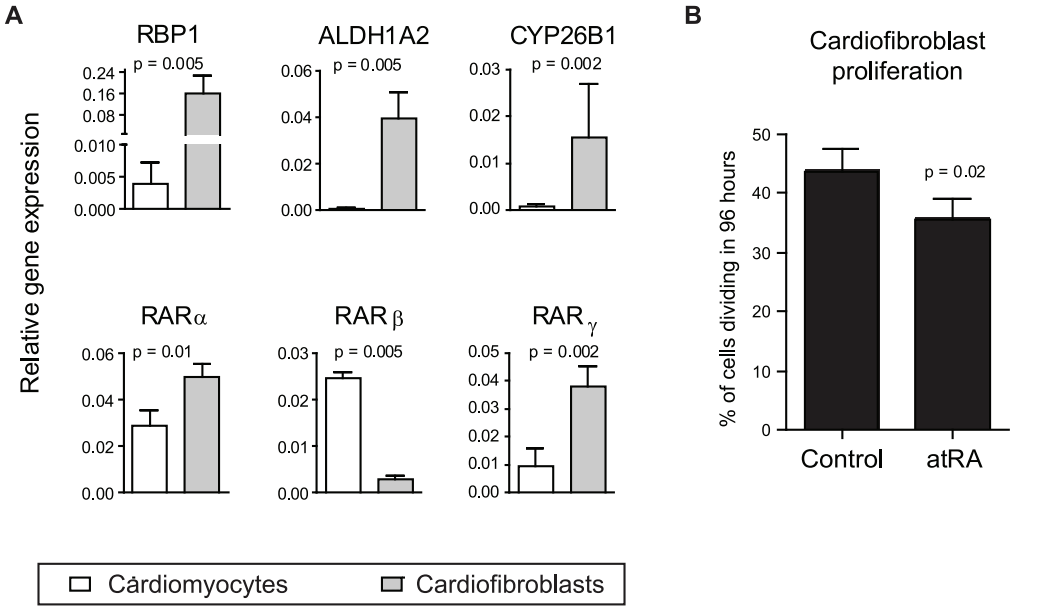

Figure 5. Expression of retinoic acid target genes in the infarct zone and effects on cardiofibroblast proliferation. A) Gene expression of retinoic acid target genes in cardiofibroblast (CF) and cardiomyocytes (CM) isolated from left ventricles of infarcted RAR-luciferase reporter hearts 1 week after infarction. RNA was extracted from cells from the infarcted zone and amplified with real time PCR using primers specific for retinol binding protein 1 (RBP1), cytochrome P450 26B1 (CYP26B1), aldehyde dehydrogenase 1A2 (ALDH1A2), and retinoic acid receptors alpha, beta and gamma (RAR α,β,γ). The figure shows test gene expression relative to expression of rpl32, which was similar in both cell types. Data are mean±SD of n = 5 experiments in each group. **B)** Cardiofibroblasts were isolated from C57BL6 hearts and cultured in medium supplemented with 1 μM all-trans retinoic acid (atRA) and 10 μM EdU, as indicator of cell division. After 96 hours, EdU incorporation was evaluated by flow cytometry. Data are presented as mean±SD of n = 4 in each group.
doi:10.1371/journal.pone.0044740.g005

operated hearts. RAR luciferase activity increases also in cardiomyocytes from infarcted and periinfarcted tissue. Fibroblasts had a higher increase of RA target genes than myocytes. Unlike the cardiomyocytes, which have low stem cell potential, cardiofibroblasts are actively dividing and differentiating.

Cardiofibroblasts stimulated with atRA had reduced cell proliferation, supporting a potential beneficial role of atRA during remodelling. Others have studied the effect of atRA on proliferation of neonatal cardiac cells: The hypertrophic response evoked by cyclic stretching of cardiomyocytes was inhibited by atRA [23]. Wu and collaborators used atRA to counteract hypertrophic responses to endothelin in neonatal cardiomyocytes [24]. In neonatal cardiofibroblasts, Wang and co-workers found that atRA dose-dependently reduced angiotensin-induced hyperplasia, and reduced the total cell protein content [9]. We confirm that anti-proliferative effects of atRA applies to adult cardiofibroblasts *in vitro*.

Conclusions

All-*trans* retinoic acid (atRA) is used in therapy and prevention of many proliferative diseases such as prostate cancer or acute promyelocytic leukemia [25]. Because of their important role as regulators of cellular growth, differentiation, morphogenesis and metabolism, retinoic acid metabolites may also be of use to modify the proliferative response of cardiac cells in the process of remodelling. However, their mechanism of action and potential use as an anti-remodelling intervention in humans remain to be investigated.

Supporting Information

Figure S1 Retinoic acid signalling is independent of hypoxia-inducible factor 1α (HIF-1α). RARE-luc activity in thorax was measured and quantified in mice treated daily with the HIF-1α inhibitor PX-478 or saline in conjunction with infarct induction. No differences were found between groups. Data are mean values of n = 4 in each group.
(EPS)

Table S1 Primer sequences of the investigated genes.
(XLS)

Methods S1 Isolation and culture of adult mouse cardiomyocytes and cardiofibroblasts.
(DOCX)

Methods S2 Evaluation of RA target genes expression using Real-time polymerase chain reaction.
(DOCX)

Methods S3 Evaluation of RA transporting and metabolizing proteins expression using western blot.
(DOCX)

Methods S4 *In vitro* evaluation of atRA effects on cardiofibroblast proliferation.

(DOCX)

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Author Contributions

Conceived and designed the experiments: DB GV. Performed the experiments: DB FH JS NB. Analyzed the data: DB FH GV. Contributed reagents/materials/analysis tools: DB FOL AS RB GV. Wrote the paper: DB. Contributed with theoretical discussion: DB FH AB FOL AS RB GV.

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