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High expression of arachidonate 15-lipoxygenase and proinflammatory markers in human ischemic heart tissue

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ABSTRACT

A common feature of the ischemic heart and atherosclerotic plaques is the presence of hypoxia (insufficient levels of oxygen in the tissue). Hypoxia has pronounced effects on almost every aspect of cell physiology, and the nuclear transcription factor hypoxia inducible factor-1α (HIF-1α) regulates adaptive responses to low concentrations of oxygen in mammalian cells. In our recent work, we observed that hypoxia increases the proinflammatory enzyme arachidonate 15-lipoxygenase (ALOX15B) in human carotid plaques. ALOX15 has recently been shown to be present in the human myocardium, but the effect of ischemia on its expression has not been investigated. Here we test the hypothesis that ischemia of the heart leads to increased expression of ALOX15, and found an almost 2-fold increase in HIF-1α mRNA expression and a 17-fold upregulation of ALOX15 mRNA expression in the ischemic heart biopsies from patients undergoing coronary bypass surgery compared with non ischemic heart tissue. To investigate the effect of low oxygen concentration on ALOX15 we incubated human vascular muscle cells in hypoxia and showed that expression of ALOX15 increased 22-fold compared with cells incubated in normoxic conditions. We also observed increased mRNA levels of proinflammatory markers in ischemic heart tissue compared with non-ischemic controls. In summary, we demonstrate increased ALOX15 in human ischemic heart biopsies. Furthermore we demonstrate that hypoxia increases ALOX15 in human muscle cells. Our results yield important insights into the underlying association between hypoxia and inflammation in the human ischemic heart disease.

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1. Introduction

A common feature of the ischemic heart and atherosclerotic plaques is the presence of hypoxia (insufficient levels of oxygen in the tissue). Hypoxia has pronounced effects on almost every aspect of cell physiology [1], and the nuclear transcription factor hypoxia inducible factor-1α (HIF-1α) regulates adaptive responses to low concentrations of oxygen in mammalian cells [2,3]. Recent evidence suggests that there is significant crosstalk between HIF-1α and the proinflammatory transcription factor nuclear factor-kappa B (NF-κB) [4,5], and that NF-κB may regulate hypoxia-mediated inflammatory responses [5,6].

In our recent work, we observed that HIF-1α co-localizes with the proinflammatory enzyme arachidonate 15-lipoxygenase (ALOX15) in human carotid plaques [7]. ALOX15 type 2 is expressed at high levels in human carotid plaque macrophages [7,8] and levels have been shown to be higher in symptomatic compared with asymptomatic lesions [8]. ALOX15 induces production of the reactive signaling molecule 15-hydroperoxytetraenoic acid (15-HPETE), a peroxidized lipid that specifically phosphorylates threonine/serine kinases [9]. Increased levels of peroxidized lipids are tightly connected with complex inflammatory diseases, such as atherosclerosis and type II diabetes [10]. ALOX15 also catalyzes production of 15-hydroxyeicosatetraenoic acid (HETE). HETE enhances the adhesion of leukocytes to endothelium by activating chemokine production which is an early event in development of atherosclerosis [11].

ALOX15 has recently been shown to be present in the human myocardium [12], but the effect of ischemia on its expression has not been investigated. Here we test the hypothesis that ischemia of the heart leads to increased expression of ALOX15 and promotes increased inflammation. We study expression of ALOX15 and its

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association to expression of HIF-1α and inflammation in ischemic and non-ischemic human heart tissue. We also use human aortic smooth muscle cells as a model to directly study the effects of hypoxia on human muscle cells.

2. Materials and methods

2.1. Human heart biopsies

Endomyocardial biopsies from the right atrium were obtained from five patients undergoing coronary bypass surgery (at the Sahlgrenska University Hospital, Gothenburg, Sweden). Samples were frozen immediately on dry ice and stored at −70 °C until analysis. The study protocol was approved by the Ethical Committee of the University of Gothenburg and all subjects gave written informed consent.

For control samples, we purchased total RNA from right atrium from normal tissue from three human adults (Invitrogen and BioChain, CA, USA).

2.2. Quantitative PCR (Q-PCR)

RNA was isolated from myocardial biopsies with the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized with the RT^2 First Strand Kit (C-03, SuperArray, SABiosciences). The RT reactions were performed with a Gene Amp PCR system 9700 (Applied Biosystems). Expression of ALOX15 mRNA was determined by real-time Q-PCR with a TaqMan Q-PCR kit (Applied Biosystems) and normalized to actin mRNA expression. Oligonucleotide primers and probes were designed with Primer Express 1.5 software and purchased from Applied Biosystems. Probes were 5′-labeled with 5-carboxyfluorescein (FAM) and 3′-labeled with tetramethylrhodamine (TAMRA).

Real time PCR amplification was set up using Taq man gene expression assays for ALOX15 (Hs00609608_m1), HIF-1α (Hs00153153_m1) and ActB (Hs99999903_m1) in combination with Universal PCR master mix (#4324018).

For gene expression analyses of inflammatory genes, the human Inflammasomes PCR Array (PAHS-097A, Qiagen) was used. All PCR amplification was performed for 40 cycles on an ABI PRISM 7700 sequence detection system (Applied Biosystems). Q-PCR data were analyzed using the comparative CT-method [13].

2.3. Global transcription analysis

Microarray gene expression data from a recently published study [12] were reanalyzed with respect to ALOX15. Affymetrix WT Gene ST1.o arrays were used to measure the transcription. This microarray dataset consists of samples of endomyocardial biopsies taken from the right atrium from patients who underwent aortic valve replacement ($n = 3$) or coronary bypass grafting (CABG, $n = 2$), as earlier described [12]. CABG samples are considered ischemic and aortic valve replacement samples are considered non-ischemic.

2.4. Aortic smooth muscle cells

Primary human aortic smooth muscle cells (Clonetics, Lonza, Basel, Switzerland) were cultured in Waymouth's medium (Gibco, Invitrogen, Carlsbad, CA, USA) with 10% human serum, 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L l-glutamine. The cells were incubated under either hypoxic (1% O_2) or normoxic (21% O_2) conditions for 24 h before extraction of RNA.

2.5. Statistics

Data are plotted as mean and SEM unless stated otherwise. Differences between groups were determined with Student's t test or one-way ANOVA using GraphPad Prism version 5.01, GraphPad Software, San Diego California USA, www.graphpad.com. P values < 0.05 (two-sided) were considered statistically significant.

3. Results

3.1. High expression of ALOX15 in human ischemic myocardium and hypoxic human vascular muscle cells

We observed an almost 2-fold increase in HIF-1α mRNA expression and a striking 17-fold upregulation of ALOX15 mRNA expression in the ischemic heart biopsies from patients undergoing coronary bypass surgery compared with non ischemic heart tissue (Fig. 1A, B). We also showed that ALOX15 expression was higher in heart biopsies taken from patients undergoing CABG (considered to be ischemic) compared with biopsies from patients undergoing aortic valve replacement (considered to be non-ischemic) (Fig 1C). To determine whether hypoxia is the driving force in ALOX15 production, we incubated human vascular smooth muscle cells in 1% oxygen for 24 h and showed that expression of ALOX15 mRNA increased 22-fold compared with cells incubated in normoxic conditions (Fig. 1D).

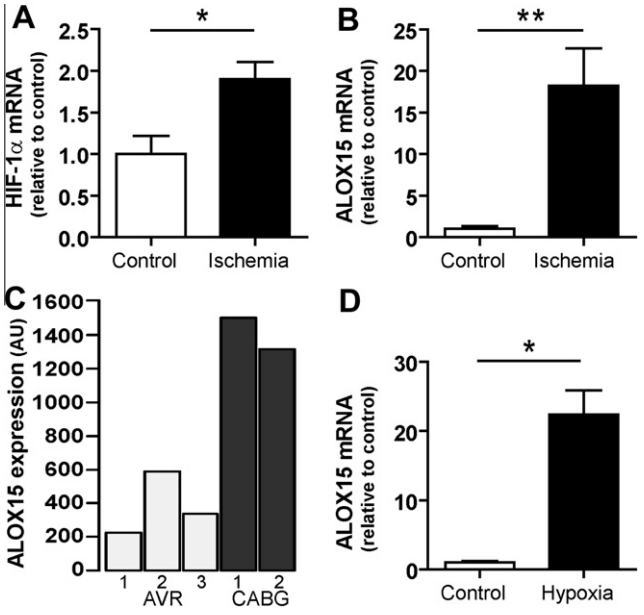

Fig. 1. Increased expression of ALOX15 in human ischemic heart biopsies. (A) HIF-1α expression is increased in human ischemic heart biopsies. Total cellular RNA was extracted from human heart biopsies and HIF–1α mRNA and α-actin mRNA levels were measured by Q-PCR. HIF–1α mRNA expression in myocardial biopsies from non-ischemic (Control) ($n = 3$) and in ischemic myocardium (Ischemia) ($n = 5$). $^*P < 0.05$. (B) Total cellular RNA was extracted from human heart biopsies, and ALOX15 mRNA and α-actin mRNA levels were measured by Q-PCR. ALOX15 mRNA expression in myocardial biopsies from non-ischemic (Control) ($n = 3$) and in ischemic myocardium (Ischemia) ($n = 5$) was normalized to α-actin. $^{**}P < 0.01$. Data are expressed as mean ± SEM. (C) Microarray data on ALOX15 mRNA expression in myocardial biopsies taken from the right atrium from patients who underwent aortic valve replacement ($n = 3$) and coronary bypass grafting (CABG) ($n = 2$). Data are expressed as arbitrary units of ALOX15 mRNA. (D) ALOX15 expression is increased by hypoxia. Primary human vascular smooth muscle cells were incubated under normoxic (21% oxygen) or hypoxic conditions (1% oxygen) for 24 h. Total cellular RNA was extracted, and ALOX15 and α-actin mRNA levels were measured by Q-PCR. Expression of ALOX15 was normalized to actin levels in cells incubated at normoxia (Control) and Hypoxia ($n = 4$). $^{**}P < 0.01$. Data are expressed as mean ± SEM.

These data suggest that ALOX15 expression is induced by low concentrations of oxygen both in human myocardium and vascular muscle cells.

3.2. Identification of ischemia-induced proinflammatory molecules

To identify which inflammatory markers are expressed in the ischemic heart, we performed Q-PCR array analysis on the ischemic heart biopsies from patients undergoing coronary bypass surgery compared with non ischemic controls. We found that ischemic heart tissue contained higher mRNA levels of TNF-α compared with non-ischemic controls (Fig. 2A). Furthermore we observed higher mRNA levels of IFN-β1, IL-1β, IL-18, and IL-33 and reduced levels of IFN-γ, IL-6, IL-12A and IL-12B (Fig. 2B). We also assessed expression of chemokines important for inflammation and recruitment of leukocytes to the inflammatory tissue. CCL2, CCL5 and CXCL1 were increased in ischemic heart while reduced expression of CCL7 and CXCL2 mRNA was seen (Fig. 2C). Cathepsin B is a lysosomal protease and plays an important role in heart failure. Given that 12/15-lipoxygenase metabolites stimulates protein kinase C-mediated release of cathepsin B from malignant cells we assessed if cathepsin B was increased in ischemic myocardium. Our data show increased catehepsin B expression in the ischemic heart tissue compared with non-ischemic heart (Fig. 2D).

The Toll-interleukin 1 receptor domain-containing adapter protein (TIRAP) and receptor interacting protein-2 (RIP-2) were significantly increased in human ischemic heart tissue compared with non-ischemic controls (Fig. 3A, B). In contrast, the adaptor protein MyD88 expression was unaffected by ischemia and other markers did not change (data not shown). We argued that the increased expression of RIP-2 might increase p38 mitogen-activated protein kinase (MAPK) and other pathways downstream of RIP-2. To test this, we measured different mitogen-activated protein kinases (MAPK) in heart tissue. MAP3K7, MAP3K7IP2 and p38MAPK1 were upregulated in the ischemic heart (Fig. 3C). In contrast, MAP3K7IP1 and p38bMAPK11 expression were downregulated (Fig. 3C).

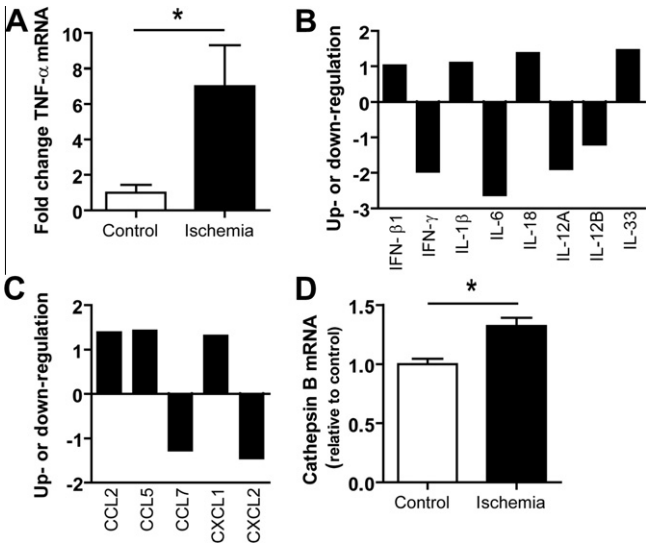

Fig. 2. Increased expression of inflammatory markers in human ischemic heart biopsies. Total cellular RNA was extracted from human heart biopsies and mRNA levels were measured by Q-PCR. (A) TNF-α mRNA expression was higher in myocardial biopsies from ischemic myocardium. Bars indicate means ± SEM. (B) Q-PCR showing the relative expression of cytokines in human heart biopsies. Expression of cytokines in ischemic myocardial biopsies is shown as fold change compared with non ischemic control. (C) Q-PCR showing the relative change in chemokines involved in inflammation and cell adhesion. Expression of chemokines in ischemic myocardial biopsies is shown as fold change compared with non ischemic control. (D) Cathepsin B mRNA expression was higher in myocardial biopsies from ischemic myocardium. Bars indicate means ± SEM. mRNA is analyzed in myocardial biopsies from Control ($n = 3$) and in ischemic biopsies ($n = 5$). $^*P < 0.05$.

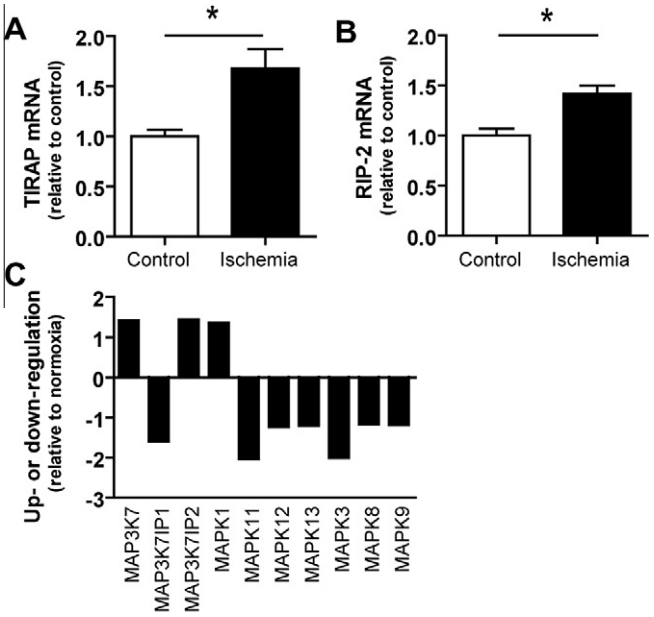

Fig. 3. Increased expression of inflammasomes in human ischemic heart biopsies. Total cellular RNA was extracted from human heart biopsies and mRNA levels were measured by Q-PCR in myocardial biopsies. (A) Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP) was increased in human ischemic heart biopsies compared with non-ischemic control. (B) Expression of receptor-interacting protein (RIP) family of serine/threonine protein kinases (RIP-2) was upregulated in ischemic heart. (C) Q-PCR showing the relative change in expression in Ischemic/Control mRNA in human biopsies of different mitogen-activated protein kinases involved in ischemia and inflammation. Data from Q-PCR showing the relative change in expression in myocardial biopsies from non-ischemic (Control) ($n = 3$) and in ischemic myocardium (Ischemia) ($n = 5$).$^*P < 0.05$.

4. Discussion

In this study, we tested the hypothesis that ALOX15 is upregulated in the human heart during ischemia, which potentially leads to increased expression of inflammatory markers. We found that ALOX15 expression was upregulated in the human ischemic myocardium compared with non-ischemic myocardial biopsies, and that ALOX15 expression was induced by hypoxia in human aortic smooth muscle cells. We also observed increased mRNA levels of the proinflammatory markers TNF-α, IL-1β, IL-18, IL-33, and chemokines such as CCL2 and CCL5 in ischemic heart tissue compared with non-ischemic controls.

Long-term expression of active HIF-1α leads to degenerative changes in the cardiomyocytes with myocyte dropout, loss of contractility, myocardial thinning and left ventricle dilatation [14]. Expression of active HIF-1α is sufficient to cause cardiomyopathy in a mouse model [14]. Thus, several HIF-1α-dependent mechanisms play a role in the pathogenesis of ischemic heart disease. Our results show increased levels of HIF-1α in ischemic heart biopsies and increased ALOX15 expression, suggesting a link between ischemia and ALOX15 in ischemic heart biopsies.

Our data show increased cathepsin B expression in the ischemic heart tissue compared with non-ischemic heart. Increased myocardial expression of cathepsin B is found in patients with heart failure indicating that cathepsin B plays a role in the genesis and development of heart failure [15]. Cathepsin B is released from malignant cells after stimulation with the 12/15-lipoxygenase enzyme products [16]. Results show that cathepsin B release is an

early event following artery occlusion and may play a role in promoting inflammatory responses and apoptotic processes [17].

Chemokine-mediated signals are important for vascular inflammation and myocardial infarction, and treatment with anti-CCL5 mAb significantly reduced both infarct size and post-infarction heart failure in a mouse model of chronic cardiac ischemia [18]. Cardioprotective effects of anti-CCL5 mAb are associated with reduction of neutrophil and macrophage infiltration within the infarcted myocardium [18]. Our previous results show that ALOX15 overexpression in macrophages induces CCL2 and CXCL10 as well as increased activation and migration of T cells [19], and inhibition of CCL2 and chemokine receptors largely abolishes atherosclerosis in hypercholesterolemic mice [20].

Previous studies have shown that hypoxia increases expression of TLR2 and TLR6 and it has been suggested that tissue hypoxia plays a role in transcriptional adaptation of innate immune responses during acute infection or inflammation [21]. Our data indicate that the toll like receptor (TLR) pathway is activated by ischemia. We found increased expression of TIRAP as well as RIP-2 in ischemic heart tissue compared with non-ischemic controls. It has also been shown that activation of RIP-2 upstream of the p38 MAPK activation plays a role in myocardial injury induced by ischemia [22]. Indeed, we found increased expression of p38 MAPK and MAP3K7IP2 in the ischemic heart compared with non-ischemic controls. TIRAP controls activation of MyD88-independent signaling pathways downstream of TLR4 [23]. Both the up-regulated genes TIRAP and RIP-2 in TLR pathway is activated leading to production of proinflammatory cytokines [24]. These data are consistent with the hypothesis that inflammation plays a role in the acute coronary syndromes. The proinflammatory IL-18 gene expression is induced under hypoxic conditions [25]. Elevated IL-18 levels have been associated with a less favorable prognosis in patients with acute coronary syndromes [26] and TNF-α correlates with myocardial perfusion defect, impairment in myocardial function, and infarct size [27]. Previous investigators have suggested that ALOX15 contribute to increased inflammation [28] and increased production of TNF-α [29].

In conclusion, our work identifies increased ALOX15 and inflammatory markers in human ischemic heart biopsies. Furthermore we demonstrate that hypoxia increases ALOX15 in human muscle cells. Our results yield important insights into the underlying association between hypoxia and inflammation in the human ischemic heart disease.

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