Hypoxia and the Renin-Angiotensin System in Atherosclerosis

Master thesis in Medicine

Cecilia Thalén Johansson

Supervisor: Lillemor Mattsson Hultén

Wallenberg Laboratory, Department of Molecular and Clinical Medicine,

Institute of Medicine, Sahlgrenska Academy, University of Gothenburg,

Sahlgrenska University Hospital, Gothenburg, Sweden

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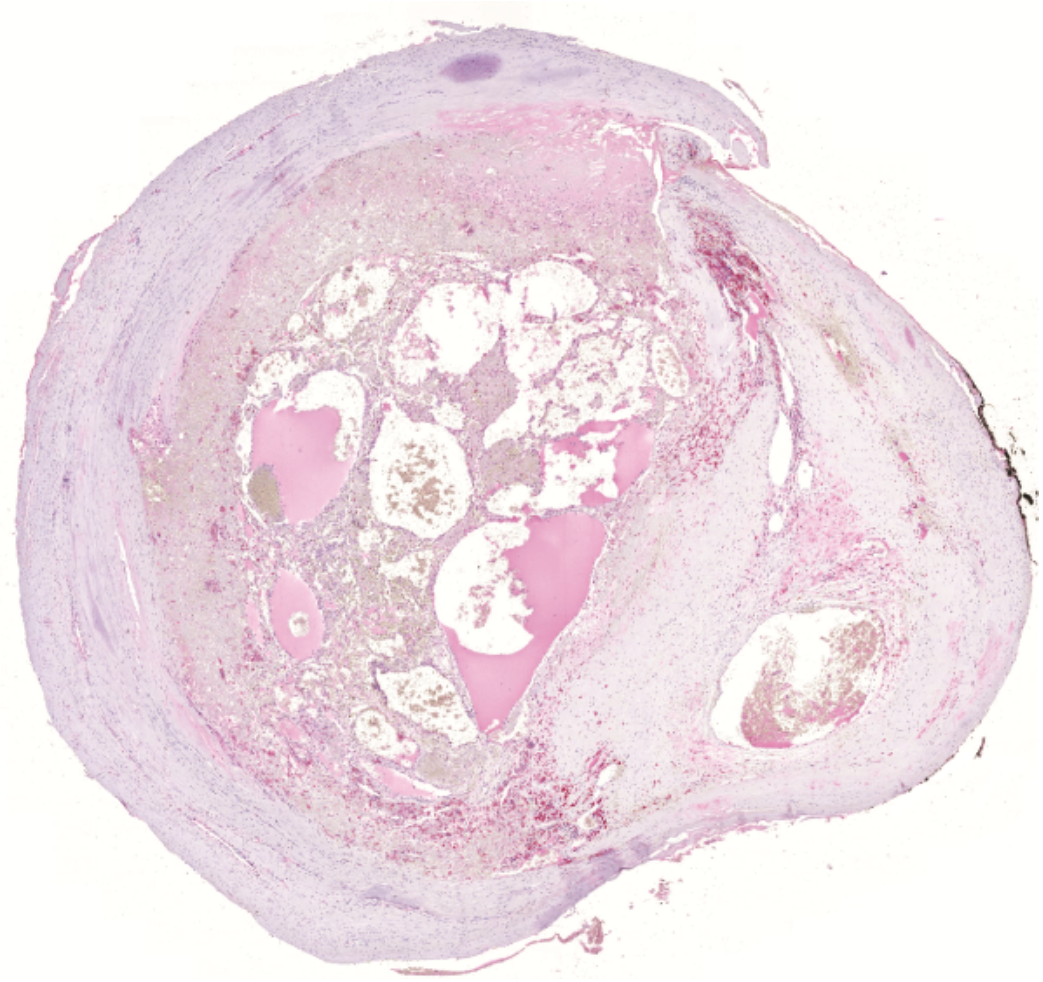


Abstract

Background – Cardiovascular disease is the leading cause of morbidity and mortality worldwide and atherosclerosis is estimated to be the underlying cause of approximately 50% of all deaths in western societies. Many aspects of the atherosclerotic disease are still incompletely characterized; it is commonly believed that inflammation is the driving force in development and progression of the disease and lately here has been increasing interest in the possible interplay between hypoxia and inflammation as well as the renin-angiotensin system (RAS) and inflammation.

Aim – To test the hypothesis that hypoxia leads to induction of the RAS, including angiotensin II receptor type 1 (AT_1), and that this results in increased inflammation in the atherosclerotic plaque. To investigate what cells would be involved in hypoxia-RAS interplay and to asses effects of RAS interfering drugs in hypoxic environments.

Methods – Histological examination of human atherosclerotic plaques and comparative analysis of plaques and serum proteins in patients treated with RAS interfering drugs and controls. In vitro cell experiments conducted on primary human smooth muscle cells and macrophages where cells were exposed to hypoxia, angiotensin II and RAS interfering drugs.

Results – Expression of AT_1 co localizes with expression of hypoxia marker HIF-1α in macrophage rich areas of atherosclerotic plaques. Statistical analysis proved strong correlation between expression of AT_1 and macrophage marker CD68 as well as between expression of HIF-1α and CD68. In vitro cell experiments confirmed expression of AT_1 in macrophages.

Conclusions – This report presents evidence implicating hypoxia-RAS interplay via stabilization of the protein HIF-1α. Further experiments are required to elucidate what effect this interplay has on inflammatory profile of the atherosclerotic plaque.

Key words – Atherosclerosis, renin-angiotensin system (RAS), angiotensin II type 1 receptor (AT_1), hypoxia inducible factor-1α (HIF-1α), macrophages.

Contents

Background

Cardiovascular disease is the leading cause of morbidity and mortality worldwide [1]. In this group of diseases we find conditions such as coronary artery disease and cerebrovascular disease; two common health problems, manifestations of which include myocardial infarction, heart failure and ischemic stroke. The underlying cause of these conditions is atherosclerosis, a disease of the large arteries characterized by the accumulation of lipids and fibrous elements in the vessel wall leading to the development of lesions known as atherosclerotic plaques or atheromata. Atherosclerosis is common and a major health problem in today´s western societies where it has been estimated to be the underlying cause of approximately 50% of all deaths [2].

The pathogenesis of atherosclerosis is complex with many contributory factors. Formation of plaques starts with the accumulation of low density lipoproteins, LDL, in the inner most layer of the vessel wall (the intima). Trapped in the intima the LDL is subjected to various modifications, including oxidation. Accumulation of oxidized LDL leads to inflammation and subsequent recruitment of monocytes and T-cells [3, 4]. Recruited monocytes differentiate into macrophages that internalize the modified lipoproteins resulting in the formation of so called foam cells; which denote lipid loaded macrophages. As the atheroma progresses the foam cells are prone to undergo apoptosis or necrosis; as they die they deposit their lipid-filled contents in the tissue thus forming the necrotic core of the plaque [2]. Foam cells and T-cells will further the inflammatory response by secretion of cytokines and growth factors mediating smooth muscle cell (SMC) migration, proliferation and extracellular matrix production. This causes SMCs to migrate from the vessel media into the growing atheroma where they undergo a phenotype shift, from a contractile phenotype to a synthetic phenotype. These intimal SMCs then secrete extracellular matrix giving rise to the

fibrous cap, a layer of fibrous tissue encapsulating the core of the atheroma, resulting in a fibrous plaque [2, 5].

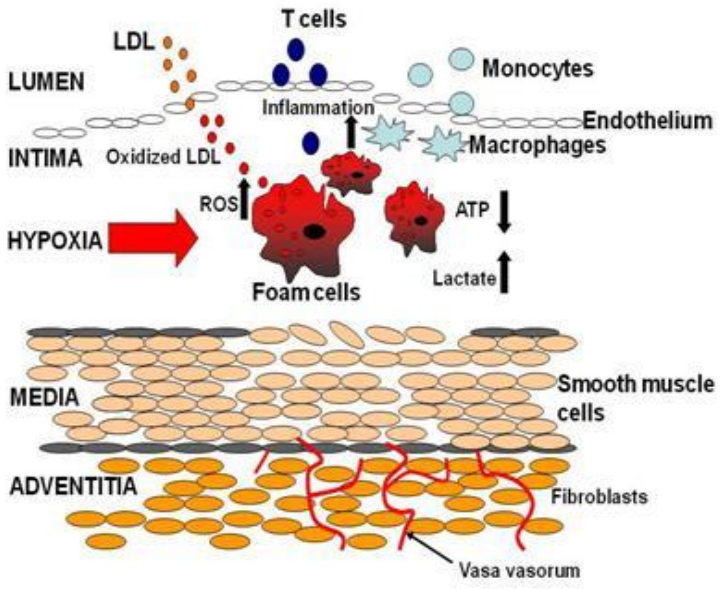
With the accumulation of fibrous elements and smooth muscle cells the plaque grows; initially the lesion expands towards the outer wall of the vessel (the adventitia) but gradually it will start expanding inwards, encroaching on the vessel lumen. Though the plaque is now starting to occlude the vessel many of these lesions are still asymptomatic. Complications in form of myocardial infarction and ischemic stroke often occur when an atherosclerotic plaque ruptures leading to the formation of an occlusive thrombosis or subsequent embolus. How prone a plaque is to rupture, i.e. the vulnerability of the plaque, depends on a large number of factors; vulnerable plaques generally display a histological composition with an increased number of inflammatory cells and a thin fibrous cap [2]. Inflammation seems to be a driving force of plaque vulnerability.

Our understanding of the process of atherogenesis (i.e. the formation of atherosclerotic plaques) has grown steadily over the years. Numerous works have highlighted the importance of inflammation in this process [1, 2, 4, 6-8] and it is commonly believed that inflammation is the driving force behind formation, progression and rupture of atherosclerotic plaque. Macrophages have been a natural area of interest in this matter. We know that macrophages are crucial in the atherogenic process, the differentiation of monocytes into macrophages in the intima is a prerequisite for development of atheromata [4]. There have, however, recently been implications that SMCs may also play an important part in the inflammatory process in atherosclerosis. It is possible that SMCs recruited to the atherosclerotic lesion may take on an inflammatory phenotype, secreting cytokines and expressing cell adhesion molecules [5]. In this area much is still unknown, but stimuli such as oxidized LDL and angiotensin II have been found to induce SMC secretion of Monocyte Chemotactic Protein-1 (MCP-1), Interleukin-6 (IL-6), IL-8 and Tumor Necrosis Factor α

(TNFα) [5]. It is possible that SMCs may play an important role in maintaining the inflammatory response in atherosclerosis. Still, on this matter, there are other contributory factors to be taken into account.

In recent years we have gained an increased understanding of the importance of hypoxia (insufficient levels of oxygen in the tissue) in plaque formation and progression. Hypoxia is a common feature of the atherosclerotic tissue due to increased oxygen consumption and decreased oxygen supply. The foam cells formed in the early stages of athrogenesis are highly oxygen consuming [9], their accumulation leads to increased oxygen consumption and oxygen demand in the tissue. As the atherosclerotic plaque grows the vessel intima grows thicker. The intima relies on diffusion of oxygen from the blood flowing through the vessel lumen for its oxygen supply and the maximum diffusion limit is approximately 100–250 μm. The thickness of the plaque often exceeds 250 μm, indeed it can often reach a thickness many times as great, and as a result oxygen supply to the tissue is hampered [10]. This combination of increased oxygen consumption and decreased oxygen supply makes hypoxia a common feature of the atherosclerotic plaque (figure 1).

Figure 1: Intimal thickening and foam cell accumulation resulting in hypoxia



The intima depends on diffusion of oxygen from the vessel lumen for its oxygen supply; thickening will increase diffusion distance and hamper oxygen diffusion. Furthermore oxygen consuming foam cells will accumulate in the growing atheroma resulting in increased oxygen demand. Increased oxygen consumption and decreased oxygen supply will lead to hypoxia

Hypoxia has pronounced effects on many aspects of cell physiology [11, 12] and has been indicated as a key factor in the progression of atherosclerotic plaques to advanced lesions, by depletion of ATP (adenosine-tri-phosphate), promotion of lipid accumulation and increased inflammation [13]. The link between hypoxia and inflammation is incompletely characterized but evidence suggests that one of many important factors may be the protein hypoxia inducible factor 1α (HIF-1α). HIF-1α is one of two subunits forming the transcription factor hypoxia inducible factor 1 (HIF-1),the predominant mediator of responses to hypoxia in all cell types [14]. The limiting factor in HIF-1 formation is the supply of HIF-1α; under normoxic circumstances HIF-1α is subjected to hydroxylation and is subsequently rapidly degraded. Under hypoxic circumstances lack of oxygen prevents hydroxylation of HIF-1α from taking place and HIF-1 can be formed [12]. HIF-1α has been shown to mediate adaptive responses to tissue hypoxia [12], however in environments of chronic continuous hypoxia or chronic intermittent hypoxia HIF-1α mediates maladaptive responses and has been implicated in development of tissue inflammation [15]. Recent evidence suggests significant crosstalk between HIF-1α and the transcription factor nuclear factor kappa B (NF-κB) [15, 16] and that NF-κB may regulate hypoxia-mediated inflammatory responses [16, 17]

Recently there has also been increased interest in the role of the Renin-Angiotensin-System (RAS) in the development and progression of atherosclerosis. The effecter molecule of the RAS is Angiotensin II, a protein formed by sequential enzymatic cleavage of angiotensinogen, a protein produced by the liver; this occurs both in the blood stream – circulation RAS, and in the tissue – tissue RAS (see figure 2). (R) Angiotensin II is a potent vasoconstrictor and the RAS is best known for its role in regulation of blood pressure, but lately attention has been directed to the RAS role in promoting atherosclerosis. Angiotensin II has been shown to directly stimulate SMC growth and production of extracellular matrix [2], important factors in development of fibrous lesions.

Figure 2: Schematic illustration of the RAS

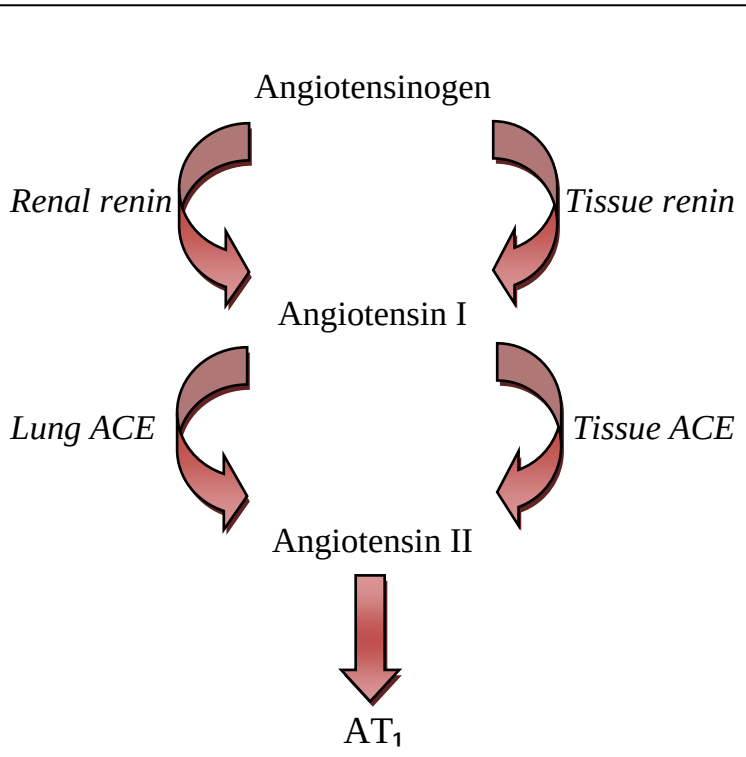


Figure 2: Angiotensinogen, formed by the liver, is sequentially cleaved to form Angiotensin II. Angiotensinogen is cleaved by renin forming Angiotensin I. Angiotensin I is the cleaved by Angiotensin Converting Enzyme (ACE) forming Angiotensin II. This process takes place locally in many tissues as well as in the blood stream.

Furthermore, the RAS and angiotensin II have been frequently implicated as mediators of inflammation [18-21]; angiotensin II has been shown to increase vascular permeability and subsequent infiltration of inflammatory cells by mechanisms including cytokine release [22]. Current literature states that the main effect of the RAS on atherogenesis is mediated by its role in promoting hypertension, insulin resistance and vascular as well as systemic inflammation [23]. Furthermore it has been observed that elevated levels of tissue RAS components are present in atherosclerosis, independently of blood pressure elevation [18]. Interestingly drugs inhibiting the RAS has been shown to reduce inflammation by mechanisms independent of blood pressure [24]. Drugs inhibiting the RAS include both sartans, which act by blockade of the Angiotensin II type 1 receptor (AT_1) – also known as

angiotensin receptor blockers (ARB) – and Angiotensin-Converting-Enzyme-inhibitors (ACEi), which act by preventing formation of angiotensin II.

The interactions between hypoxia and the RAS have previously been addressed in the context of hypertension caused by periods of intermittent hypoxia in obstructive sleep apnea. It has been shown that exposure to intermittent hypoxia can increase arterial blood pressure in humans through RAS dependent mechanisms [25], one might argue that there seems to exist some sort of connection. This raises the question of a possible interaction between tissue hypoxia and the different components of the RAS locally in the atherosclerotic plaque. A question this study means to address.

Aim

This study means to address the possibility of interactions between hypoxia and the RAS in the process of atherogenesis; the hypothesis being that hypoxia leads to induction of the RAS and that this leads to increased inflammation in the hypoxic atherosclerotic plaque. As part of investigating this hypothesis the question of which cells that would mediate this possible proinflammatory effect will be addressed, the hypothesis being that SMCs, in addition to macrophages, may play an important part in mediating inflammation induced by angiotensin II. Furthermore, effects of angiotensin II type 1 receptor blockade will be assessed in the context of its possible effect modulating properties and anti-inflammatory effects in hypoxic environments, the hypothesis being that RAB treatment may alter the composition of the atherosclerotic plaque and reduce occurrence of proinflammatory molecules in vivo as well as in vitro.

Material and methods

Histological examination of atherosclerotic plaques – and – comparative analysis of plaque morphology and serum markers of inflammation between patients receiving ARB treatment and controls

Study population

The study population consisted of 121 patients with symptomatic carotid stenosis recruited within the Gothenburg Atheroma Study Group. Patients were characterized with respect to medical history, physical examination, target organ damage and cardiovascular risk factors. Information was collected through a medical history questionnaire and examination of patient records. The study protocol was approved by the Ethical Committee of the University of Gothenburg and all participating subjects gave written informed consent.

Study design

To investigate plaque morphology histological examination of human atherosclerotic plaques was performed. 123 plaques were obtained from arteria carotis of the 121 patients constituting the study population. Stained sections were characterized with respect to expression of AT_1, hypoxia marker – HIF-1α, macrophage marker – CD68 and SMC marker – β-actin; furthermore localization of said expression was characterised. Analysis was performed to determine possible correlations between location as well as levels of expression.

To investigate effects of ARB treatment on plaque morphology and serum markers of inflammation in patients with atherosclerotic disease comparative analysis of different patient groups was performed. A retrospective cohort design was used. Two cohorts were defined, all patients in the study population receiving ARB treatment constituted the first (n=23); the second consisted of matched controls receiving no treatment interfering with the RAS, neither ARB nor ACEi (n=23). Controls were individually matched

for each subject in the exposed group. When matching consideration was taken to possible confounding factors, specifically: gender, age, diagnosed hypertension, diagnosed diabetes, previous and present smoking habits as well as treatment with statins and acetyl-salicylic-acid (ASA); table I show characteristics of patients used in comparative analysis. Comparison of plaque morphology and serum markers of inflammation was performed.

Table 1: Characteristics of patients used for comparative analysis

Variable	ARB therapy	
	Yes (n=23)	No (n=23)
Women, n (%)	6 (26)	11 (48)
Age, years	68±14	68±12
mean	69	69
median	69	70
Clinical event		
Stroke, n (%)	10 (43)	6 (26)
TIA*, n (%)	8 (35)	7 (30)
Amaurosis fugax, n (%)	5 (22)	10 (44)
Time since clinical event, days	124±114	105±77
mean	106	79
median	101	66
Hypertension, n (%)	22 (96)	19 (83)
Diabetes, n (%)	5 (22)	6 (26)
Previous smoker, n (%)	6 (26)	6 (26)
Current smoker, n (%)	3 (13)	3 (13)
ASA** therapy, n (%)	21 (91)	22 (96)
Statin therapy, n (%)	20 (87)	19 (83)
Blood pressure, mmHg		
Systolic	155±25	160±40
Diastolic	80±10	80±20

* TIA = transient ischemic attack ** ASA=acetyl-salicylic-acid

Atherosclerotic plaques

123 atherosclerotic plaques from arteria carotis obtained during surgical endartherectomi (i.e. removal of the atherosclerotic plaque) were used for histological examination. Specimens were divided into 3 mm sections, fixed in formalin for 24 hours and embedded in paraffin before 4 µm thick sections were taken for immunohistochemistry. Sections were deparaffinised and antigens retrieved using DIVA Decloaker buffer (BioCare, Concord, CA, USA). Thereafter sections were washed with Tris-buffered saline buffer (TBS – a solution of 0,20M Tris(hydroxymethyl)-aminomethane and 0,73M NaCl in Milli-Q H_2O, pH set to 6.8) and incubated with blocking buffer (TBS with addition of 0.1 % Tween and 1 % Bovine Serum Albumin (Sigma-Aldrich, St. Louis, Missouri, USA)) for 30 minutes before incubation with primary antibody in blocking buffer or DaVinci Green (PD 900 M) (Biocare Medical, California, USA) for one hour. The samples were washed before detection with MACH3 kit (M3M532 H)(Biocare Medical, California, USA) and subsequently washed again before detection with Vulcan Fast Red Chromogen kit 2 (FR805M) (Biocare Medical, California, USA). Sections were stained with Mayers hematoxylin for 45 seconds before dehydration.

Primary antibodies used were: for AT1 – mouse monoclonal Anti-Angiotensin II Type 1 Receptor antibody [1E10-1A9] (ab9391) (abcam, Cambrige Science Park, Cambrige, UK) at dilution 1:30 in DaVinciGreen (PD 900 M) (Biocare Medical, California, USA); for CD68 – lyophilized mouse monoclonal anti-CD68 (NCL-CD68-KP1) (Lecia Novocastra, Kista, Sweden) at dilution 1:500 in blocking buffer; for HIF-1α – mouse monoclonal anti-HIF-1 alpha antibody (ESEE122) (NB100-131) (Novus Biologicals, Atlanta, USA) at dilution 1:400 in DaVinciGreen (PD 900 M) (Biocare Medical, California, USA); β-actin – mouse monoclonal anti-human Muscle Actin (M0635, IR700) (DAKO, Glostrup, Denmark) at dilution 1:400 in blocking buffer.

The BioPix 2.0 software (BioPix AB, Gothenburg, Sweden) was used to quantify expression of AT_1, HIF-1α, CD68 and β-actin in stained serial sections of carotid plaques. For each section the signal of interest was identified and the area of plaque were signal was found calculated; total plaque area was calculated and by dividing area of signal expression by total plaque area and multiplying by 100 the percentage of signal expression per plaque was attained.

Furthermore, sections were characterized with respect to histological appearance and classified according to the American Heart Association (AHA) classification [26] as lesion type I-VI, where a higher number indicates a more advanced lesion.

Serum samples

Serum samples were collected from all patients and analyses of inflammatory markers were preformed. Analyzed parameters were: Ultra sensitive C-reactive Protein (U-CRP) by use of CRP High Sensitivity (981798) (Thermo Fischer Sientific, MA, USA); Monocyte Chemotactic Protein-1 (MCP-1) by use of Quantikine ELISA human CCL2/MCP-1 (DCP00) (R&D systems); IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) by use of Human ProInflammatory 9-Plex Ultra-Sensitive Kit (K15025C-1) (Meso Scale Discovery, Gaithersburg, Maryland, USA).

In vitro examination of the effects of hypoxia and ARB treatment on expression of AT_1 and markers of inflammation in cell culture

To elucidate the effects of hypoxia on the RAS and on inflammation at a cellular level, cell culture experiments were conducted on primary human aortic SMCs and primary human monocyte derived macrophages; these cell types were chosen as SMCs and macrophages were primarily considered to be the cells of interest.

Cell culture – 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. During experiments two different groups were used. The test group was challenged with incubation under hypoxic conditions (1% O_2); Controls were incubated at normoxic conditions (21% O_2). In both groups a subset of cells were incubated with the sartan Candesartan (Astra Zeneca AB, Södertälje, Sweden), an AT_1 - blocker, at the concentration of 10^{-7}M. Experiments lasted for 24 hours before collection of medium and extraction of RNA. In total 4 experiments were conducted on cells of passage 2, 4, 5 and 6 respectively.

Cell culture – monocyte derived macrophages

Buffy coats were obtained from the local blood bank at Kungälv Hospital, Sweden, and human mononuclear cells were isolated by centrifugation in a discontinuous gradient of Ficoll-Paque (GE Healthcare). Cells were seeded in Macrophage-SFM medium (Gibco) containing granulocyte macrophage colony stimulating factor (GM-CSF). After 3 days, the medium was changed to RPMI medium without GM-CSF and cells were cultured for 6 days before experiments were started. During experiments cells were challenged with incubation under hypoxic conditions, controls were incubated under normoxic conditions; in both the hypoxic test group and the normoxic control group a subset of cells were stimulated with either angiotensin II or Candesartan. Experiments lasted for 24 hours before collection of medium and extraction of RNA.

Real time reverse transcriptase polymerase chain reaction (Real Time RT-PCR)

RNA was isolated from SMCs and macrophages with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). For SMCs expression of AT_1, HIF-1α and β-actin mRNA was

determined, for macrophages expression of AT_1 and β-actin mRNA was determined. For analysis all parameters were normalized to β-actin mRNA expression. The reverse transcription reaction was set up using a cDNA reverse transcription kit (#4368814, Applied Biosystems, Foster City, CA, USA) and performed with a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Real time PCR amplification was set up using Taq man gene expression assays for AT_1 (Hs99999095_m1), HIF-1α (Hs 00153153_m1) and ActB (Hs99999903_m1) respectively, in combination with TaqMan Universal PCR master mix (#4324018) (Applied Biosystems, Foster City, CA, USA) and performed for 40 cycles on an ABI PRISM 7700 sequence detection system.

Enzyme-linked immunosorbent assay (ELISA)

Secreted cytokines were analyzed in medium from cultured primary human SMCs. Analysis of GM-CSF, interferon- γ (IFN-γ), Interleukin-1β (IL-1β), IL-10, IL-12 p70, IL-2, IL-6, IL-8, Tumor necrosis factor- α (TNF-α) and IL-18 was performed using Human ProInflammatory 9-Plex Ultra-Sensitive Kit (K15025C-1) (Meso Scale Discovery, Gaithersburg, Maryland, USA) and Human IL-18 ELISA Kit (7620) (MBL, Woburn, USA) according to manufacturer's instructions. Furthermore analysis of C-reactive protein (CRP), Intercellular Adhesion Molecule-1 (ICAM-1), Serum Amyloid A (SAA) and Vascular Cell Adhesion Molecule-1 (VCAM-1) was performed using Human Vascular Injury II Kit (K15136C-1) (Meso Scale Discovery, Gaithersburg, Maryland, USA) according to manufacturer's instructions.

Analysis for secreted components of the RAS (Angiotensin II and ACE) was performed using Angiotensin II Human/Rat ELISA (RA05880R) (BioVendor) and Human ACE Immunoassay (DACE00) (R&D Systems) according to manufacturer's instructions.

Western Immunoblotting

To investigate the occurrence of the protein AT_1 in macrophages Western Immunoblotting technique was used. As the AT_1 protein and the β-actin protein were suspected to be approximately the same size (40-45 kDa and 42 kDa respectively) sequential analysis was used starting with detection of AT_1 followed by detection of β-actin.

Primary human monocytes exposed to hypoxia or normoxia were used. Samples and ladder were loaded onto NuPAGE 4-12% Bis-Tris Gel 1.0mm X 10 well (Invitrogen, Carlsbad, CA, USA) along with loading buffer (a solution of 50mM Tris(hydroxymethyl)-aminomethane, 50mM dithiothreitol and 315mM sodium dodecyl sulfate in Milli-Q H_2O 10% glycerol and 0,005% bromphenol blue) and separated by electrophoresis at 200V for one hour. Proteins were subsequently transferred by electro transfer at 30V for one hour to Immuno-Blot polyvinylidene fluoride (PVDF) membrane (162-0177) (Bio-Rad, California, USA) using NuPAGE Transfer Buffer 20X (Invitrogen, Carlsbad, CA, USA). The membrane was thereafter washed with TTBS (TBS with addition of 0.1 % Tween 20) and incubated in blocking buffer (TTBS with addition of 5 % non fat dry milk) over night at 4°C. Following day the membrane was washed with TTBS before incubation with primary antibody in antibody solution (TTBS with addition of 2 % non fat dry milk) for one hour at room temperature with gentle shaking. For primary antibody mouse monoclonal Anti-Angiotensin II Type 1 Receptor antibody [1E10-1A9] (ab9391) (abcam, Cambrige Science Park, Cambrige, UK) was used at dilution 1:400. The membrane was washed and incubated with secondary antibody in antibody solution for one hour at room temperature with gentle shaking. For secondary antibody sheep anti-mouse IgG, peroxidase-linked speies-specific whole antibody (ECL) NA931(GH healthcare life sciences, Little Chalfont, UK) was used at dilution 1:5000. After washing, AT_1 was detected using

chemiluminiscence reaction with Immobilon Western Chemilumiscent HRP Substrate (WBKLS0500) (Merck Millipore, Billerica, USA).

After detection of AT_1 the membrane was stripped from antibodies by incubation with 0.2M NaOH solution for one hour at room temperature. After washing, the membrane was incubated in blocking buffer for one hour at room temperature with gentle shaking. There after detection of β-actin was performed by following the same protocol as for AT_1 and by use of primary antibody: rabbit polyclonal anit-actin A2066 (Sigma-Aldrich, St. Louis, Missouri, USA) at dilution 1:1250; and secondary antibody: Goat polyclonal anti-rabbit IgG – H&L (HPR) (ab6721) (abcam, Cambrige Science Park, Cambrige, UK) at dilution 1:3000.

Statistics

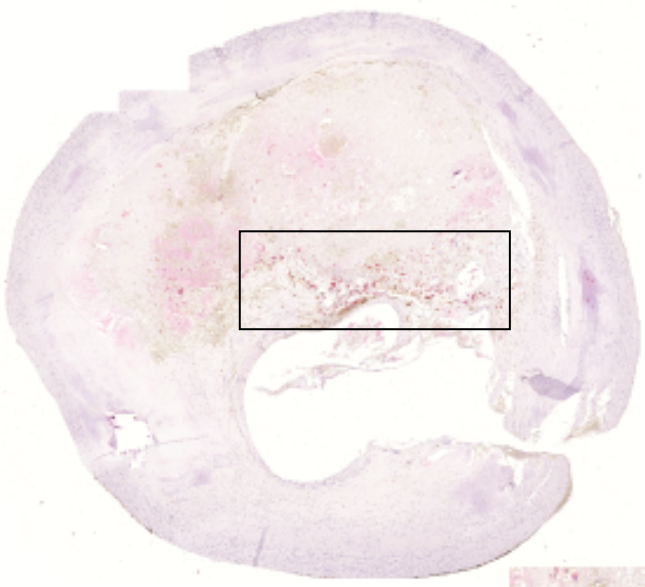
Data are plotted as mean and SEM unless stated otherwise. All analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA); a 95% confidence interval was used and P-values ≤ 0.05 were considered significant. Differences between groups were determined using non-parametric two tailed T-test (Mann-Whitney two tailed T-test). Correlations between groups were determined using non-parametric two tailed correlation (Spearman two tailed correlation).

Results

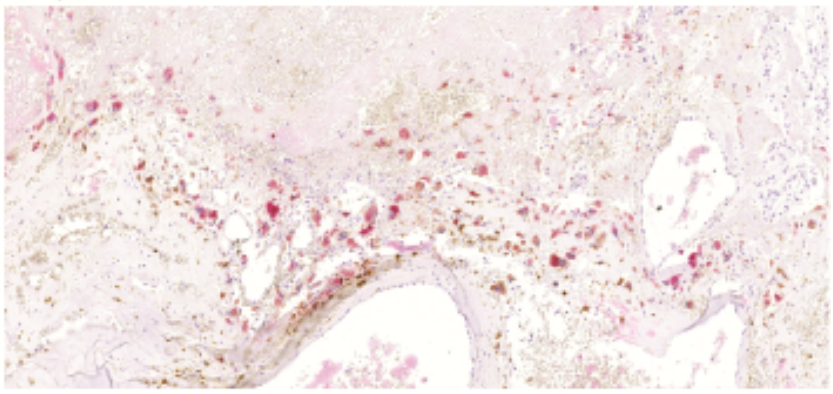
Expression of AT_1 co localizes with CD68 positive macrophages and HIF-1α expression in human carotid atherosclerotic plaques

Examination of carotid plaques proved expression of AT_1 to be co localized with expression of CD68, a protein used as a macrophage marker, and with expression of HIF-1α, a protein expressed under hypoxic conditions. No similar association was seen with β-actin, used as a marker of SMCs. As can been seen in figures 3 and 4 signals on AT_1-stained sections appear to coincide with signals on CD68 and HIF-1α stained sections.

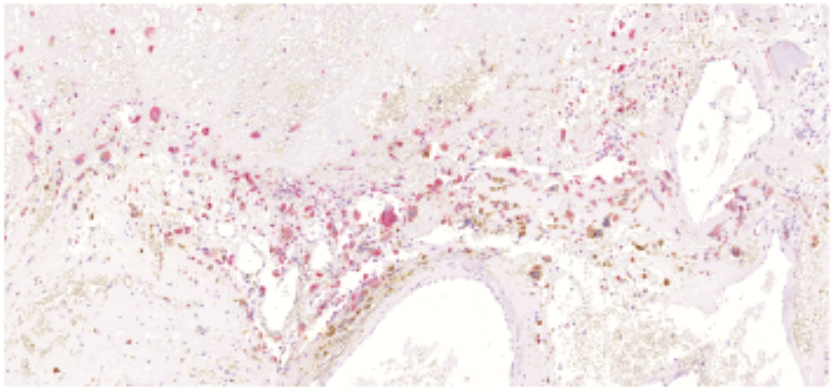
Figure 3: Serial sections of an atherosclerotic plaque



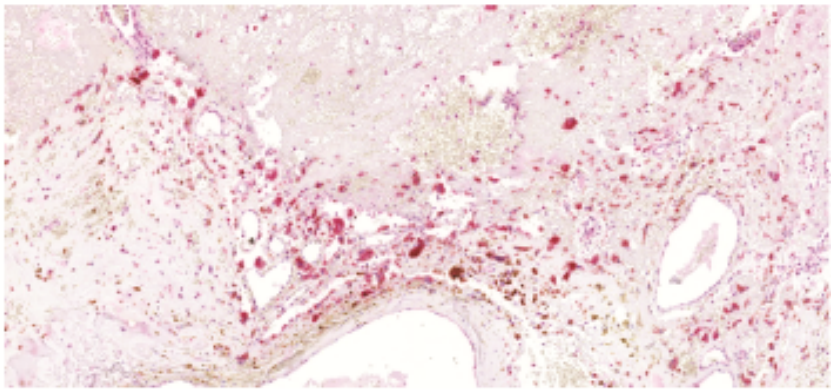
3A: Section of a human atherosclerotic plaque from arteria carotis stained for expression of AT1. Pictures 3B-D shows enlargement of the marked area of the plaque.



3B: Three times magnification of area of AT1 expression in the plaque pictured in 3A.

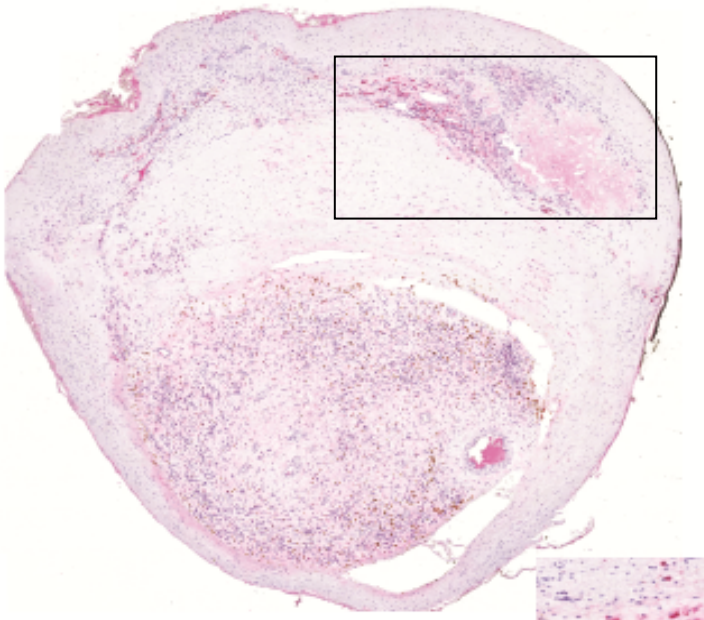


3C: Three times magnification of area of CD68 expression in the plaque pictured in 3A.

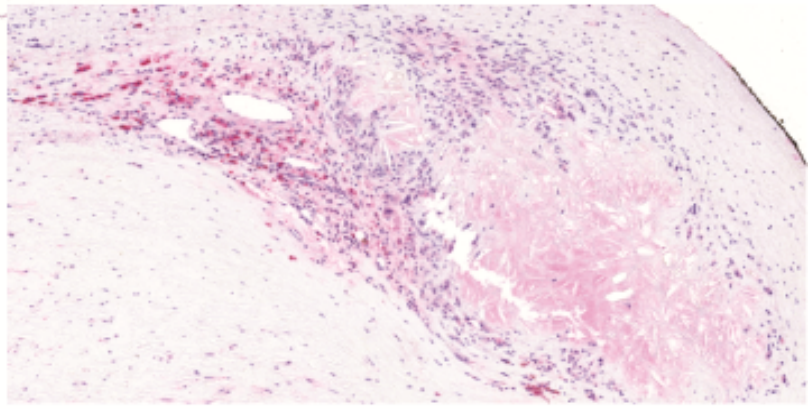


3D: Three times magnification of area of HIF-1α expression in the plaque pictured in 3A.

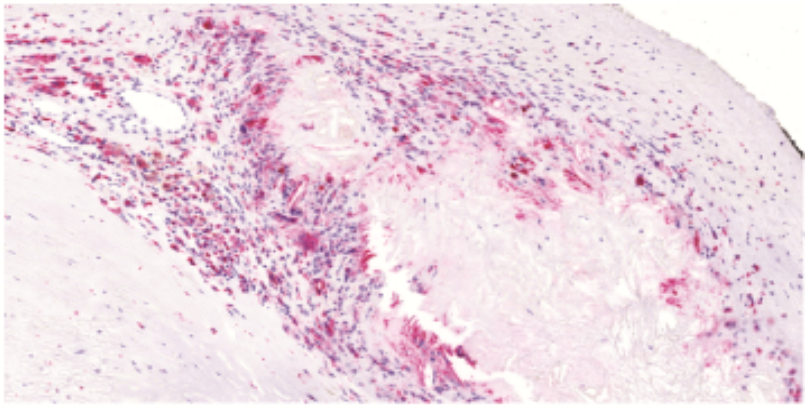
Figure 4: Serial sections of an atherosclerotic plaque



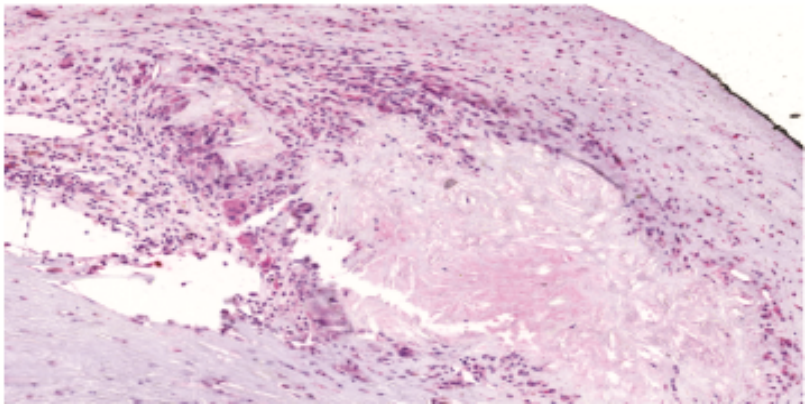
4A: Section of a human atherosclerotic plaque from arteria carotis stained for expression of AT1. Pictures 4B-D shows enlargement of the marked area of the plaque.



4B: Two times magnification of area of AT1 expression in the plaque pictured in 4A.



4C: Two times magnification of area of CD68 expression in the plaque pictured in 4A.

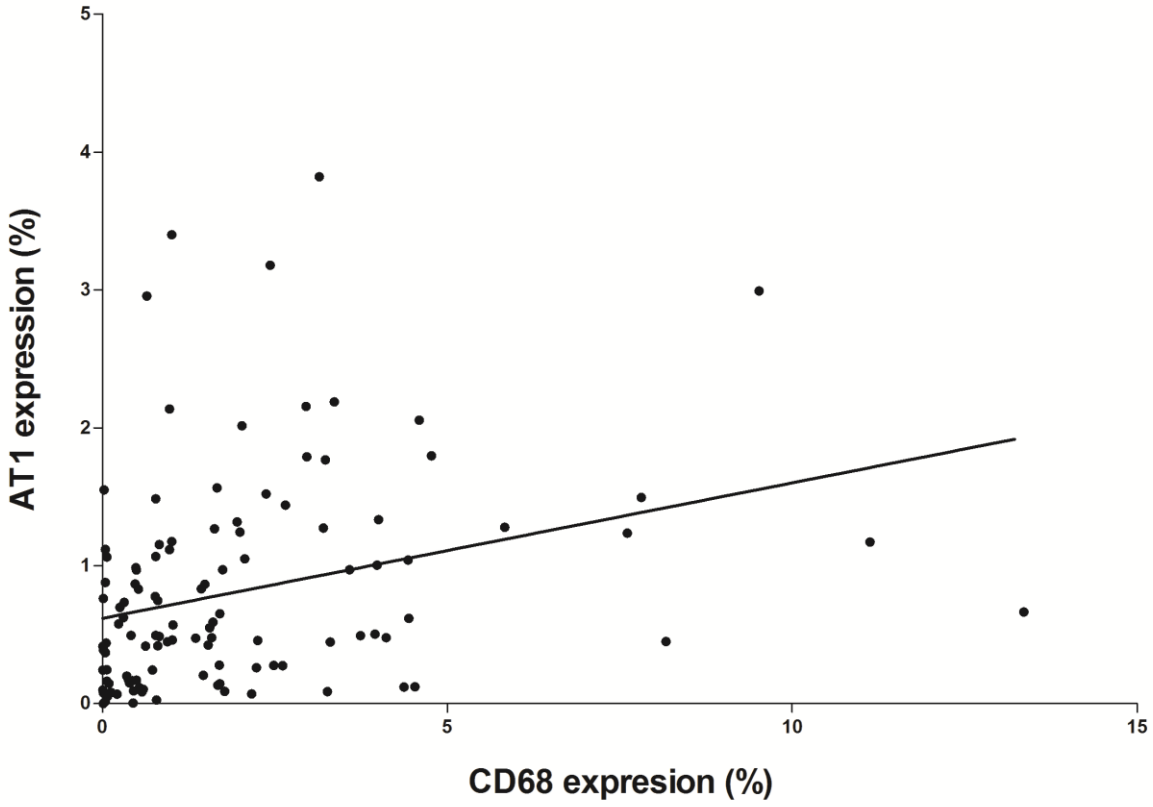


4D: Two times magnification of area of HIF-1α expression in the plaque pictured in 4A.

Significant correlation between CD68 and AT_1 expression as well as HIF-1α and CD68 expression in human carotid atherosclerotic plaques

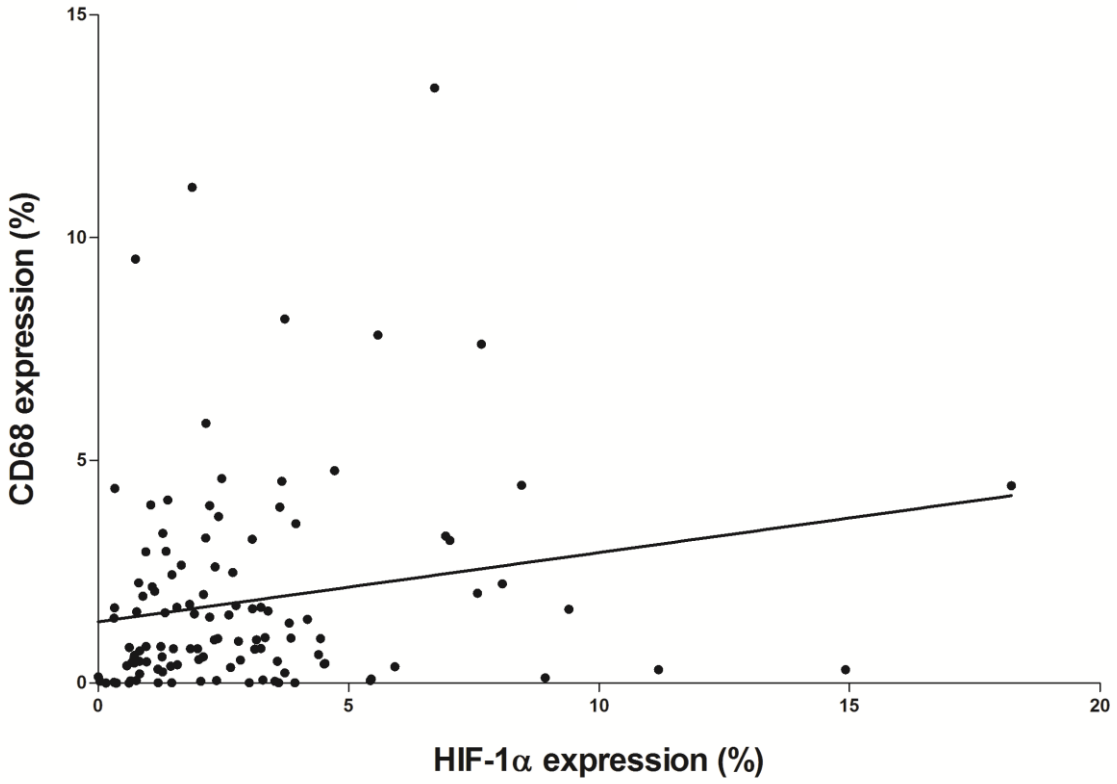
Expression of AT_1, CD68 and HIF-1α was quantified as percentage of expression per total plaque area. Subsequent correlation analysis proved significant correlation between CD68 and AT_1 expression: $p < 0.0001$, Spearman R = 0.4252 (fig. 5). As well as between HIF-1α and CD68 expression: $p = 0.0097$, Spearman R = 0.2393 (fig. 6).

Figure 5: Correlation between expression of CD68 and AT_1



Levels of CD68 expression and AT_1 expression plotted against each other with CD68 expression in percent on the X-axis and AT_1 expression in percent on the Y-axis. Correlation analysis using Spearman two tailed correlation revealed significant correlation: $p < 0.0001$, 95% confidence interval 0.2566 to 0.5687, Spearman R = 0.4252

Figure 6: Correlation between expression of HIF-1α and CD68



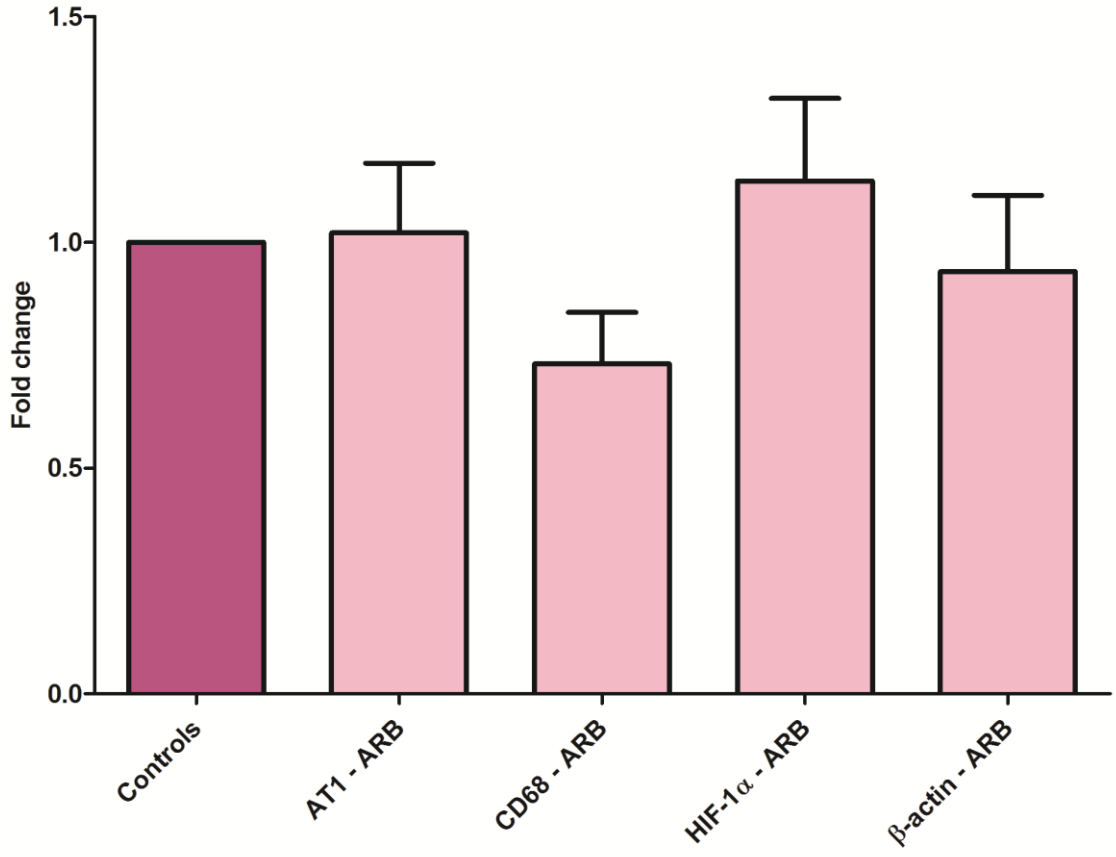
Levels of HIF-1α expression and CD68 expression plotted against each other with HIF-1α expression in percent on the X-axis and CD68 expression in percent on the Y-axis. Correlation analysis using Spearman two tailed correlation revealed significant correlation: p = 0.0097, 95% confidence interval 0.05405 to 0.4085, Spearman R = 0.2393

Similar plaque morphology and levels of serum markers of inflammation in ARB treated patients and controls

Statistical analysis of collected patient and plaque data revealed no statistically significant differences between the patient groups. Figure 7 shows differences in expression of AT_1, HIF-1α, CD68 and β-actin between groups illustrated as fold change were the mean expression, of

each protein respectively, in the control group was set to one and then compared to the expression in the ARB treated group. Distribution of morphological parameters between groups is also shown in table II. Figure 8 shows levels of serum markers of inflammation illustrated as fold change were the mean level, of each protein respectively, in the control group was set to one and then compared to the expression in the ARB treated group. Distribution of serum markers of inflammation between groups is also shown in table III.

Figure 7: Differences in expression of AT_1, CD68, HIF-1α and β-actin between studied groups



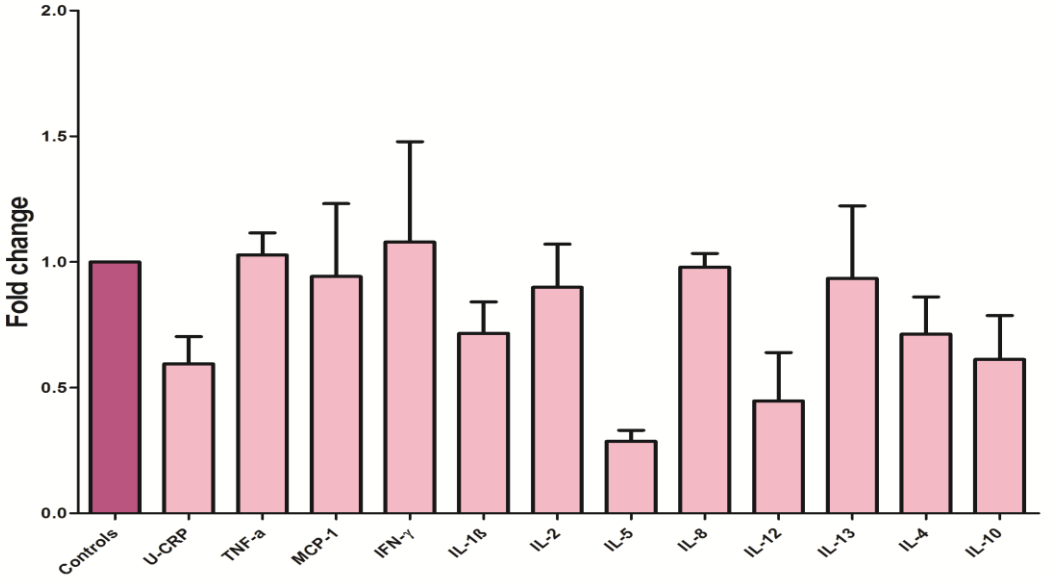
Comparison of plaque morphology. The diagram shows differences in expression of AT_1, CD68, HIF-1α and β-actin between ARB treated patients and controls. Differences are illustrated as fold change. No statistically significant differences were found.

Table II: Distribution of morphological parameters between studied groups

Variable	ARB therapy	
	Yes (n=24*)	No (n=24*)
AHA-class		
class 3, n (%)	2 (8)	1 (4)
class 4, n (%)	7 (29)	9 (39)
class 5, n (%)	4 (17)	2 (8)
class 6, n (%)	11 (46)	9 (39)
Area of AT_1 expression (%)		
mean	0,72	0,71
medaian	0,54	0,56
standard deviation	0,52	0,55
Area of CD68 expression (%)		
mean	1,46	2
medaian	1,39	0,82
standard deviation	1,12	2,9
Area of HIF-1α expression (%)		
mean	2,92	2,57
medaian	2,24	1,32
standard deviation	2,21	3,3
Area of β-actin expression (%)		
mean	4,29	4,58
medaian	3,56	3,74
standard deviation	3,79	3,65

* There were 23 patients in both groups; in each group one patient contributed two plaques, from both the left and right carotid artery.

Figure 8: Differences in serum markers of inflammation between studied groups



Comparison of serum markers of inflammation. The diagram shows differences in serum markers of inflammation between ARB treated patients and controls. Differences are illustrated as fold change. No statistically significant differences were found.

Table III: Distribution of serum markers of inflammation between studied groups

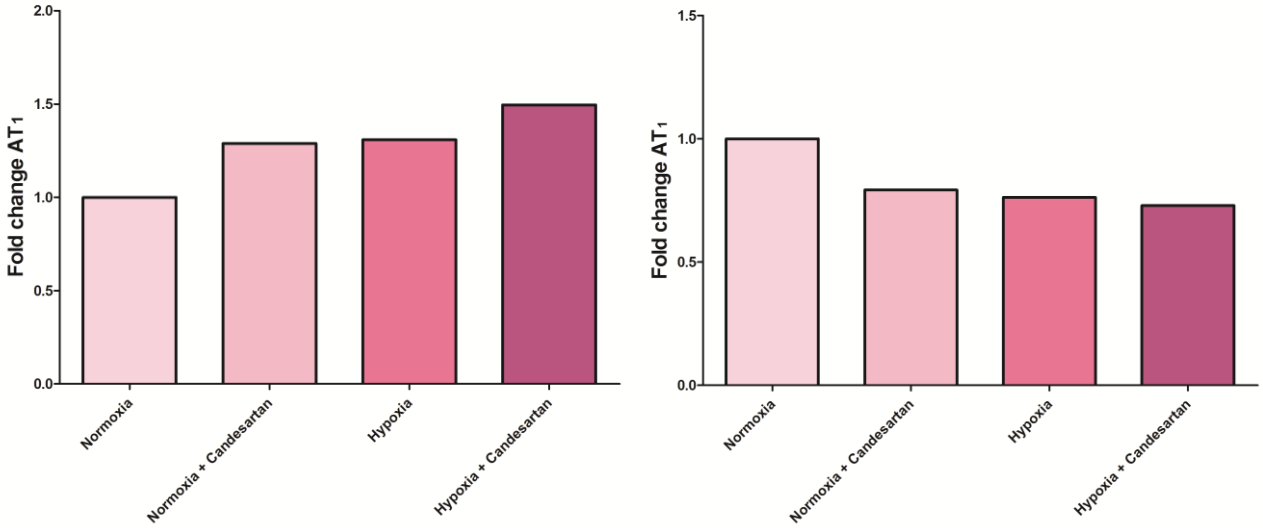
Group	**U-CRP**	**TNF-α**	**MCP-1**	**IFN-γ**	**IL-1β**	**IL-2**
ARB						
Mean	2,04	3,46	264,76	0,54	0,28	0,17
Median	1,4	3,06	176,7	0,28	0,22	0,13
SD	1,78	1,4	380,54	0,93	0,23	0,15
Controls						
Mean	3,43	3,37	280,62	0,5	0,39	0,19
Median	2,03	3,09	267,56	0,29	0,32	0,16
SD	4,68	1,21	121,11	0,83	0,3	0,18
Group	**IL-5**	**IL-8**	**IL-12**	**IL-13**	**IL-4**	**IL-10**
ARB						
Mean	0,35	3,55	1,31	2,24	0,07	1,05
Median	0,28	3,53	0,63	1,03	0,05	0,54
SD	0,26	0,93	2,65	3,23	0,07	1,41
Controls						
Mean	1,24	3,63	2,93	2,39	0,1	1,72
Median	0,31	3,36	0,45	1,13	0,03	0,83
SD	3,05	1,58	9,34	4,47	0,16	3,31

SMC cell-culture experiments proved inconclusive as to the effects of hypoxia and ARB treatment on AT_1 mRNA expression

Real Time RT-PCR was used to detect expression of AT_1 mRNA in SMCs exposed to hypoxia, hypoxia in combination with ARB, normoxia and normoxia in combination with ARB respectively. No significant effects of exposure to neither hypoxia nor ARB were detected when all results were put together. Results varied notably between experiments conducted on cells of different passage; figure 9 shows the results of measurements from the first and last experiments respectively; as can be seen exposure to hypoxia and Candesartan lead to increased AT_1 mRNA expression in experiment one (fig. 9A) and to decreased AT_1 mRNA expression in experiment four (fig. 9B).

Figure 9: Differences in AT_1 mRNA expression in SMC experiments

9A: SMC experiment 1 **9B: SMC experiment 4**



Primary human aortic smooth muscle cells were used. Diagrams show differences in AT_1 mRNA expression between experiments conducted on cells of different passage. Cells in experiment 1 were of passage 2 (shown in 9A); cells in experiment 4 were of passage 6 (shown in 9B). When analyzing, levels of AT_1 expression was normalized to levels of β-actin expression.

Expression of HIF-1α mRNA was reduced in SMCs exposed to hypoxia

Real Time RT-PCR was used to detect expression of HIF-1α mRNA in SMCs exposed to hypoxia, hypoxia in combination with ARB, normoxia and normoxia in combination with ARB respectively. In all four experiments expression of HIF-1α mRNA was reduced by exposure to hypoxia; exposure to Candesartan induced no difference in HIF-1α mRNA expression. Results are shown in figure 10.

Figure 10: Difference in HIF-1α mRNA expression between SMCs exposed to hypoxia and normoxia

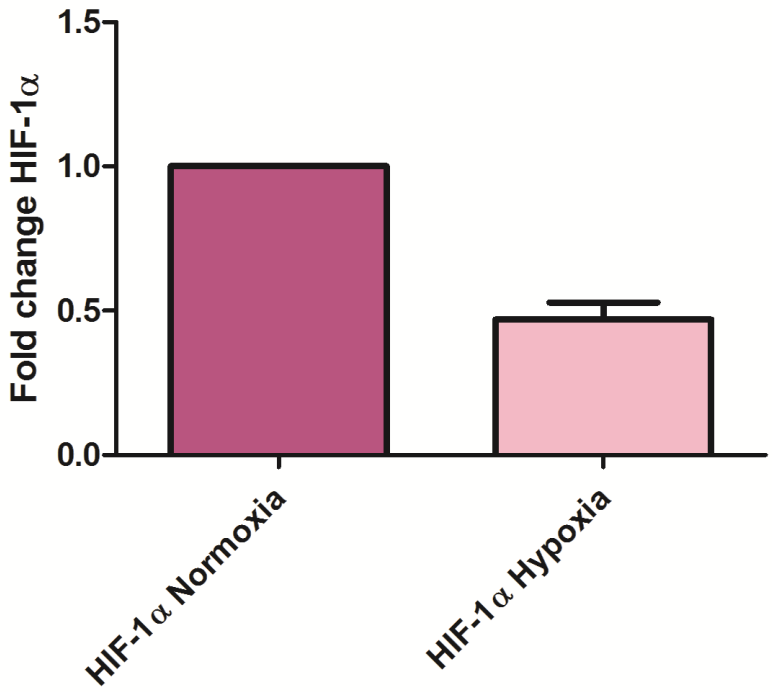


Diagram shows differences in HIF-1α mRNA expression between SMCs exposed to normoxia and hypoxia respectively. As can be seen exposure to hypoxia markedly reduced expression of HIF-1α mRNA. When analyzing, levels of HIF-1α expression was normalized to levels of β-actin expression.

No change in secretion of cytokines, ACE or angiotensin II from SMCs exposed to hypoxia or Candesartan compared to controls

ELISA was used to analyze levels of proinflammtory substances in medium from SMC cell-culture experiments. No significant differences could be seen between medium from different groups of cells; though, as with expression of AT_1 mRNA, occurrence of proinflammatory substances varied notably between experiments conducted on cells of different passage (not shown).

Analysis of secreted components of the RAS proved low levels of ACE and angiotensin II in all examined samples. No effect was seen of exposure to hypoxia or Candesartan.

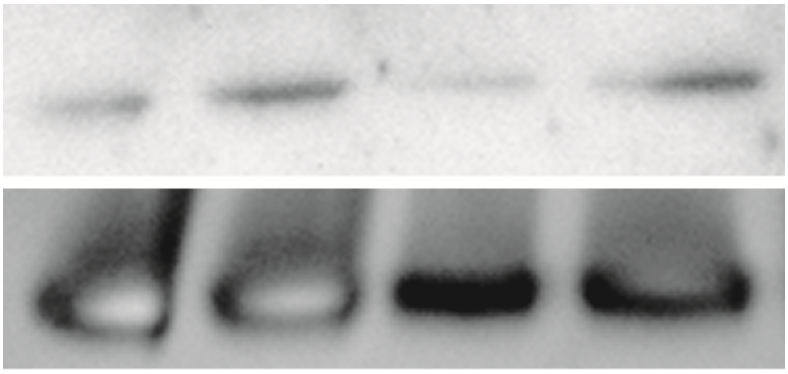
Macrophage cell-culture experiments proved inconclusive as to the effects of exposure to hypoxia, angiotensin II or Candesartan in AT_1 mRNA expression

Real Time RT-PCR was used to detect expression of AT_1 mRNA in primary human monocyte derived macrophages; cells had been incubated at either hypoxia or normoxia and a subset of cells in environments were treated with angiotensin II or Candesartan. No significant effects of exposure to hypoxia, angiotensin II or Candesartan were detected. In 21 out of 24 samples mRNA levels were too low as to be determined. In the three samples that contained sufficient levels too be detected cycle threshold (CT) levels were high, average 42 and 50 cycles were used for detection.

Expression of AT_1 protein in macrophages verified with Western Immunoblotting

As expression of AT_1 in macrophages could not be shown by analysis of mRNA expression with Real Time RT-PCR, Western Immunoblotting was performed to verify the expression of the AT_1 protein. As can be seen in figure 11A a clear signal was found corresponding to a protein size of approximately 60 kDa, which is consistent with expected size of the AT_1 protein (approximately 40,45 or 60 kDa depending on the glycosylation of the protein) . Figure 11B shows detection of β-actin on the same membrane after stripping of the AT_1 antibodies.

Figure 11: Detection of AT_1 protein and β-actin protein in human monocyte derived macrophages by Western Immunoblotting



11A: Detection of AT_1

11B: Detection of β-actin

Here shown is the detection of AT_1 protein (11A) and β-actin protein (11B) in primary human monocyte derived macrophages by Western immunoblotting. The same membrane was stained for detection of AT_1 and subsequently stripped of antibodies before staining for β-actin.

Discussion

In summary, the main findings related in this thesis are: the observation that expression of AT_1 co-localizes with expression of HIF-1α (a marker of hypoxia) in macrophage rich areas of human atherosclerotic plaques, and the discovery of correlation between expression of CD68 and AT_1 as well as HIF-1α and CD68 in the human atherosclerotic plaque. Verification of expression of AT_1 protein in primary human monocyte derived macrophages by Western immunoblotting further supports these findings.

Comparative analysis of the effects of ARB treatment on histological appearance of carotid atherosclerotic plaques and markers of inflammation in serum samples, performed on a study population consisting of patients with symptomatic[*] atherosclerotic disease, proved no conclusive evidence supporting the hypothesis that ARB treatment would affect the composition of the plaque and the occurrence of proinflammatory proteins in vivo. Investigation of the potential role of SMCs in mediating hypoxia induced up regulation of the RAS and subsequent inflammatory responses proved no conclusive evidence supporting the hypothesis that hypoxia leads to induction of the RAS and that this leads to increased inflammation nor for the hypothesis that exposure to ARB would decrease occurrence of proinflammatory molecules in vitro.

Related results indicate that the primary cell type involved in RAS-inflammation interplay in the atherosclerotic plaque would be macrophages. The co localization of macrophages and expression of AT_1 in sections of atherosclerotic plaques has been previously observed by others [27] and furthermore, other components of the RAS, such as ACE and angiotensin II, have been found to be similarly expressed mainly in macrophage rich areas of the plaque [27, 28]. In this thesis it is further observed that the macrophage rich areas expressing AT_1 are also high in HIF-1α expression; this would indicate that hypoxia may have

[*] Symptomatic being defined as occurrence of transitory ischemic attack (TIA), amaurosis fugax or stroke

a part in inducing AT_1 expression in macrophages via stabilization of HIF-1α. This scenario does not appear to be entirely farfetched as others have shown that hypoxia induces expression of AT_1 in a HIF-1α dependent manner in other primary human cells, namely pulmonary fibroblasts [29].

Methodological considerations – problem analysis

Comparative analysis of histological plaque appearance and serum markers of inflammation were unable to establish any differences between patients treated with ARB and patients receiving neither ARB nor ACEi treatment. This does not correspond well with other findings that indicate that differences in inflammatory profile and plaque character does exist [24, 30]. However, our study contains a number of weaknesses that need to be addressed that might explain these discrepancies. Firstly: our control group consisted of patients with symptomatic atherosclerotic disease, which means to say they have all shown clinical manifestations of unstable plaques; it is therefore unlikely that any difference in AHA class should be observable. And as most unstable plaques display similar morphological features it is unlikely that we should be able to detect any differences regarding histological appearance; it is possible that small differences in plaque morphology does exist between the two studied groups but the methods available for analysis are far to blunt for any small differences to be detectable. The problem with the control group is however not very easy to get around, assuming the study involves examination of atherosclerotic plaques. It is not ethically justifiable to imperil asymptomatic patients by subjecting them to possibly lethal surgery; this makes it difficult to retrieve plaques from asymptomatic patients. If one should only wish to study ARB effect on serum markers it would however not be unmanageable to acquire a more suitable control group. Or one can of course study plaque morphology indirectly by examining occurrence of clinical manifestations indicating vulnerable plaques; to study this one would ideally use a prospective cohort trial. Secondly: we used small cohorts which make

it further unlikely that any small differences would become apparent. Thirdly: no consideration was taken to the time of ARB treatment. The statistical material collected simply states whether or not the patient received ARB treatment at the time of the study. The same is true for the statistical data on most of the possible confounders that were considered. Ideally information on exposure to different drugs and risk factors would be more detailed. Finally: we must also consider the fact that our patients were not randomized to specific treatments. It is therefore likely that the selection of patients to treatment may be a confounding factor in our study. In summary: the comparative study of ARB treatment effect on plaque morphology and serum markers of inflammation was not ideally designed and perhaps it is not surprising that it proved inconclusive.

The experiments conducted on primary human aortic SMCs proved difficult to interpret due to the substantial differences in outcome between the different experiments, indeed, outcomes of the first and the last experiments were completely opposite (fig. 9). However, we must consider the fact that the environment in cell culture differs widely from the one of the human aorta in vivo. It is well known that cells taken from their natural habitat and placed in culture will adapt to the new environment; from literature we know that SMCs possesses a high level of plasticity and are prone to change their phenotype in response to outer stimuli [5]. It would not seem unlikely that the reason for the varying outcomes to our experiments is the fact that the cells were of different passage in each experiment. Considering that cells of a lower passage are most likely possessed a phenotype similar to that of SMCs in vivo our conclusion was that the results of the first experiment (cells of passage 2) are likely to be the most reliable ones; this indicates that the clear trend seen in the first experiment, i.e. that expression of AT_1 is induced by Candesartan and further induced by hypoxia, might be worth investigating. However no similar trend could be found for secreted proinflammatory substances when analyzing the results of ELISA analysis of medium from

the first SMC experiment. Our results show no indication that the increased AT_1 expression would lead to increased secretion of proinflammatory substances; however, this might possibly be explained by lack of angiotensin II stimuli. Others have shown that angiotensin II can stimulate SMC proliferation and migration as well as expression of IL-18 receptor [5]. It is possible that new experiments, conducted on cells not exposed to cell culture environment for too long, were cells are stimulated with angiotensin II would prove interesting in further elucidating the possible role of SMCs in hypoxia-RAS-inflammation crosstalk.

When analyzing outcome of the SMC experiments we meant to use Real Time RT-PCR detection of HIF-1α mRNA to establish that the cells were indeed in a hypoxic state; however expression HIF-1α mRNA was consequently lower in cells exposed to hypoxia (fig. 10). We know that hypoxia stabilizes HIF-1α by preventing proteosomal degradation of the protein [12], so most likely expression of the HIF-1α protein is increased in the SMCs subjected to hypoxia – though we cannot be sure as we did not investigate this. Supposing HIF-1α protein levels are higher in SMC subjected to hypoxia this would correlate with a decrease in HIF-1α mRNA, which in turn would suggest some kind of negative feedback regulation of HIF-1α mRNA synthesis by the HIF-1α protein or other mechanisms related to hypoxia. This might be something worth investigating but the value of such an investigation in elucidating potential interactions between hypoxia and the RAS is questionable. But what we can learn from this is that we cannot rely on Real Time RT-PCR quantification of HIF-1α mRNA expression to establish that cells are expressing a hypoxic phenotype; perhaps detection of the HIF-1α protein by, for example, western immunoblotting would better serve this purpose.

Western immunoblotting confirmed that macrophages do indeed express AT_1, results consistent with those of others [31]; but unlike many others we did not succeed in conclusively establish AT_1 mRNA expression in macrophages in Real Time RT-PCR analysis.

The fact that we were unable to detect AT_1 mRNA but were able to detect the AT_1 protein with Western immunoblotting lead us to believe that the primer we used for Real Time RT-PCR might not be suitable to use in experiments conducted on primary human monocyte derived macrophages. For potential future experiments one could try using another primer that has been found adequate in similar situations by others, for example Guo F. et al [31]. We also feel that, just as with the SMCs, one has to be aware of the fact that the macrophages we conduct our experiments on are exposed to conditions that widely differs from the conditions found in the human body and in the atherosclerotic plaque. One could try to minimize the confounding effect this has by producing an environment more similar to the one of the atherosclerotic plaque. In this study we used angiotensin II to stimulate macrophages; angiotensin II has been shown to induce several components of the RAS, including AT_1 [27, 31]. It might of course seem strange to use a substance know to induce increased expression of AT_1 when expression of AT_1 is the very thing we aim to study; but since our primary interest lies in finding out how hypoxia effects AT_1 expression this might not be a big problem. If our hypothesis holds true and hypoxia does induce increased expression of AT_1 this should be detectable even if cells are treated with angiotensin II. Other stimuli that might be used could be inducers of inflammation, for example lipopolysacaride (LPS) and hypoxia. Another possible solution to the fact that we were unable to detect sufficient levels of mRNA might be to adjust the Real Time RT-PCR method used; possibly increasing the number of cycles used for detection would prove helpful.

In summary

We set out to investigate the possibility of interactions between hypoxia and the RAS in the process of atherogenesis as well as what cells would seem to participate in such an interaction. Histological examination of human atherosclerotic plaques proved very useful in this task; and we now feel we have clear indications suggesting that such an interaction does

exist and that the primary cells of interest are macrophages. In vitro cell-culture experiments did however not prove very useful; though we now have a clear idea of how experiments may be improved for future research.

We also wished to evaluate effects of ARB drugs in hypoxic environments. It is likely the study population used for this comparative analysis was too small to detect any differences in plaque morphology. As to the question of ARB effects on systemic inflammation measured as serum markers of inflammation we can in retrospective see that our patient material was not ideal for addressing this question; as earlier stated another study design using a different study population should probably be used to address this question. Cell culture experiments could prove helpful in further addressing our questions and, as previously stated, we now have a clear idea of how experiments may be improved for future research.

Future studies

There is need for further studies to fully understand the implications of hypoxia in the atherosclerotic tissue and in what way the RAS is involved in mediating hypoxia induced effects. We would like to continue investigating the potential interplay between hypoxia and the RAS in macrophages as we feel this might prove useful in furthering our understanding of atherogenesis. The hypothesis we intend to address is that hypoxia leads to induction of the RAS and that this leads to increased inflammation, and we now feel we should focus our interest on macrophages. The first step would be to establish if AT_1 expression in macrophages is in fact increased by exposure to hypoxia and that this does in fact result in increased inflammation. We hope to be able to conduct new in vitro experiments using primary human monocyte derived macrophages and stimulate them in different ways to mimic the environment of the atherosclerotic plaque. Thereafter we hope to be able to detect expression of AT_1 mRNA with Real Time RT-PCR, using an adjusted protocol, and

subsequently quantify expression. Medium from cell experiments would be examined for occurrence of pro-inflammatory substances and quantification of said substances preformed by use of ELISA. If we can establish that our hypothesis holds true we can then examine potential ways of preventing hypoxia induced inflammation mediated by the RAS.

Conclusions and Implications

In this thesis it is concluded that angiotensin II receptor type 1 is expressed in hypoxic, macrophage rich areas of the human atherosclerotic plaque; this conclusion was made by histological examination of atherosclerotic plaques. This is further supported by the finding that levels of AT_1 expression are significantly correlated to CD68 expression ***, and that levels of HIF-1α as well are correlated to CD68 expression **. This might perhaps lead to increased interest in investigating the role of hypoxia in atherogenesis which might in turn lead to important advances in our understanding of this complex process that is the underlying cause of approximately 50% of all deaths in today's western society.

Populärvetenskaplig sammanfattning

Hjärt-kärlsjukdom är den ledande orsaken till död och sjuklighet i dagens samhälle [1]; åderförkalkning, på medicinskt fackspråk kallat ateroskleros, är den underliggande orsaken till många manifestationer av hjärt-kärlsjukdom och har uppskattats vara orsak till ungefär 50 % av alla dödsfall i vårt västerländska samhälle [2]. Ateroskleros är en sjukdom som karakteriseras av bildandet av plack i kroppens blodkärl, det är en långsam process som drabbar de flesta av oss så småningom. Det hela börjar med att LDL-kolesterol (populärt kallat ”det onda kolesterolet”) ansamlas i det innersta lagret av kärlväggen; detta leder till en skada på kärlet och som resultat uppstår inflammation i vävnaden. Vi tror oss idag veta att det är inflammationen som är den drivande faktorn i bildandet av och utvecklingen av aterosklerotiska plack [1, 2, 4, 6-8]. Inflammatoriska celler i immunsystemet kommer dra sig till området för att försöka reparera skadan; bland dessa celler återfinner vi makrofagerna (”storätarna”), en viktig komponent i immunsystemet som bl.a. verkar genom att utsöndra signalmolekyler till andra celler i immunsystemet och agera som en koordinator av det inflammatoriska svaret. Normalt sett är inflammationen en viktig del i kroppens läkningssystem, när en vävnad skadas uppstår inflammation som bidrar till att skadan läks och när läkning har skett klingar inflammationen av; men eftersom denna skada inte kan repareras kommer inflammationen att fortsätta, öka och leda till större vävnadsskada – inflammatoriska celler och LDL fortsätter ansamlas. Den brännande frågan i sammanhanget är – vad är det som driver inflammationen? Detta är en stor och komplex fråga där mycket har gjorts för att föra kunskapen framåt. Vi vet idag mycket om hur och varför inflammationen uppstår, men långt ifrån allt. Det är just denna fråga som vår studie berör.

Vi har i denna studie funnit att det i områden av syrebrist i det humana aterosklerotiska placket återfinns makrofager (inflammatoriska celler) som uttrycker angiotensin II receptor typ 1 (AT_1). Angiotensin II och AT_1 är viktiga komponenter kroppens

system för att reglera blodtrycket (lägg till referens); men man har också på senare tid börjat inse att stimulering av AT_1 med angiotensin II kan leda till inflammation (lägg till referens). Det skulle alltså förefalla som om makrofager i aterosklerotiska plack uttrycker AT_1 vilket skulle kunna bidra till inflammationen i placket. Det mest intressanta i vår upptäckt är att dessa makrofager som uttrycker AT_1 uppvisar tecken på syrebrist. Detta antyder att det skulle kunna finnas en koppling mellan syrebris och uttryck av AT_1. Något som ytterligare stödjer detta är att när vi utför våra analyser ser vi att uttryck av syrebristmarkören HIF-1α (hypoxia inducible factor-1α) korrelerar väl med förekomst av makrofager. Likaså korrelerar förekomst av makrofager ytterst väl med uttryck av AT_1. Detta öppnar för spännande möjligheter till ny forskning kring vilka effekter syrebrist och AT_1 uttryck i makrofager kan ha på inflammation och utvecklingen av ateroskleros. Om vi kan förstå dessa processer är det möjligt att vi kan lära oss att motverka dem och minska dödligheten och sjukligheten i hjärt-kärlsjukdom. Redan idag finns det läkemedel som blockerar AT_1; om vi kan visa att stimulering av AT_1 driver utveckling av ateroskleros skulle användningen av dessa läkemedel kanske komma fler till gagn.

Tillvägagångssätt

I vårt arbete har vi undersökt humana aterosklerotiska plack från frivilliga studiedeltagare som genomgått operation med kirurgiskt avlägsnande av plack i halskärlen. Till vårt förfogande hade vi 123 plack, dessa delade vi i mycket tunna snitt (4 mikrometer) som vi sedan färgade in med specifika antikroppar och färger för att detektera uttryck av makrofager, AT_1 och syrebristmarkören HIF-1α. Till varje färgning behöver man ett nytt snitt men eftersom snitten är så tunna blir det fortfarande samma del av placket man tittar på trots att det är ett nytt snitt. Efter färgning kunde vi fotografera placken i mikroskop för att sedan analysera dem med hjälp av ett datorprogram specialframtaget för forskningsanalys av olika

vävnader. Genom att inspektera de olika färgningarna för varje plack kunde vi se att AT_1 och syrebristmarkören HIF-1α båda uttrycks i områden där det finns rikligt med makrofager. När vi sedan med hjälp av vårt datorprogram gör en uträkning för att se hur mycket AT_1, HIF-1α och makrofager det finns i varje plack ser vi att mängden AT_1 korrelerar oerhört väl med mängden makrofager, ju fler makrofager desto mer AT_1. Vi ser också att mängden HIF-1α korrelerar väl med mängden makrofager, ju mer HIF-1α – det vill säga ju mer syrebrist – desto mer makrofager.

För att försäkra oss om att det vi ser i placken verkligen skulle kunna stämma undersökte vi humana makrofager som donerats av frivilliga givare och som vi placerade i cell odling (dvs. cellerna tas från sin naturliga miljö i kroppen och odlas i värmeskåp i näringsrik vätska). På dessa makrofager kunde vi sedan testa om de uttryckte AT_1 med hjälp av en teknik som kallas western immunoblotting, som i korthet går ut på att proteiner från celler separeras beroende på storlek och sedan färgas in med specifika antikroppar så att de kan detekteras. På detta sätt kunde vi fastslå att humana makrofager mycket riktigt uttrycker AT_1 och det vi observerat när vi undersökte placken skulle mycket väl kunna stämma.

Framtiden

Vi hoppas kunna forska vidare på vilken betydelse syrebrist, makrofager och AT_1 har för utvecklandet av ateroskleros. Förhoppningsvis kan även andra tänkas intressera sig för ämnet och hjälpa oss att undersöka detta. Visionen är givet vis att vi i framtiden skall kunna erbjuda bättre behandling för denna vanliga och svåra sjukdom som ateroskleros är.

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