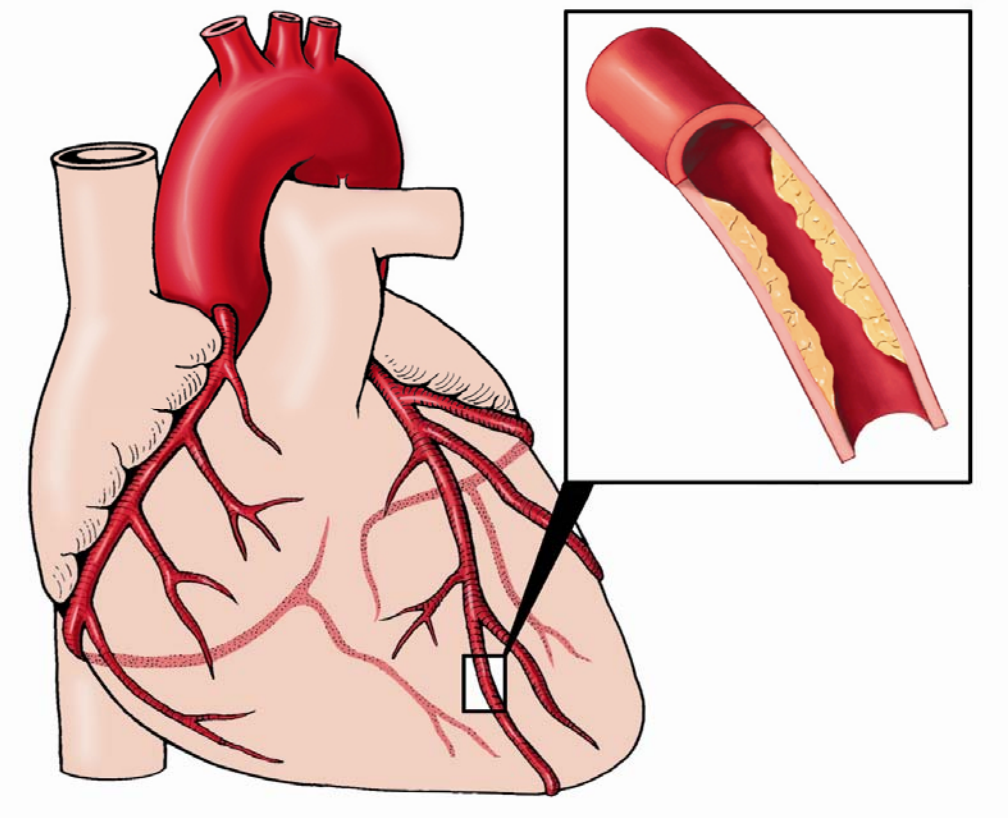
The road from atherosclerosis to myocardial infarction –studies in an experimental model

Anne-Louise Hemdahl



Stockholm 2005

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ABSTRACT

Atherosclerosis is a slow progressing disease with continuous lipid deposition and inflammation in the arterial wall. Stenotic arteries may cause angina pectoris but it is the sudden formation of an occluding thrombosis on the atherosclerotic plaque that leads to myocardial infarction. In a majority of cases such complications derive from plaque rupture or plaque erosion. Several steps in the series of events leading from silent atherosclerosis to myocardial infarction have remained unclear, in part due the lack of suitable animal models. This thesis has aimed to develop a mouse model in which myocardial infarction is triggered in order to study the physiological and molecular mechanisms that are important in the development of myocardial infarction.

Isoflurane anesthetized apoE -/- x LDLR -/- and C57BL/6J mice were exposed to systemic hypoxia by reducing the inhaled oxygen concentration to 10% for 10 minutes. Mental stress was induced in conscious mice by blowing pressurized air into the cage. Physiological parameters were recorded every 30 minutes for 2-6 days by implanted transmitters. To investigate whether thrombosis was involved a thrombin inhibitor was administered in a bolus dose before the stress and after the hypoxic stress by continuous administration by osmotic mini-pumps. Controls were given PBS. Infarction development was determined by troponin T analysis and histology. The plaques and hearts were isolated at different time points after hypoxia and analysed by transcriptional profiling and quantitative real time RT-PCR. On selected genes involved in plaque vulnerability, protein expression and activity was assessed by immunohistochemistry, western blot, zymography

The results show that myocardial infarction can be induced by hypoxic stress in a two-phase pathway: an initial phase comprising a transient ischemic response which triggers a delayed second phase of ischemia and myocardial infarction. Previous results show that the first phase is endothelin dependent and in our present studies we show that the first ischemic phase triggers a delayed phase which involves thrombin. Furthermore our data show that hypoxia induces expression of inflammatory, proteolytic and pro-coagulant genes that may promote a vulnerable and pro-thrombotic plaque environment and we hypothesise that these factors may lead to endothelial or plaque damage and an activation of haemostasis. This model should be a useful tool to investigate plaque activation, thrombus formation and infarction development.

Key words: atherosclerosis, myocardial infarction, ischemia, hypoxia, matrix metalloproteinase, remodelling, electrocardiogram, gene expression, inflammation

ABBREVIATIONS

apoE	*apolipoprotein E*
ATP	*adenosine triphosphate*
BSA	*bovine serum albumin*
cAMP	*cyclic AMP*
cDNA	*complementary DNA*
CMV	*cytomegalovirus*
CREB	*cAMP response element binding protein*
cRNA	*complementary RNA*
CSF	*colony-stimulating factor*
CXCL CXC	*chemokine ligand*
DMSO	*dimethylsulfoxide*
DNA	*deoxyribonucleic acid*
DNase	*deoxyribonuclease*
EC	*endothelial cell*
ECG	*electrocardiogram*
EDTA	*ethylenediaminetetraacetic acid*
ET	*endothelin*
FCS	*fetal calf serum*
IFN	*interferon (e.g., IFN-g)*
Ig	*immunoglobulin*
IκB	*inhibitory NF-κB*
IL	*interleukin*
i.v.	*intravenous*
JAK	*janus kinase*
JNK	*c-Jun N-terminal kinase*
kbp	*kilobase pair*
kDa	*kilodalton*
KO	*knock-out*
LDL	*low density lipoprotein*
LDLR	*low density lipoprotein receptor*
LPS	*lipopolysaccharide*
mAb	*monoclonal Ab*
MAPK	*mitogen-activated protein kinase*
MCP-1	*monocyte chemoattractant protein-1*
M-CSF	*macrophage CSF*
MEK	*mitogen-activated protein kinase kinase*
MI	*myocardial infarction*
mRNA	*messenger RNA*
NAD	*nicotinamide adenine dinucleotide*
NADH	*reduced NAD NADP, NAD phosphate*
NADPH	*NAD phosphate (reduced)*
NF	*nuclear factor*
NFAT or NF-AT	*nuclear factor of activated T cells*
NF-κB	*nuclear factor κB*
NGAL	*neturophil gelatinase-associated lipocalin*
NO	*nitric oxide*
oxLDL	*oxidized LDL*
PCR	*polymerase chain reaction*
RNA	*ribonucleic acid*
RNase	*ribonuclease*
RT-PCR	*reverse transcriptase polymerase chain reaction*
s.c.	*subcutaneous*
SD	*standard deviation*
SEM	*standard error of the mean*
SMC	*smooth muscle cell*
STAT	*signal transducer and activator of transcription*
TLR	*toll-like receptor*
TNF	*tumor necrosis factor*
TTC	*triphenyl tetrazolium chloride*
VCAM	*vascular cell adhesion molecule*

LIST OF PUBLICATIONS

This thesis is based on the following original papers and manuscript, which will be referred to by their Roman numerals:

I.

Hemdahl AL, Caligiuri G, Hansson GK, Thoren P.
Electrocardiographic characterization of stress-induced myocardial infarction in atherosclerotic mice.
Acta Physiol Scand. 2005 Jun;184(2):87-94.

II.

Hemdahl AL, Falk E, Thoren P, Hansson GK.
Thrombin inhibitor reduces myocardial infarction in apoE-/- x LDLR-/- mice.
Am J Physiol Heart Circ Physiol. 2004 Aug;287(2):H872-7.

III.

Hemdahl AL, Gabrielsen A, Thelin A, Thorén P, Hansson GK
Hypoxia-induced gene expression in atherosclerosis
Manuscript

IV.

Hemdahl AL, Gabrielsen A, Zhu C, Eriksson P, Hedin U, Kastrup J, Thorén P, Hansson GK
Expression of Neutrophil Gelatinase-Associated Lipocalin in atherosclerosis and myocardial infarction
Arterioscler Thromb Vasc Biol. 2005 In press

RELATED ARTICLES

NOT INCLUDED IN THIS THESIS:

Lowbeer C, Forsberg AM, Tokuno S, **Hemdahl AL**, Gustafsson SA, Valen G.
Cardiac troponin T content in heart and skeletal muscle and in blood samples from ApoE/LDL receptor double knockout mice
Clin Chim Act. 2004 Jun;344(1-2):73-8.

Madeddu P, Emanueli C, Spillmann F, Meloni M, Bouby N, Richer C, Alhenc-Gelas F, Van Weel V, Eeefting D, Quax PHA, Hu Y, Xu Q, **Hemdahl AL**, van Golde J, Huijberts M, de Lussanet Q, Struijker Boudier H, Couffinhal T, Douplà C, Chimenti S, Latini LR, Baumans V, Levy B.
Models of Myocardial and Limb Ischemia: Diagnostic Endpoints, Therapeutic Targets and Relevance to Clinical Problems.
Submitted for publication

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INTRODUCTION

ATHEROSCLEROSIS

Atherosclerosis is the underlying cause of myocardial infarction, stroke and peripheral arterial disease which are together the leading causes of death in developed countries[1]. Atherosclerosis is characterized by inflammation and accumulation of lipids in the subendothelial layer[2,3] that progress over decades. The atheroma is preceded by fatty streaks that are prevalent in young people but may or may not cause symptoms or progress into vulnerable plaques susceptible to rupture and thrombosis[4].

Risk factors

The initial steps of atherosclerosis, so called fatty streaks, have been reported in infants[5] and as for today there is no complete protection against atherosclerosis. However, some factors play significant roles in the development of atherosclerosis and may be modified in order to reduce the progression. Risk factors can be separated into two groups; modifiable and fixed. In the modifiable group lifestyle and behavioural factors are accounted for and includes cholesterol, blood pressure, smoking, diabetes and stress. The fixed group represents sex, age and family history.

Cholesterol High cholesterol (hypercholesterolemia) has in numerous studies been associated with the development of atherosclerosis. Cholesterol is taken up from the food in the intestine by chylomicrons and transported to the liver. In the liver free cholesterol, cholesterol esters, triglycerides and free fatty acids are encapsulated into VLDL that transports the lipids to muscle cells, cardiomyocytes and other tissues. While high levels of LDL are detrimental and plasma concentrations over 3mmol/L justify administration of lipid lowering drugs such as statins the fraction of HDL in plasma is capable of reverse cholesterol transport from atherosclerotic plaques and is therefore beneficial. Statins lower cholesterol levels but may also have direct effects on the vessel wall, thus rendering anti-inflammatory properties and stabilizing vulnerable plaques[6-9].

Blood pressure High blood pressure is over-represented in patients with ischemic heart disease. Although the connection is evident the mechanisms are not completely clear.

Smoking Smoking is considered a major external risk factor for a cardiac event and it is unusual that people younger than 50 years suffer from myocardial infarction unless they are smokers. Cigarette smoking generally doubles the risk for a cardiac event[10] and passive smoking has been shown to increase the risk by 30%[11].

Diabetes Approximately 20% of patients with myocardial infarction also have diabetes, in most cases type-2 diabetes. The incidence of cardiovascular disease is 2-4 fold higher in persons with diabetes as compared to non-diabetics and the risk is further increased by smoking. The prognosis after a heart attack is also worse in a diabetic as compared to a non-diabetic person. Statistics from the Swedish Heart Intensive care Admissions registry, RIKS-HIA, shows that one year after a myocardial infarction 13% of the men without diabetes and 22% of the diabetic men have died. In women the number is 14% and 26% respectively[12].

Family history Predisposing genetic factors include mutations, such as in familial hypercholesterolemia and allelic variants that make the patient more susceptible to atherosclerosis and its clinical complications. For example the apolipoprotein E4 allele is present in approximately 24% of the population and carries a relative risk for coronary heart disease of 1.5; this has led to the suggestion that this gene variant accounts for approximately 10-15% of all coronary heart disease[13,14].

Sex and age In Sweden cardiovascular disease accounts for approximately 26% of deaths in men and 21% in women[15]. Myocardial infarction is the most common complication of cardiovascular disease with approximately 25000 patients hospitalised each year, in which 2/3 represents men. Since 1995-2003 the average age for first infarction in men has increased from 69 to 72 years and for women from 74 to 78 years[15]. Mortality after myocardial infarction has declined and between 1996-2002 the one-year mortality after myocardial infarction decreased from 8% to 5% in ages <65 years and 20% to 14% for ages 65-75 years and in the age category over 75 years, a reduction from 38% to 34% was observed. Better pharmacological treatment and initiation are in part explanatory for this positive trend[16].

ATHEROGENESIS

Atherosclerosis is a slow progressing disease that starts in early adolescence preferentially as sites with hemodynamic strain[4]. The process is initiated by infiltration of lipids into the arterial intima[17]. In the intima LDL is retained by proteoglycans in the extracellular matrix[18,19] where it accumulates and undergoes modification such as oxidation (oxLDL)[20]. Oxidised LDL activates the endothelium[21,22] to express surface molecules such as vascular cell adhesion molecule-1 (VCAM) responsible for leukocyte adhesion[23]. Monocytes and lymphocytes adhere to the receptors and migrate into the subendothelial space where they become activated and differentiated. Macrophage differentiation leads to secretion of chemotactic proteins such as monocyte chemotactic protein (MCP-1) and growth factors such as macrophage colony-stimulating factor (M-CSF) which will enhance monocyte recruitment and migration and thus accelerate the inflammatory process[24,25]. Monocyte-derived macrophages express scavenger receptors (CD36, SR-A)[26] and toll-like receptors[27] on their surface for internalisation of various molecules to be destroyed such as pathogen-like molecules, bacterial toxins, apoptotic cell fragment and oxLDL. If, however, the macrophages removal of noxious particles is insufficient, LDL will accumulate as cytosolic droplets and ultimately turn the macrophages into foam cells[28].

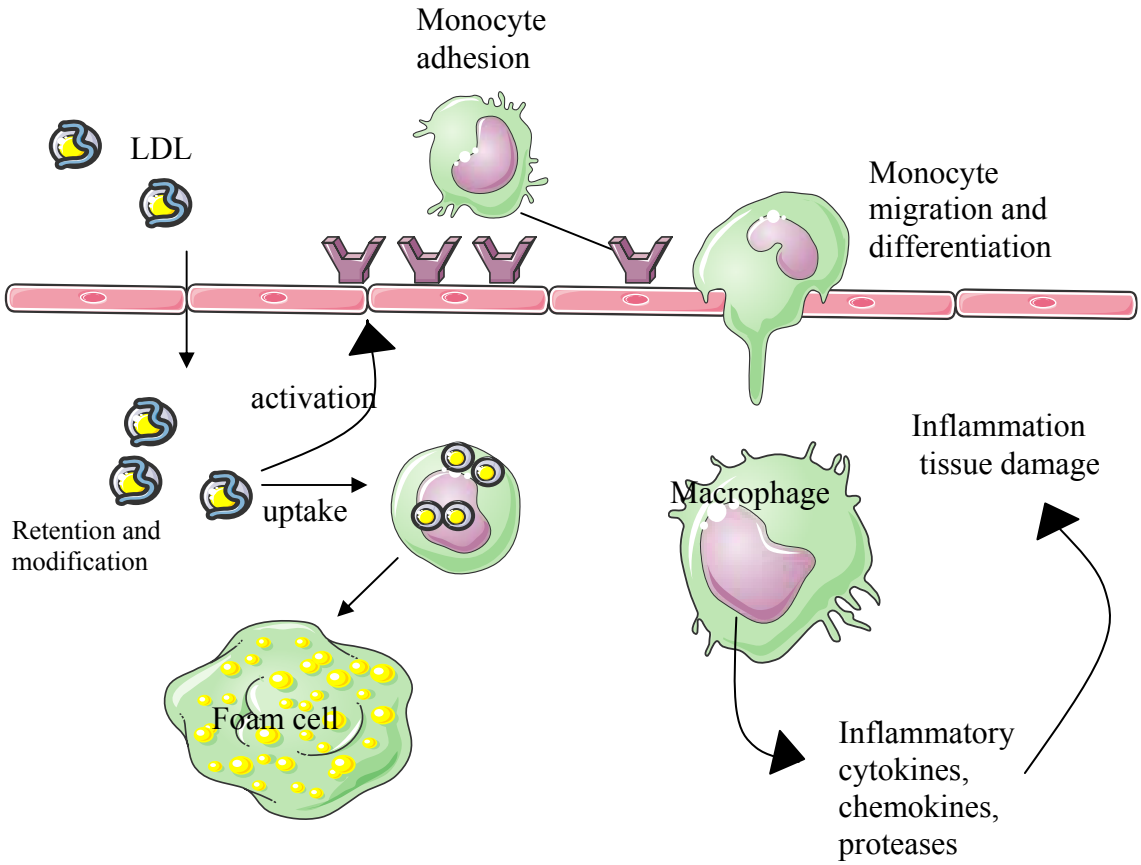


Figure 1. Schematic illustration of atherosclerosis initiation. Lipid retention (LDL) and modification leads to monocyte recruitment, macrophage differentiation and activation. Lipids are engulfed by macrophages which develop into foam cells.

Other cells present in the atheroma are T-cells[29], antigen presenting dendritic cells and mast cells[30]. Together with monocytes and macrophages these cells produce inflammatory cytokines[31] which activate macrophages and vascular cells leading to inflammation and progression of the disease.

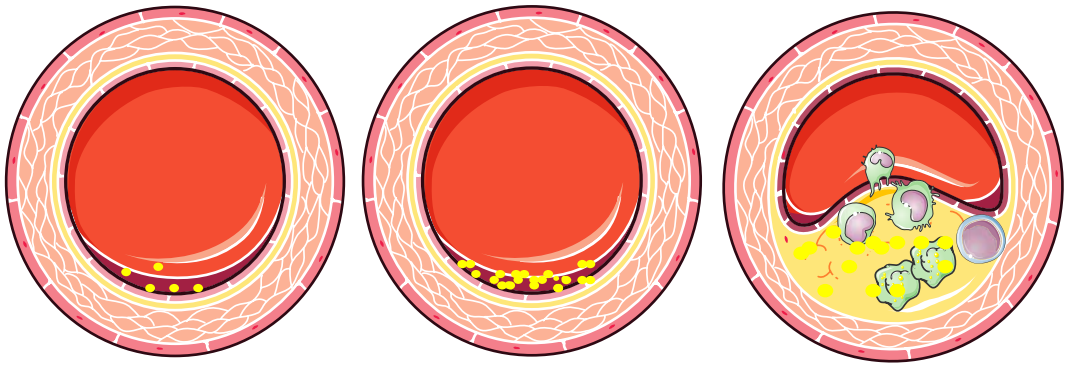


Figure 2. Atherosclerosis development in the arterial intima. Lipids accumulate in the arterial intima and the accumulation of foam cells and inflammatory cells will lead to progressive narrowing of the lumen.

MYOCARDIAL INFARCTION

Precipitation

Myocardial infarction occurs when blood supply does not meet the myocardial demand. It was previously believed to be caused by the mere stenosis caused by large plaques. However, several studies have shown that it is not the atherosclerotic narrowing of the lumen that causes the infarction but instead a thrombus at the surface of an activated plaque which causes a sudden coronary occlusion leading to myocardial infarction. Vulnerable plaques are characterized by a large lipid rich core[32,33], high content of calcification[34], high expression of inflammatory cells[35] and a thin fibrous cap[36]. Numerous studies have reported highly activated T-cells[37,38], macrophages[38,39] and mast cells[40,41] in vulnerable plaques that secrete proteases and pro-inflammatory cytokines that will enable a vulnerable plaque phenotype[42,43]. One of these cytokines is IFN-γ which inhibits collagen production and smooth muscle proliferation[44]. The reduced collagen production and the increased degradation of existing collagen by proteinases results in an imbalance of collagen and a degradation of the fibrous cap.

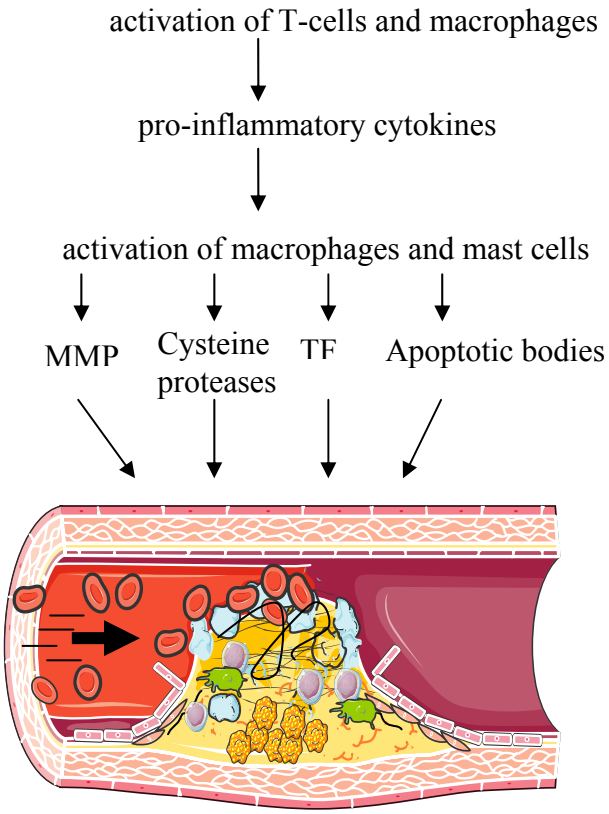


Figure 3. Hypothetic sequence of events leading to plaque rupture. T-cells and macrophages produce pro-inflammatory cytokines which activate macrophages and mast cells to produce factors such as MMPs, cysteine proteases and tissue factor (TF) as well as apoptotic bodies which may all lead to vulnerable plaques prone to rupture.

Coronary thrombosis may occur from plaque rupture or endothelial erosion[45]. Superficial erosion or denudation of the endothelial cells lining the plaque is sought to represent 25% of all fatal coronary thromboses and is though to occur in severely stenotic plaques and areas of highly activated macrophages[38]. Plaque rupture accounts for approximately 75% of major coronary thrombi and may occur at a critical moment when a threshold combination of hemodynamic, pro-thrombotic and vasoconstrictive forces is rapidly generated by external stressors such as emotional upset or physical activity during a period of plaque vulnerability[46-48]. Unlike denudated plaque the ruptured plaque is not necessarily the most stenotic one but is morphologically characterized by a large lipid core containing foam cells and calcified lipid droplet with a thin fibrous cap of collagen[49-52]. Ruptured plaque often exhibit signs of inflammatory/immune cell activation[45,51-53] and remodelling[54] which contributes to a vulnerable plaque phenotype. When a plaque ruptures the lipid gruel and underlying connective tissue matrix is exposed to the blood stream and can activate platelets and the coagulation cascade with simultaneous release of vasoactive substances that cause thrombus formation and vasoconstriction[55,56]. A severe disruption of the plaque may precipitate a thrombus sufficiently large to form an occlusive coronary thrombosis leading to myocardial infarction. A minor disruption may lead to a mural thrombus which may fail to produce symptoms or lead to unstable angina. Minor plaque disruptions and fissures may also be incorporated in the plaque, contributing to the expansion of the atheroma[57,58].

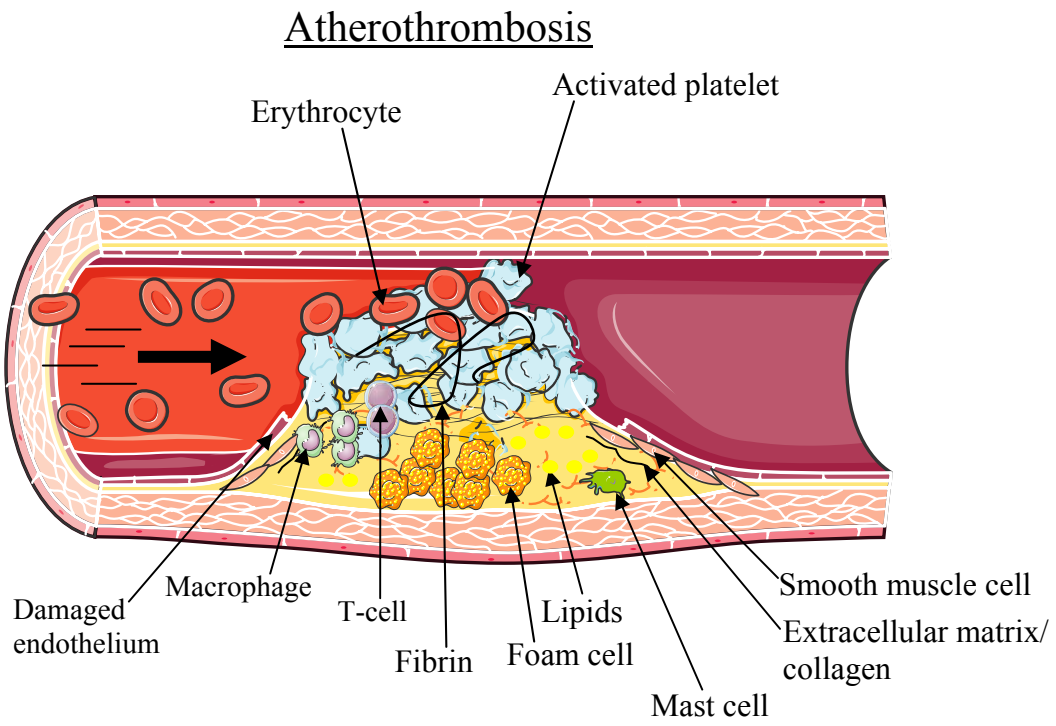


Figure 4. Cell components of atherothrombosis. Plaque rupture is the major cause of athero-thrombosis which exposes pro-coagulant factors on the extracellular matrix to the blood, thus leading to thrombus formation that may cause coronary occlusion and myocardial infarction.

Symptoms and diagnosis

Acute myocardial infarction usually gives rise to chest pain often radiating toward the left arm. Symptoms may vary, however, individuals and men and women, the latter experiencing more subtle symptoms. Myocardial infarction as a result of atherothrombosis is difficult to predict since the triggering factors for plaque rupture are unknown and the morphology of the plaque as imaged by angiography does not provide information about the vulnerability of the plaque. Intracoronary thermography can visualize the temperature of the plaque and may therefore provide an estimate of the inflammatory component which is indicative of a vulnerable plaque. However, the different imaging techniques are still under evaluation. Myocardial infarction prediction is difficult also since only 14% of myocardial infarction patients experience symptoms prior to their first infarction.

After cell death myocardial proteins leak out into the circulation. One of the earliest measurable markers of cardiomyocyte damage is Creatine phosphokinase (CK-MB) which can be detected within 4-6 hours of the onset of acute myocardial infarction. A protein with longer half-life is troponin T where an elevation can be measured at 3-9 hours at myocardial infarction, peaks at 12-18 hours at reperfusion and at 72 hours at permanent occlusion and stays high up to 14 days. Several studies have shown that troponin T correlates well with the size of infarction[59,60].

Electrocardiographic analysis aids the diagnosis of myocardial infarction by complementing the clinical and biochemical criteria. ST changes point to ischemia in the myocardium while pathological Q waves are diagnostic for permanent myocardial damage.

Treatment

Lysis of a small coronary thrombosis may occur spontaneously but for acute coronary events such as complete coronary artery obstruction immediate thrombolytic therapy or Percutaneous coronary intervention are necessary to minimize myocardial cell death. Streptokinase[61] and tissue-type plasminogen activator (t-PA)[62] are two widely used fibrinolytic agents. Alterations and modifications of the native t-PA molecule is today the subject of research in order to develop more effective and easily administered drugs[63]. Occlusive thrombosis may also be removed by Percutaneous Coronary Intervention (PCI) by dilating the narrowed artery. PCI involves a small catheter with a deflated balloon at the tip that is inserted into the narrowed part of an artery. The balloon is inflated, compressing the plaque and enlarging the inner diameter of the blood vessel so that blood can flow easily again.

Coronary thrombosis depends on platelet activation and humoral coagulation. Mediators of platelet activation and aggregation are thrombin, vasopressin, serotonin, tromboxane A_2, adenosinediphosphate (ADP), noradrenalin and adrenalin, collagen, platelet activating factor and for therapeutic interventions, inhibitors and antagonists of these mediators are used. To prevent thrombus formation by platelet aggregation cyclooxygenase (COX) inhibitors such as acetylsalicylic acid (ASA) are administered to irreversibly inhibit cyclooxygenase activity by acetylation of prostaglandin synthase and prevention of thromboxane A2 formation.

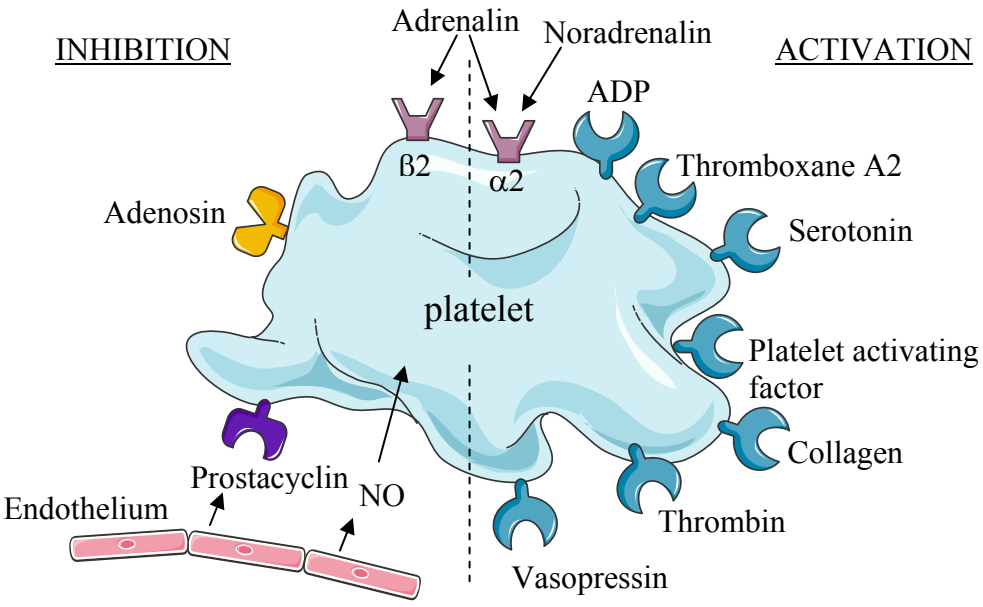


Figure 5. Regulation of platelet activity. Platelets display several receptors for ligands such as noradrenalin, thromboxane A2 and thrombin to name a few. Activation of platelets causes conformation changes that will cause platelet aggregation.

ADP-receptor antagonists give a selective reduction in the amount of available ADP-receptors on platelets but do not alter the affinity of the receptors. Glycoprotein (GP) IIb/IIa acts as a platelet aggregation receptor that upon stimulation binds to fibrinogen, fibronectin, vitronetin and von Willebrand factor. GP IIb/IIIa antagonists block the final common pathway of platelet aggregation and may therefore have therapeutic effects irrespectively of activation mechanism. A reduced mortality in myocardial infarction has also been observed after administration of NO-donors or nitroglycerine which inhibits platelet aggregation by increasing cyclic GMP in the platelet and by its vasodilatory effects[64].

Under normal conditions the arterial endothelium exhibit anti-thrombotic properties with release of nitric oxide and prostacyclin, which possess platelet inhibiting and vasodilatory effects. However, as described in the myocardial infarction chapter, damage to the arterial wall exposes the underlying matrix that is rich in pro-thrombotic material such as phospholipids, tissue factor and platelet adhesive matrix molecules to the blood and result in fibrin clot formation. The humoral coagulation system involves the extrinsic and the intrinsic pathways which are composed of distinct groups of coagulation factors but share the final sequence of activation which starts with conversion of factor X (X) to factor Xa (Xa). The generation of Xa initiates a common pathway that leads to the production of thrombin. Activation of thrombin enables the conversion of fibrinogen to fibrin which is the backbone of the clot. Coagulation can be inhibited at different steps in the cascade by inhibition of thrombin, factor Xa, tissue factor, fVIIa, or vitamin K-dependent factors[65] or by increased antithrombin or activated protein C[66].

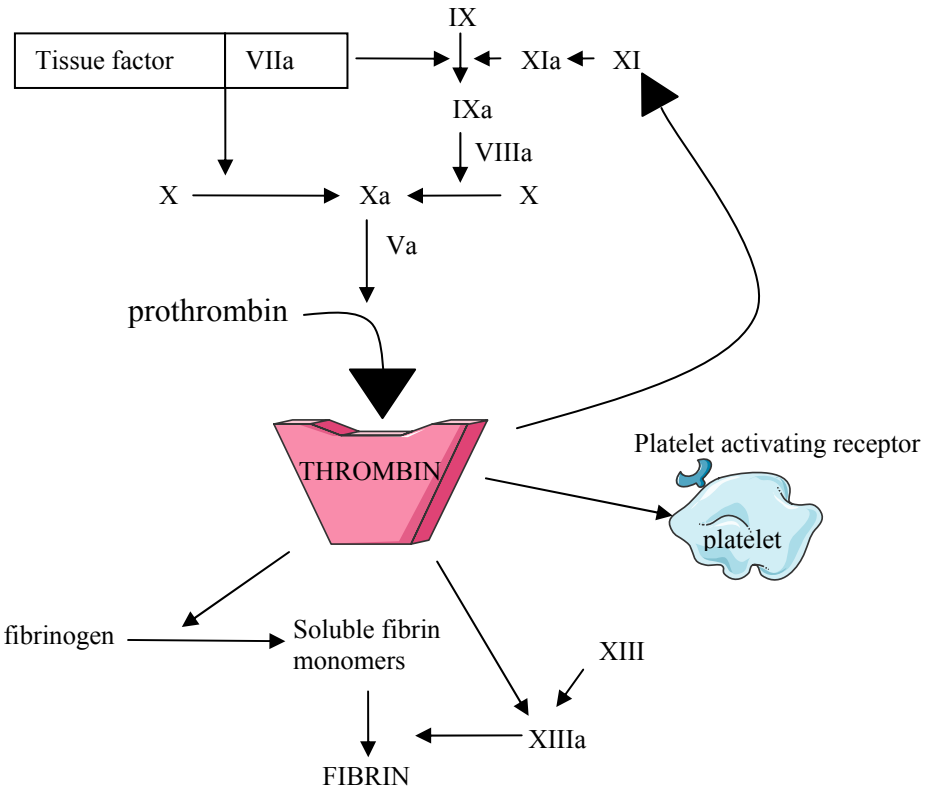


Figure 6. Schematic illustration of the coagulation cascade. The activation of coagulation factors leads to thrombin activation and fibrin formation.

MOUSE MODELS OF ATHEROSCLEROSIS AND MYOCARDIAL INFARCTION

A number of animal models have provided valuable insights into the progression of atherosclerosis, such as the rabbit, pig and non –human primate[67-71]. In recent years atherogenic mechanisms have been largely studied in mouse models, mainly due to the extensive genetic information available and the possibility to genetically engineer the mouse genome by insertion of new genes, insertions of mutants and removal or alteration of a specific gene.

Atherosclerotic inbred mouse strains

During normal feeding, mice carry their cholesterol in the HDL fraction but by enriching the diet to contain more saturated fat, cholesterol and cholate the plasma lipoprotein profile can be changed to carry the cholesterol in the VLDL fraction; this promotes atherosclerosis development in some inbred strains such as the C57BL/6J mice[72,73]. The lesions are fatty streaks characterized by deposition of cholesterol and macrophages. However the lesions are small and in contrast to humans the plaques are restricted to the aortic root and do not evolve beyond lesions containing lipid-laden macrophages. While C57BL/6Jl6 mice are most susceptible for atherosclerosis some strains such as the C3H and BALB/c strain are more resistant to atherosclerosis strain and SV129 is an intermediate[72].

Gene deletion and/or over expression as models of atherosclerosis

To modulate the lipoprotein profile various modifications have been made in genes regulating cholesterol metabolism. The most successful modulation has been on apolipoprotein E, which mediates lipoprotein interactions with several receptors. ApoE is present on the surface of chylomicrons, VLDL and HDL particles and a deficiency leads to hyperlipidemia, with an elevation of VLDL particles and atherosclerosis even when fed a normal diet [74,75]. On normal diet monocyte adhesion to endothelium was observed in 2 months old apoE -/- mice. At 10-30 weeks lesions contained predominately macrophage foam cells while fibro-lipid lesions became more apparent at 15 weeks and onward. The lesions were observed at sites typically affected in humans such as the aortic root, ascending aorta at branch points of several arteries[76,77] with similar morphological features as in humans[75,78,79]. Feeding the apoE -/- mice a diet enriched in saturated fat greatly accelerated the lesion progression but even though complex lesions were associated with media degeneration. Other perturbations of the apoE gene involve over-expression of mutant forms of the apoE gene and different isoforms of the human apoE gene[80,81].

Another knock-out model which induces atherosclerosis is a deletion in the LDL receptor. In contrast to humans where a deletion of the LDL receptor leads to dramatic phenotype and myocardial infarction in the second decade of life[82-84] the murine deletion leads only to modest hypercholesterolemia when fed a normal diet. However subsequent studies have demonstrated large atherosclerotic lesions even in the coronary arteries when fed a high

cholesterol diet[85] . No such changes were seen in wild-type mice that were fed a high-fat diet or LDL receptor knockout fed a normal diet. The LDL receptor knockout has been crossed with other genetically modified mice to examine the role of genetic modifiers in atherosclerosis. A combination of the apoE and LDLR knockout was made by the group of Ishibashi[86] where lesion progression was increased as compared to the individual knockouts. The apoE -/- x LDLR -/- compound knockout developed fatty streaks already after 4 weeks and atherosclerotic lesions at 2 months.

Mouse models of myocardial infarction

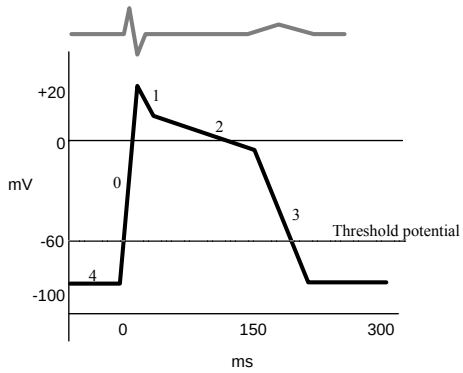
Cardiovascular ischemic events such as myocardial infarction and stroke are often due to thrombotic occlusion of atherosclerotic arteries. Although complex atherosclerotic plaques have been created in various mouse models, spontaneous thrombosis has been more difficult to precipitate. Spontaneous thrombosis formation in apolipoprotein E deficient (apoE -/-) mice on high cholesterol diet and older apoE -/- on normal diet has been reported[87,88] however the origin of the thrombosis have remained unclear and no model have so far been able to precipitate spontaneous thrombus formation. Thrombosis can however be induced artificially by chemical initiation of the coagulation cascade by thrombin injection, adenovirus or $FeCl_3$[88-91] or by mechanical denudation of the endothelial surface[92]. Myocardial infarction can be induced surgically in rodents when the left anterior coronary artery is ligated. These models are reproducible but do not show any resemblance to the human situation.

THE HEART

The action potential

The cardiac action potential depends on transmembrane movement of sodium, potassium and calcium and the membrane potential depends on concentration differences in Na^+, K^+, and Ca^{2+} across the cell membrane and opening and closing of channels that transport these cations. Most channels are opened and closed by changes in the membrane voltage and are called voltage-gated channels while others are opened by neurotransmitters, hormones, metabolites and/or drugs and are called ligand-gated channels. In the resting ventricular cells mostly K^+ channels are open.

A)



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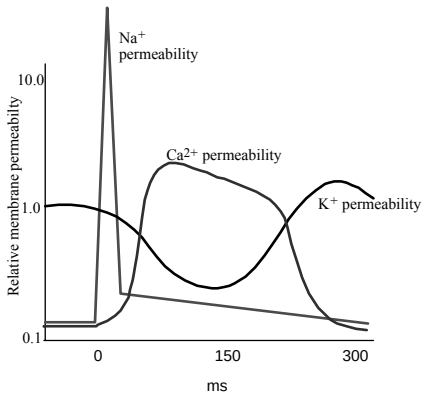

Figure 7. Action potential and corresponding ion channel activity. **A)** Illustrates the depolarisation and repolarization phase of the action potential and **B)** the ion channel activity during depolarisation and repolarization.

Depolarization occurs when the membrane potential moves away from the K^+ equilibrium and towards the Na^+ potential via voltage gated Na^+-channel (fast I_{Na}). This causes a rapid rise in voltage and represents phase 0 in the action potential. When Na^+ approaches equilibrium the inside of the cell becomes positively charged as compared to the outside and will therefore decrease the number of open Na^+ channels and open the outward transient rectifying K^+ channels (I_{to1}) again. This will repolarize the membrane and represent phase 1 in the action potential. In phase 2 the opening of long lasting Ca^{2+} channels (I_{CaL}) in combination with the closing of K^+ channels (I_{K1}) give rise to a plateau in the action potential. Phase 3 reflects that Ca^{2+} channels have subsequently closed and particular outward delayed rectifying K^+ channels (I_{Kr} and I_{Ks}) have opened in a delayed response to depolarisation and the membrane potential drives toward the K^+ equilibrium state. In phase 4 the resting membrane potential is

maintained primarily by K^+ channels that are open at highly negative membrane potentials (I_{K1}). They are called inward rectifying K^+ channels because when the membrane is depolarized (by the opening of Na^+ gated channels) they do not permit outward movement of K^+.

Electrocardiogram (ECG)

The electrocardiogram represents the electrical activity of the heart. The cardiac electric impulse is generated in the sinoatrial (SA) node by pacemaker cells and is rapidly conducted through the atrium to the atrioventricular (AV) node. From the SA node approximately 80 beats per minute are transported to the AV node. In the AV node the action potential is delayed to 60 beats per minute since the tissue is fibrous and does not transmit the signals as well as the specialized cells. From the AV node the signals is conducted through the His bundle and bundle branches, finally leading to excitation and contraction in the ventricular myocytes.

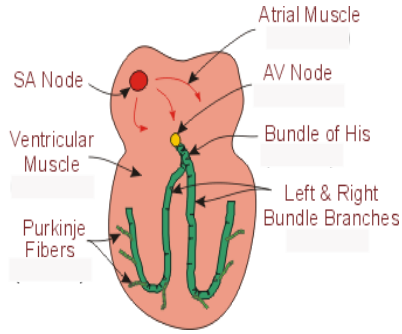

Figure 8. The myocardial conductance system. The electrical activation of the heart starts in the SA node in the atrium and is transported via the AV node to the bundle branches and purkinje fibers in the ventricular wall.

By placing sensing electrodes on the surface of the body the continuous depolarisation and repolarization of the heart can be recorded. As the myocardium depolarizes and repolarizes the membrane potential gives rise to a dipole that can be measured. An electrode facing an approaching wave of depolarisation records a positive potential, which registers an upright deflection in the ECG while if depolarisation moves away from the recording electrode a down ward deflection is recorded. The positive and negative deflections in the electrocardiogram show the direction and the amplitude of the depolarisation and repolarization, respectively, and the sensors amplify the signal that spreads through the cells in the atrium to the ventricle and gives a plot of the voltage as a function of time.

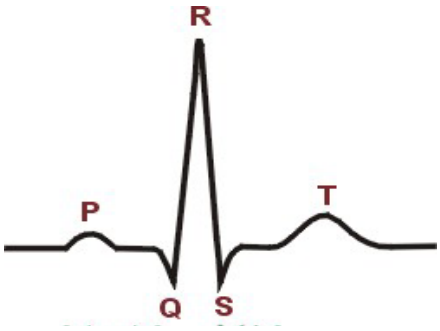

Figure 9. Electrocardiogram describing the depolarisation and repolarization of the atrium and ventricle. The P wave represents atrial depolarisation. The QRS complex corresponds both to the repolarization of the atrium and the depolarisation of the ventricle however, since the ventricle wall is thicker than the atrial wall a positive deflection is registered. The T wave represents the ventricular repolarization.

The voltage change associated with atrial excitation appears on the ECG as the P wave. The QRS segment incorporates both the repolarization of the atrium and the depolarisation of the ventricles but since the mass of the ventricles is bigger than the atrium the small repolarization currents is hidden in the large positive wave. The ST segment is the period between the end of the S wave and the beginning of the T wave. In normal conditions the ST segment is isoelectric, or nearly so because all ventricular muscle is depolarised which means that all cells are in phase 2 of the action potential. The T wave represents a long action potential in the subendocardial cells as compared to the subepicardial cells. The long depolarisation of the subendocardial cells results in an epicardial repolarization before the subendocardium although the endocardium was the first to depolarise, therefore the dipole will give rise to a positive deflection of the T wave as seen in the ECG.

Electrocardiogram in myocardial infarction

The three main ECG changes that occur in myocardial infarction are pathological Q waves, ST-segment elevation and T-wave depression, which mirror different pathological changes. Pathological Q waves are due to myocardial necrosis, which gives an electrically inactive area in which the electrode "sees through the dead myocardium" and looks at the other wall of the ventricle where the electrical force moves in the opposite direction from the endocardium through the ventricular wall. The pathological Q wave appears early in infarction and remains throughout life. Acute myocardial ischemia may alter ventricular action potentials by inducing lower resting potential, decreases amplitude and velocity of phase 0 and an abbreviated action potential duration (pathologic early repolarization). These electrophysiological effects create a voltage gradient between ischemic and normal cells during different phases of the cardiac electrical cycle. The resulting current of injury is reflected on the electrocardiogram by deviation of the ST segment. T wave depression is believed to occur as an effect of oxygen depravation on the course of repolarization. Inverted T waves often succeed ST elevations and the deep coronary T waves may remain for weeks or months before they normalize. Transmural infarctions that cover the whole ventricle wall most often exhibit all three ECG changes at different times. If only a part of the ventricle wall is affected by the infarction it is a subendocardial infarction and may display a reduction in the amplitude of the R wave and ST- and T wave changes.

Human versus mouse electrocardiogram

The electrocardiogram in mice differs some from the human one. To enable a heart rate of 500-600 beats per minute, the mouse relies mainly on rapid I_{to} repolarization currents while humans rely on the I_{Kr} and I_{Ks} ion channels. Mice do express the I_{Kr} and I_{Ks} ion channels but the specific ion channels become inactive after embryonic development[93]. In the ventricular repolarization phase the inactivity of I_{Kr} and I_{Ks} ion channels results in a short and steep two-phased notch in the monophasic action potential in the mouse as compared to the plateau phase in humans (illustrated in figure 10). The lack of plateau phase is reflected on the mouse ECG where the T wave starts immediately after the end of the S-segment and in the work of Danik et is called the b and c area[94].

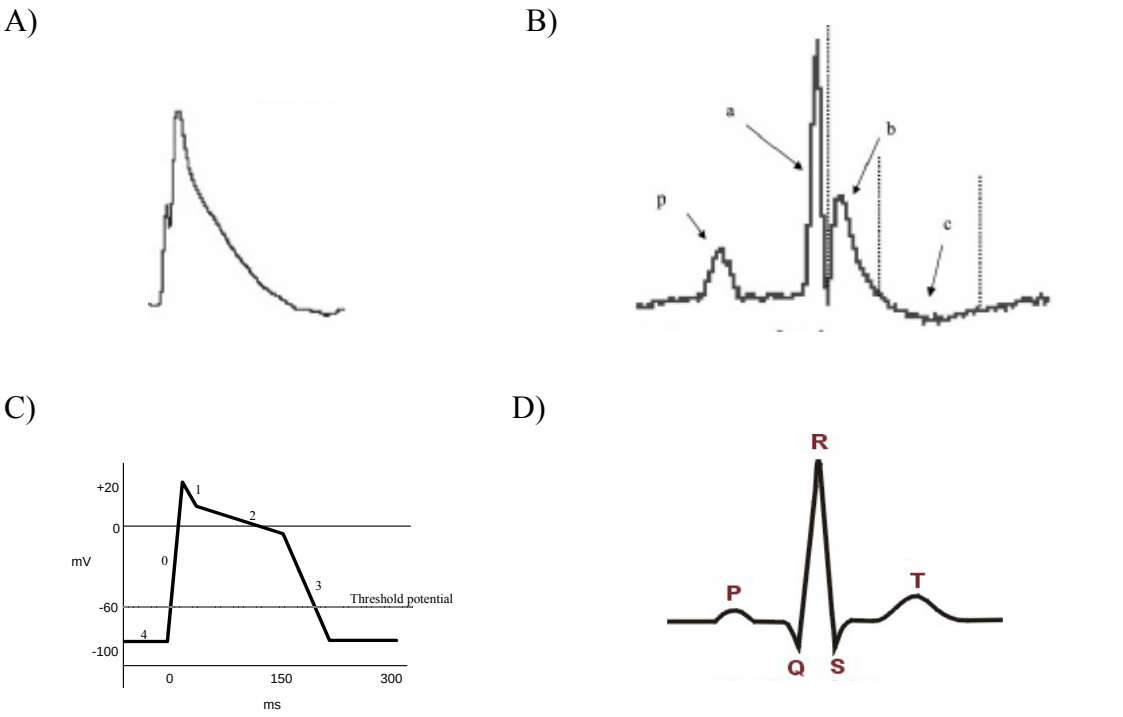

Figure 10. Action potentials and ECG recordings in mouse and human. **A)** Depicts a monophasic action potential and **B)** ECG from mouse as modified from Danik et al[94].
C) Illustration of a monophasic action potential and **D)** ECG of human origin. The frequency of the mouse ECG is approximately ten times the human.

HYPOXIA

Acute responses to hypoxia are mainly due to O_2 sensitive ion channels which mediate adaptive changes in the cellular excitability, contraction and secretory activity. Chronic responses depend on the modulation of hypoxia-inducible transcription factors, which determine the expression of numerous genes encoding enzymes, transporters and growth factors[95].

Episodes of low oxygen supply activates several chemosensory systems to rapidly modulate pulmonary ventilation and perfusion as well as blood circulation to optimize the supply of O_2 to metabolizing tissue. These responses involves specialized chemosensory cells such as the carotid bodies present in the arterial circulation and the neuroepithelial bodies present in the airways and the direct response of vascular smooth muscle cells[96,97]. Peripheral vessels dilate in response to low oxygen whereas the vessels of the pulmonary vasculature contract to shunt blood away from poorly ventilated regions[98-102]. Constriction is initiated by inhibition of one or several K^+ channels which set the membrane potential[103-105]. The resulting depolarisation activates voltage gated Ca^{2+} channels, which raises the cytosolic calcium and leads to myocyte constriction. Although K^+ channels are the effectors of hypoxic pulmonary vasoconstrictor it is not clear whether they are under the control of a O_2 sensor or if they can sense O_2 changes *per se*. Carotid and neuroepithelial bodies responds to hypoxia by initiating activity in efferent chemosensory bodies by closure of K^+ channels which leads to membrane depolarisation and Ca^{2+} influx. Increased cytosolic calcium concentration provokes neurotransmitter release and activation of efferent sensory fibres[106,107]. Cell death occurs when oxygen supply fails to meet the demand and thus leads to membrane damage or initiation of death program[108]

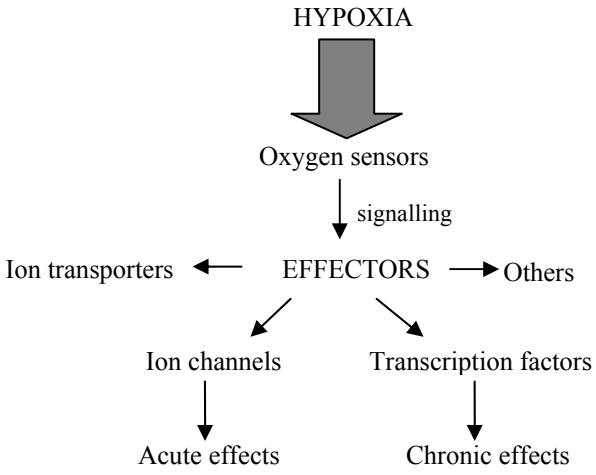

Figure 11. A schematic illustration of hypoxia sensing. Hypoxia may induce a rapid response modulating pulmonary ventilation and perfusion or an adaptive response with transcriptional activation. Illustration modified from Lopez et al[95].

Several responses are developed by cells and tissues to adapt to low oxygen. Increased ventilation and cardiac output and switch from aerobic to anaerobic metabolism. Most of these changes take place early at the onset of hypoxia but later on there is a transcriptional response mediated in large part by the action of the transcription factor hypoxia-inducible factor 1 (HIF-1) to induce expression of genes important for adaptation to low oxygen. HIF-1 is a heterodimeric transcription factor consisting of an inducible α and a constitutively expressed β subunit of basic helix loop helix (bHLH) character[109]. During normoxia the HIF-1α protein is rapidly degraded by von Hippel-Lindau protein (pVHL)[110,111] while during hypoxia the protein accumulates to form a heteromdimer complex with HIF-1β/Arnt, translocates into the nucleus and binds to the promoter region of targeted genes. The most well known target gene is vascular endothelial growth factor (VEGF)[112] which in numerous studies has been shown to play an important role in angiogenesis and tumour growth[113]. Other down-stream genes of HIF-1α are erythropoietin, endothelin-1, glucose transporter -1, -3, heme oxygenase-1, insulin-like growth growth factor 2 (IGF-2), LDL receptor related protein-1, inducible nitric oxide synthase, phosphofructokinase, plasminogen activator inhibitors-1, transferrin and the VEGF receptor FLT-1[114,115].

AIMS OF THIS STUDY

Cardiovascular disease has for long been the primary causes of death in developed countries, and it is rapidly becoming the number one killer in the developing countries. In Sweden 50% of the population dies from manifestation of atherosclerosis and every year more than 19 million people world wide experience an acute cardiovascular event most commonly myocardial infarction. Therefore there is a considerable demand for diagnosis and treatment of the pathologic conditions that underlie these dramatic clinical events.

This thesis aims to:

- Introduce an experimental model to study the development of myocardial infarction.

- Investigate physiological mechanisms involved in infarction development in this system.

- Screen for potential target genes that as a result of hypoxic stress promote plaque vulnerability, thrombosis and myocardial infarction.

METHODOLOGICAL CONSIDERATIONS

EXPERIMENTAL MODEL

Animals and diet

In our study we used male apoE -/- x LDLR -/- [79,86] mice from M&B Taconic. To enhance atherosclerosis progression the mice were fed a "Western-type" diet (Lactamin AB, Kimstad, Sweden) containing corn, starch, glucose, sucrose, cocoa butter, cellulose, minerals, vitamin mix, 0.15% cholesterol and 21% total fat (wt/wt) for 6-8 months. The long time on diet was necessary to obtain the severe atherosclerotic plaques that could precipitate myocardial infraction in the double apoE -/- x LDLR -/- mice. Studies from our group showed that an equally long diet in apoE -/- single-knockout mice did not result in myocardial infarction until after 1 year on diet (unpublished data). In studies where normal C57BL/6J mice were used they were fed a normal diet to be able to compare atherosclerotic mice with healthy non-atherosclerotic mice (paper I, II and IV).

Mental and hypoxic stress

Mental stress was induced by blowing pressurized air (100 mmHg) from an air compressor into the cages of conscious mice. The sound from the air caused anxiety and the provocation lasted 30 minutes. Hypoxic stress was performed in isoflurane anaesthetized mice. In the breathing mask the oxygen concentration was reduced gradually from 21% to 16, 14, 12 and finally 10%, each for 2 minutes. After the hypoxic stress, oxygen supply was returned to 21% and the mice were allowed to regain consciousness and return to their normal habitat. Control atherosclerotic and C57BL/6J mice were exposed to anaesthesia for 30 minutes. In most studies the mice were anesthetised and euthanized after 48h, a time point chosen because biochemical markers start to become measurable in the blood and morphological signs such as cell oedema and necrosis in tissue have become visible[59,116-118].

A) B)

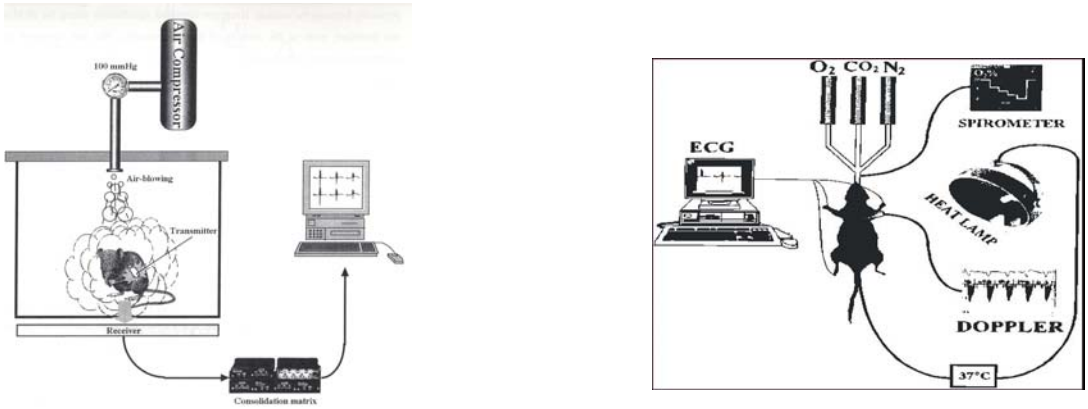

Figure 12. Experimental setup of mental and hypoxic stress. **A)** Pressured air is blowing into the cage of the conscious mouse. **B)** Oxygen concentration is reduced in the breathing mask of an anesthetised mouse. The electrocardiogram, blood flow and temperature are monitored simultaneously.

Diagnosis of myocardial infarction

To diagnose which apoE -/- x LDLR -/- mice developed myocardial infarction after hypoxic stress, serum was analyzed to measure troponin T concentrations. In humans troponin T concentration > 0.2 μg/L is the threshold for diagnosing infarction but for mice acute myocardial infarction was considered present if troponin T concentration was ≥ 0.01 μg/L since all levels above this threshold were associated with electrocardiographic and histological signs of myocardial infarction. In humans troponin T elevation is seen after 3-9 hours, peaks at 12-18 hours at reperfusion and at 72 hours at permanent occlusion and stays high up to 14 days. An earlier marker of infarction in humans is Creatine phosphokinase of cardiac origin (CK-MB), which can be detected within 4-6 hours after the onset of acute myocardial infarction. We also analyzed CK-MB in some of our samples but the results were not consistent with ECG changes or morphological signs of myocardial infarction, therefore we chose not to use CK-MB for diagnosing myocardial infarction. While the specificity of CK-MB in mice remains unclear, several reports have shown troponin T levels to correlate well with infarction size[59,60].

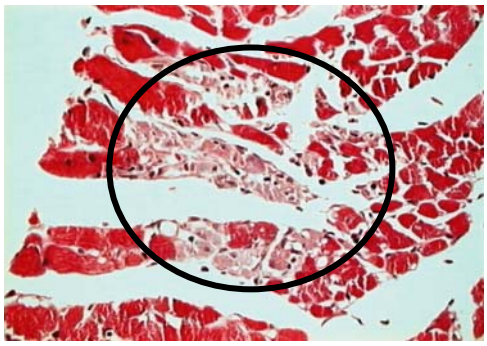

Figure 13. Masson tri-chrome staining of a recent MI in an apoE -/- x LDLR -/- mouse. The infarction area (circle) is characterized by pale staining and high cell density (from paper I).

Histological analysis was also performed by using elastin-trichrome staining which renders elastin black, collagen blue, and muscle and blood components red. The earliest signs of myocardial cell death swelling of the cardiomyocytes and neutrophil infiltration in the infarcted area and can be appreciated after approximately 1-2 days[116,118]. In old myocardial infarctions however the cells have been replaced by fibrous tissue and scar formation which lacks the contractile function. Therefore the early signs of stress-induced infarctions could be separated from spontaneous infarctions which had occurred previously and independent from our stress treatment.

PHYSIOLOGICAL ANALYSIS

Electrocardiogram

The electrocardiogram was recorded by a two limb (upper right leg – lower left leg) lead connection during the acute hypoxic stress. To allow long-term monitoring of physiological parameters such as heart rate, body temperature and ECG a telemetry system was used.
The telemetry transmitters, with a two-lead limb connection attached subcutaneously, were implanted in the abdominal cavity one week before data collection[119]. Data was recorded once every half hour and transferred to a microcomputer. ECG analysis was performed with PcLaB software v.5.0 [120].

A) B)

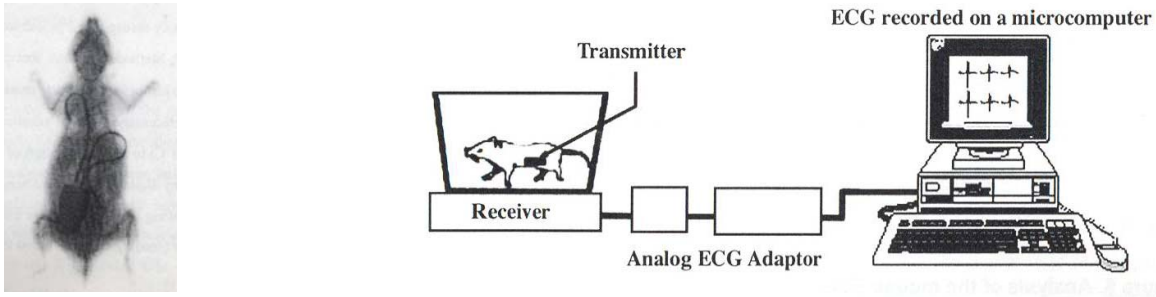

Figure 14. Telemetry setup. **A)** Radiographic image showing the position of the transmitter in an anesthetised mouse. **B)** The mouse cage is placed on top of a receiver that transmits the signal to a computer which records data on ECG, heart rate and body temperature. Pictures are modified from Kramer et al[119].

Data sampling and evaluation

Data on ECG, heart rate and body temperature was collected every half hour during 24 hours before the mental and hypoxic stress and for 48 hours afterwards. The measured area was called the STU segment area and corresponded to the b and c area described by Danik et al[94] as the mouse ventricular repolarization phase. The STU segment surface area was analyzed in PcLaB software[120] and calculated as the total area deviating from the isoelectric line, defined as the average level in an interval from 10 msec before the Q wave, until the end of the STU segment when the ECG returned to the isoelectric line. To asses the variability after hypoxic stress, the standard deviation was calculated of all STU segment area recordings in the baseline state and compared to the standard deviation of all STU segment area measurements after the stress challenge. The change in standard deviation was defined as Δ-variability (Δ-variability = Abs (SD^{STU}_{after} - SD^{STU}_{before}, in which SD^{STU} is the standard deviation of STU segment area). Δ-variability was calculated as the absolute (abs) change, thus including both STU segment area elevations and STU segment area depressions.

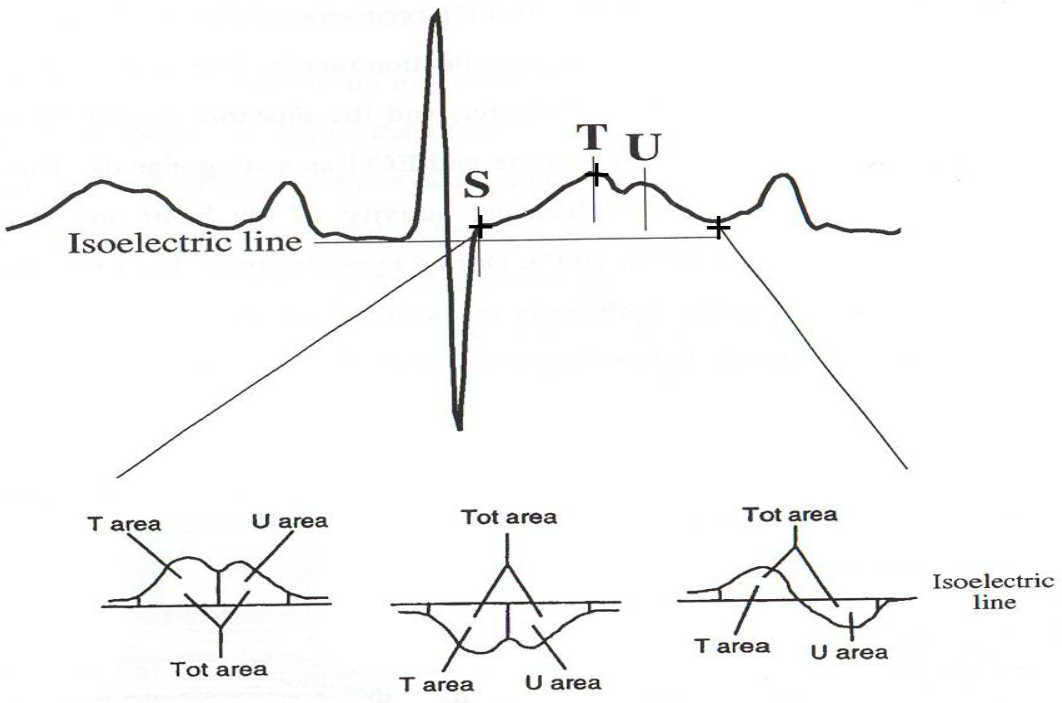

Figure 15. Analysed area of the mouse ECG. From the PcLab software the operator manually defined the STU segment with precursors. Both STU segment elevations and deflections as compared to the isoelectric line were included.

DRUG TREATMENT

Melagatran is the active form of the oral, direct thrombin inhibitor ximelagatran (Exanta™) which is used for prevention and treatment of thromboembolism. Melagatran is a potent, rapidly binding, competitive inhibitor of human α-thrombin which inhibits thrombin activity [121,122]. It effectively inhibits both free and clot-bound thrombin. In comparison with the widely used warfarin, ximelagatran has several advantages including a more shallow dose-response curve and consequently a wider therapeutic index between the anticoagulant effect and bleeding, a rapid onset of action, a low potential for drug-drug interactions and no food-drug interaction[123]. Therefore we used melagatran to investigate if thrombin was involved in the development of myocardial infarction after hypoxic stress.

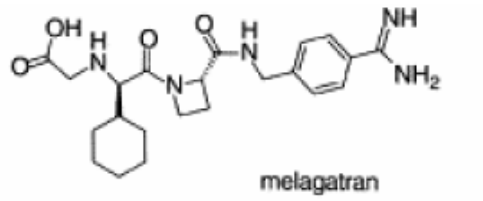

Figure 16. Chemical structure of melagatran.

GENE- AND PROTEIN EXPRESSION

Immunohistochemistry and quantitative real-time RT-PCR

Immunohistochemistry was used to localize specific proteins in 5-10μm thin sections by using monoclonal or polyclonal antibodies recognising the protein of interest. This method was used for protein localisation in the tissue but also for semi-quantitative analysis, as in paper II where fibrinogen positive cells were quantified in cardiomyocytes of a heart section to determine the infarction area.

To analyse total tissue expression of genes we used the quantitative method real-time RT-PCR in which the mRNA of a specific gene is analysed by a fluorescent detection principle. While this method is precise, it does not provide information about the cellular localisation of the expressed gene.

In situ zymography

Plaque remodelling may play an important role in the development of myocardial infarction therefore proteolytic activity of MMP-9 in murine atherosclerotic plaques was analyzed by zymography. Degradation of quenched fluorescein-labeled gelatin was visualized in a fluorescence microscope and to ensure that the signal originated specifically from MMP-9 activity and not from MMP-2, a MMP-2-inhibitor was added to the gel-mix at preparation. Generation of proteolytic activity was prevented in controls by addition of phenanthroline and EDTA.

Microarray analysis

Global microarray chips for the murine genome were used to screen the transcript profile of atherosclerotic mouse hearts and plaques at different time points after hypoxia. Two different chips were used because during the course of our studies the Affymetrix MG_U74Av2 chip was replaced by the MG_430A 2.0 chip. The two chips contain oligonucleotides complementary to the same 20,000 transcripts of the murine genome. In a bioinformatics analysis no significant mismatch was detected between sequences in the two chips. Previous studies have estimated Affymetrix chip arrays to contain as many as 5% false positive and false negative genes[124], therefore array results should be interpreted with caution. In our study the arrays were used as hypothesis generators and genes of interest were verified by quantitative real time RT-PCR to demonstrate differences between groups.

RESULTS AND DISCUSSION

The stress model first described by Caligiuri et al[125] showed that mental stress and also brief systemic hypoxic stress induce a reversible Endothelin-dependent ischemic phase which results in myocardial infarction in approximately 50% of atherosclerotic mice. In this thesis, we have found that the first ischemic phase triggers a second and irreversible phase that involves thrombin generation[126], since administration of a thrombin inhibitor reduced infarction. This stress model resembles several components of coronary occlusion presaging clinical myocardial infarction, with pre-existing coronary atherosclerosis, vasospastic events, coronary thrombosis, electrocardiographic signs and leakage of intracellular cardiomyocyte proteins. It may therefore be an important tool to study the mechanisms leading from silent atherosclerosis to acute myocardial infarction.

There are some limitations of this model however, making it laborious to use for example for infarction area measurement since infarctions are developed in a scattered pattern and are small in comparison to infarctions from the ligature model. Also the direct location of the infarction can only be observed post mortem by thorough histological examination of serial sections from the heart. To analyse myocardial changes after infarction other models such as ligation of the coronary artery may be more suitable since the infarction areas is easy to assess. Although the ligation model enables quantification of tissue and cell-specific changes occurring after myocardial infarction, it does not provide much information about the series of events precipitating myocardial infarction, and infarction experiments with this model far too often exclude the complexity of atherosclerosis when performed in non- atherosclerotic mice. In the stress model, however, atherosclerosis is necessary for infarction development and without severe atherosclerosis myocardial infarction cannot be precipitated. This shows that advanced atherosclerosis is indeed necessary for myocardial infarction development in this mouse model. In conclusion the stress model illustrates an authentic infarction development. It thus resembles the human situation.

A TWO-PHASE PATHWAY TO INFARCTION (*paper I*)

In the initial study of the model[125], hypoxia was shown to induce ECG changes in the atherosclerotic mice as a sign of ischemia[125]. The acute stress phase was mediated by endothelin, since administration of an endothelin-receptor antagonist reduced troponin T release.

Histological analysis of the infarcted hearts showed an early phase of cell death (day 1-3), followed by inflammatory infiltration (day 3), removal of dead myocytes, development of granulation tissue (day 7) and subsequent healing by a dense collagenous scar (day 21)[125]. Brief ischemia induced myocardial infarction in these mice but interestingly the acute ECG changes were returned to baseline when oxygen was normalized, thus suggesting that the infarction occurred in a later phase. To further investigate the series of events leading from stress to myocardial infarction, we implanted transmitters into the abdominal cavity of the mice to continuously record ECG, heart rate and body temperature after hypoxic and mental stress.

The results showed that after mental stress 50% (4/8) of the apoE -/- x LDLR -/- died after developing severe and irreversible STU segment elevation approximately 12-24 hours after the stress. The signs after hypoxic stress were more subtle and were characterized by irreversible STU segment variability. The variability consisted of steep shifts between STU segment elevation and T wave inversion (STU segment area depression). To calculate the variability change after stress we used Δ-variability, which was defined as: Δ-variability = $Abs(SD^{STU}_{after} - SD^{STU}_{before})$, in which SD^{STU} is the standard deviation of all recorded STU segment areas. The Δ-variability was calculated as the absolute (abs) change, thus including both STU segment area elevations and STU segment area depressions. It was also shown that high Δ-variability was indicative of cell death since Δ-variability correlated to the amount of released troponin T in the atherosclerotic mice. Interestingly, a delay between plaque rupture and onset of myocardial infarction has been described in humans[127] suggesting that the observed ECG variability in our study reflected vasospasms or dynamic turnover of thrombosis and thrombolysis. In non-atherosclerotic mice acute hypoxia induced a non-significant STU segment change as compared to atherosclerotic mice and did not result in STU elevations, high STU segment Δ-variability or troponin T release.

In essence, the mental stress exposure resulted in death of 50% of the mice while hypoxia displayed a more subtle reaction. Overall, the two stress models shared the ECG pattern of a two-phase pathway for the infarction development: an initial phase comprising a transient ischemic response, which triggered a delayed second phase of ischemia and myocardial infarction. This shows that increased STU segment area variability was indicative of myocardial infarction development in atherosclerotic mice following ischemic stress.

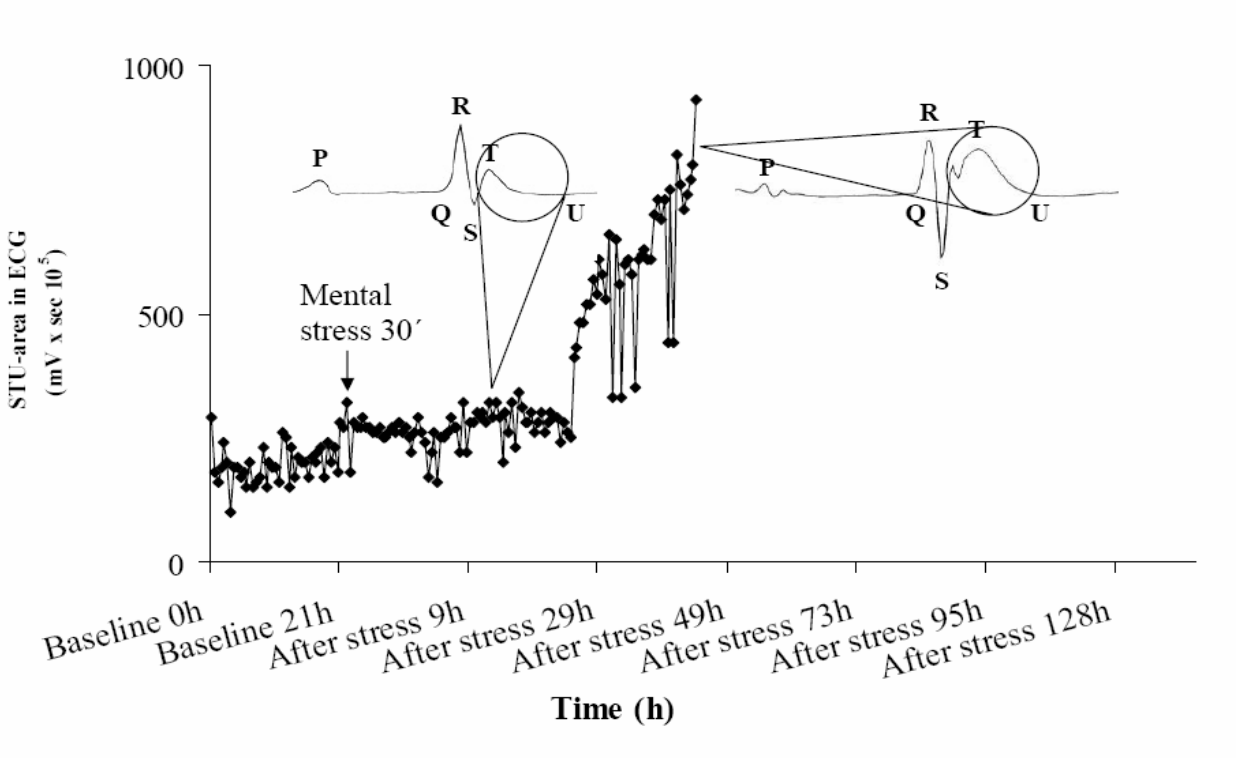

Figure 17. Graph displaying the STU segment area 24h before stress and during 30 minutes of mental stress and the following 6 days. Mental stress triggered a transient ischemic response followed by a second irreversible phase of ischemia and death before the end of experiment.

THROMBIN ACTIVATION IN STRESS-INDUCED INFARCTION (*paper II*)

Myocardial ischemia is characterized by vasoconstriction and thrombosis, which may be the cause and consequence of each other. Activated platelets can induce vasoconstriction by releasing thromboxane A2 and serotonin[55,128] while vasoconstriction may promote plaque rupture and thrombosis[129,130]. Inhibition of the vasoconstrictor endothelin, by endothelin receptor antagonists have demonstrated beneficial effects on pulmonary artery hypertension[131] while clinical trials on heart failure have given mixed results[132]. In our model we have previously shown that endothelin is responsible for the initial ischemic response to hypoxic stress[125]. This suggests that endothelin-mediated vasoconstriction may act as a stimulus for thrombosis. Endothelin can be generated as a response to hypoxia[133] and may in our setting be released either locally from the coronary arteries or from the lungs to promote vasoconstriction.

Hypoxia has been shown to trigger the pro-coagulant pathway and induce expression of tissue factor and plasminogen activator inhibitor-1[134-136] which may be particularly important in atherosclerotic arteries. Therefore we investigated the involvement of coagulation and thrombosis in the stress model by administrating a thrombin inhibitor before and after stress.

Thrombin plays an important role as a key enzyme of the coagulation pathway by cleaving fibrinogen to fibrin but it is also as a mediator of inflammation. Several anticoagulants are available such as heparin and low molecular weight heparins (LMWH). We chose to use melagatran which is a direct thrombin inhibitor acting independent of antithrombin, in contrast to heparins[137]. Melagatran was administered as a bolus dose before the stress and continuously administered afterwards from an osmotic pump implanted on the back of the mouse.

No difference in the STU segment of the ECG was observed in the group receiving melagatran as compared to the PBS group during hypoxia suggesting that thrombin was not involved in the first ischemic phase.

Infarction size was measured by immunohistochemical staining followed by counting the number of fibrinogen positive cells. Accumulation of fibrinogen in cardiomyocytes is a marker for irreversible cell membrane damage and can be detected as early as 3h after coronary occlusion[138]. We found this method more accurate than mitochondrial dehydrogenase staining of viable cardiomyocytes with triphenyl tetrazolium chloride (TTC), which is a widely used method in larger animals. In our experience using mice, TTC often gave a false positive infarction area at perfusion, merely reflecting the severity of stenosis than the actual infarction area. Histological examination of the coronary arteries of the apoE -/- x LDLR -/- mice verified advanced plaques obstructing the lumen.

Vulnerable plaques are characterised by several features such as a large lipid core, thin fibrous cap and inflammation, but also by intraplaque haemorrhage suggestive of plaque rupture[52]. Such advanced lesions were also observed in the coronary artery of the apoE -/- x LDLR -/- mouse, in which plaques often contained incorporated erythrocytes intermixed with foam cells. Although intraplaque haemorrhage has been used as an indicator of plaque rupture[139,140] we did not use this criterion as a diagnostic marker of plaque rupture in the absence of endothelial discontinuity (paper II). In coronary circulation of the mouse, the fibrous cap of atherosclerotic plaques is not as well defined as in humans and the lipid core is often covered only by a thin endothelial layer, which makes a clear cut distinction between endothelial erosion and plaque rupture difficult.

Although thrombin was not involved in the acute phase of reversible ischemia, thrombin inhibition resulted in a 46% reduction in infarction size, 60% reduction in infarction incidence and significantly reduced troponin T release. These data suggests that hypoxic stress generated an endothelin-dependent ischemia which triggered a delayed second phase involving thrombin, probably via activation of the humoral coagulation system, platelets, endothelial cells or a combination thereof. Histological findings of atherothrombosis and haemorrhaged plaques in non-treated mice support the hypothesis of thrombin generation as an important component in the development of myocardial infarction in this model.

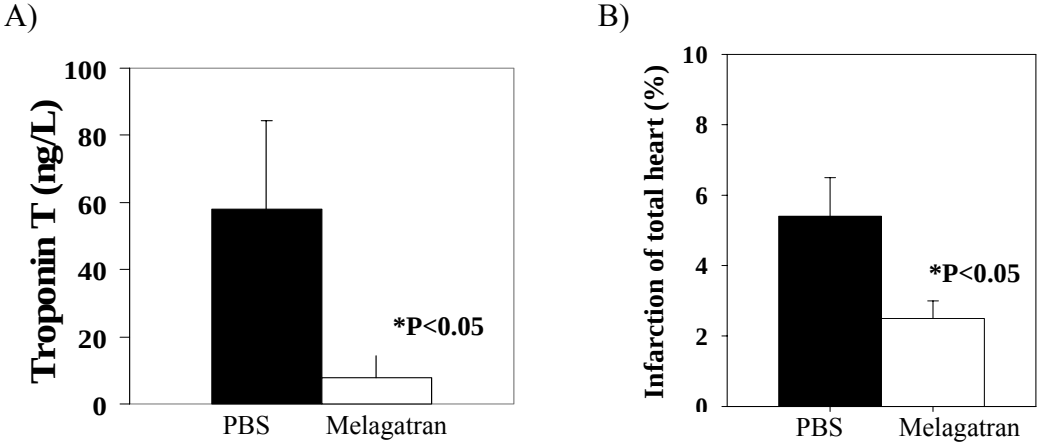

Figure 18. The effects of thrombin inhibition by melagatran **A)** The biochemical marker for myocardial infarction troponin T was significantly reduced. **B)** Infarctions were also significantly reduced by thrombin inhibition.

HYPOXIA-INDUCED GENES *(paper III)*

To investigate the mechanisms triggered by ischemia we performed transcript profiling at different time points after the stress.

Transcriptome analysis was performed on plaques from hypoxia stressed mice to find genes that may promote plaque vulnerability. The myocardium was analysed to assess the cardiac response to infarction. Three time points were analysed: 3h, 24h and 48h. At the last time point, mice were divided into an infarction group and a non-infarction group based on circulating troponin T levels.

Signal transduction pathways were analysed in silico using a bioinformatics approach to the transcriptome. It revealed early activation of the hypoxia signalling cascade in the plaque and the heart and later on an induction of NO signalling. These results are consistent with the notion of instant accumulation of the transcription factor hypoxia inducible factor-1α (HIF-1α) to the nucleus at hypoxia[141] and that NO is increased in heart failure [142,143] and ischemic preconditioning[144] and in vascular dysfunction[145]. No change in mRNA levels of HIF-1α itself was observed after hypoxia, which is in line with the notion that HIF-1α is regulated mainly on the protein level rather than the transcriptional level. HIF-1α is constitutively expressed but it is rapidly degraded in the proteasome during normoxia. In hypoxia, HIF-1α accumulates and forms a complex with HIF-1β (Anrt)[109]. Interestingly Arnt-2 was induced in the heart at 3h after hypoxic stress. Although the hypoxia signalling pathway displayed a specific set of genes, several signalling cascades share pathways and transcription factors such as Egr-1 and c-fos which in our study were represented in 4 and 5, respectively, of the 7 analysed cascades. This shows that signalling pathways display redundancy. Bioinformatic data suggesting activation of a particular signalling pathway should therefore be interpreted with caution.

Genes of interest identified in arrays were verified by quantitative real time RT-PCR. It confirmed that hypoxia induced up-regulation in the plaque of several genes involved in the coagulation cascade such as fX and tissue factor. Tissue factor is a membrane anchored cell-surface bound protein that initiates coagulation when blood contacts damaged tissue. Tissue factor is expressed by leukocytes, intima SMC, macrophages and endothelial cells[146,147]. Plasma levels of tissue factor have been reported in patients with unstable angina[148,149] and myocardial infarction[150,151] and several clinical studies have reported an increased risk for myocardial infarction with increasing numbers of tissue factor expressing leukocytes[152-155]. In the plaque fX mRNA was found to be increased directly after stress and also in plaques from mice that developed myocardial infarction.

Acute changes within the plaque such as intraplaque haemorrhage, cap rupture and cap ulceration, are preludes to the onset of clinically important ischemic events[52] and previous studies have shown that the site of rupture is characterized by intense inflammatory infiltrate[38,39] consisting predominantly of macrophages and T-lymphocytes. It has been

hypothesized that this inflammatory infiltration may play a key role in the destabilization of the plaque[4] by their release of cytokines and proteolytic enzymes[4,156]. In our study, hypoxia-exposed plaques exhibited gene expression of the inflammatory cytokine cxcl16 and of tissue inhibitor of metalloproteinase -1 (TIMP-1) at all time points except in mice which had not developed myocardial infarction at 48h. Interestingly, the array analysis displayed several upregulated matrix metalloproteinases (MMPs) such as MMP-3 in infarcted hearts and MMP-13 in the plaque 24h after hypoxia. mRNA of MMP-9 was verified by quantitative real-time RT-PCR and was significantly higher in plaques originating from mice with myocardial infarction. These data suggest that hypoxia brings on pro-inflammatory, pro-thrombotic and matrix degrading genes which may precipitate coronary occlusion in a vulnerable plaque.

THE ACTIVE PLAQUE AND WOUNDED HEART *(paper III and IV)*

The atherosclerotic plaque is a dynamic structure which undergoes continuous remodelling of the extracellular matrix on which its structural integrity depends. Several factors such as oxidative stress, apoptosis and inflammation play important roles in plaque activity and in reorganizing the existing plaque shape and structure. In paper IV we examined an interesting protein that may be important for plaque stability by protecting inactivation of a proteolytic enzyme, thereby ensuring the persistence of matrix proteolysis which may render a vulnerable plaque phenotype.

Matrix metalloproteinases play a major role in degradation of collagen and other extracellular matrix macromolecules and are upregulated in pathological remodelling processes[157,158]. The occurrence of MMPs in coronary lesions was first discovered by Henney et al who localized MMP-3 mRNA expression in macrophages[159]. Subsequent studies have demonstrated the importance of MMP-3 and other MMPs in plaque destabilization and aneurysms[160-163]. A clinical study demonstrated a four-fold increase in MMP-9 expression in patients undergoing endarterectomy with recent symptoms from unstable carotid plaques[164]. There was also a significant increase in MMP-9 levels in plaques with histological features of acute disruption[164,165]. Plasma levels of MMP-9 has also been described to be higher in coronary artery disease patients as compared to healthy volunteers[166,167] and may be an independent predictor of cardiovascular mortality[166].

In paper III, several genes were found to be upregulated after hypoxic stress. In the infarcted group, one of the most prominently increased genes was 24p3, the mouse homolog of human Neutrophil Gelatinase Associated Lipocalin (NGAL). NGAL is a 25kDa protein that binds covalently to gelatinase B/MMP-9. NGAL and MMP-9 can form a complex within the granules of neutrophils or form heterodimers after being independently secreted. The hydrophobic and cone shaped structure of NGAL protects MMP-9 from degradation by TIMP-1, which prolongs the proteolytic effects of MMP-9[168] and supports its activation [169]. This suggests that NGAL in the atherosclerotic plaque, where de novo collagen synthesis is reduced[44,170,171] and collagen degradation by matrix metalloproteinases is high, may promote increased and prolonged proteinase activity. This could potentially contribute to decreased stability of the collagen cap, rendering the plaque more susceptible to erosion and rupture with subsequent thrombosis and infarction. Our data (paper IV) show that plaques from infarcted apoE -/- x LDLR -/- mice express high levels of 24p3 and MMP-9 transcripts and that these proteins co-localize in areas with high prevalence of proteolytic activity.

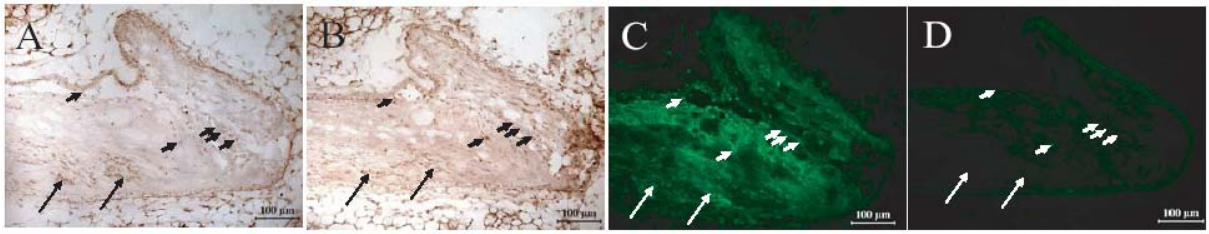

Figure 19. The photomicrographs illustrate the correlation of NGAL and MMP-9 protein with proteolytic activity by zymography (figure 4 in paper IV). **A)** Shows the NGAL expression (arrows) and **B)** MMP-9 protein. **C)** Displays the proteolytic activity of MMP-9 which is dampened in **D)** the control.

MMP-9 exists as a pro-form and an active proteolytic form. Its expression and activity are tightly regulated, on the transcriptional level, by conversion from pro-MMP-9 to its active form and by endogenous inhibitors (TIMPs). In our study (paper III) we found high expression of TIMP-1 immediately after hypoxia while MMP-9 expression was low. In the infarcted plaque however the ratio between TIMP-1 and MMP-9 mRNA was decreased suggesting that proteolysis was favoured. Such an imbalance between degradation and repair of the extracellular matrix may render the plaque vulnerable[172].

In our study NGAL/24p3 and MMP-9 transcripts were gradually increased in the heart after hypoxic stress and peaked in the infarction group, although no-significantly for MMP-9. In the plaque however the hypoxic stress response was modest while a dramatic increase was observed in the group with myocardial infarction. While the function of NGAL sill remains unclear several possible hypotheses can be discussed. Firstly, NGAL may promote plaque instability in by protecting MMP-9 from degradation and prolonging its proteolytic properties. Elevated expression of NGAL and MMP-9 may also be a result of tissue damage due to infarction. Therefore, matrix degradation may lead to thrombus formation and myocardial infarction but it may also be part of the repair process. Post-mortem studies have identified old thromboses within the atherosclerotic plaque which have become incorporated into the lesion[173]. This process requires extensive plaque remodelling and MMP-9 is one of the important proteolytic enzymes that are involved in this process.

We also found NGAL/243p3 to be expressed by endothelial cells, macrophages and smooth muscle cells in the human atherosclerotic plaque (figure 5 and 6, paper IV). NGAL has previously been described in neutrophils and epithelial cells in organs continuously exposed to micro-organisms, such as the stomach and the lungs[174,175]. NGAL can also bind to pro-inflammatory ligands such as bacterial formyl peptides, leukotriene B4 and platelet-activating factor[176].

In summary NGAL/24p3 is highly increased in the heart and plaque after hypoxia-induced myocardial infarction and is associated with high proteolytic activity in the plaque. However, the function of NGAL remains unclear; therefore further studies are necessary to investigate the beneficial or detrimental properties of NGAL in myocardial infarction development.

CONCLUDING REMARKS

Atherosclerosis is a slowly progressing disease with focal lipid deposition and inflammation in the arterial wall. Stenotic arteries may cause episodes of angina and an occluding plaque thrombus may lead to myocardial infarction. In a majority of cases such complications derive from plaque rupture or erosion and may occur at a critical moment when a threshold combination of hemodynamic, pro-thrombotic and vasoconstrictive forces is rapidly generated by external stressors such as emotional upset, physical activity or infections in concert with plaque vulnerability. Several steps in the series of events leading from silent atherosclerosis to myocardial infarction have remained unclear, in many cases due to lack of suitable animal models. This thesis has aimed to develop a mouse model where spontaneous myocardial infarction can be triggered in order to study the physiological and molecular mechanisms in the development of myocardial infarction.

We have shown that myocardial infarction can be induced by hypoxic stress in a two-phase pathway: an initial phase comprising a transient ischemic response which triggers a delayed second phase of ischemia and myocardial infarction. Previous results showed that the first phase is endothelin dependent and in our present studies we have shown that this phase triggers a delayed phase which triggers a response which involves thrombin generation and causes myocardial infarction. Furthermore our data show that hypoxia induces expression of inflammatory, proteolytic and pro-coagulant genes that may promote a vulnerable and pro-thrombotic plaque environment and we hypothesise that these factors may lead to plaque damage, activation of platelets, humoral coagulation or a combination thereof after hypoxia. This model should be a useful tool to investigate plaque activation, thrombus formation and infarction in an experimental setting.

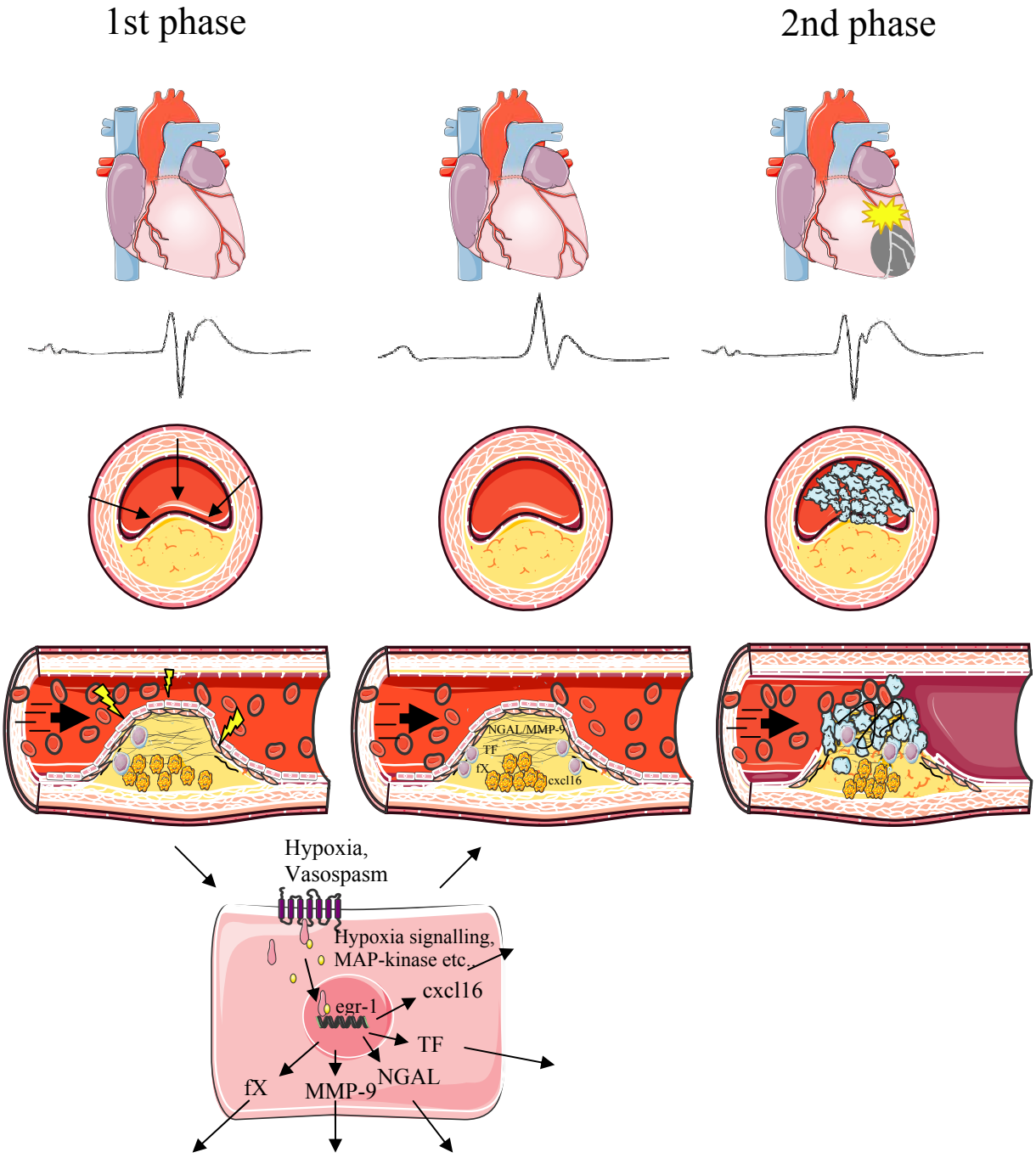

Figure 20. Hypothetic scheme of the series of events leading from hypoxia to myocardial infarction. Hypoxia activates the first phase characterized by endothelin-dependent vasospasm and STU segment elevation as a sign of ischemia. This activates several signalling pathways with egr-1 as one of the transcription factors set in motion to produce genes such as TF, fX, NGAL, MMP-9 and cxcl16. The presence of these factors may contribute to a vulnerable plaque phenotype and lead to thrombin generation and thrombus formation with subsequent myocardial infarction in a second irreversible phase with STU segment changes, elevation of infarction markers and histological signs of myocardial infarction.

FUTURE AIMS AND STUDIES

● Thrombin inhibition reduced myocardial infarction size. Future studies should include therapeutic treatment, targeting inflammation and/or plaque vulnerability in this model.

● The infarction model has provided a wealth of data on genes that may be important for infarction development. In this thesis we focused our attention on genes involved in inflammation, plaque instability and thrombosis. However, more work should be done to further investigate genes that may play a role in hypoxia adaptation, wound healing, angiogenesis, remodelling and collateral formation. Transcripts found on the array should be verified not only by quantitative real time RT-PCR but also by immunohistochemistry and western blot to validate protein expression and its localisation.

● In the plaque, NGAL was found to be expressed by endothelial cells, macrophages and smooth muscle cells; it would be interesting to look solely at the endothelial derived NGAL expression and separate the signal from other cell types.

● The large increase of NGAL transcript in plaques and myocardium from apoE -/- x LDLR -/- mice with myocardial infarction and the notion that NGAL is a predictor of renal failure suggest that NGAL may also be an important factor for predicting myocardial infarction. To elucidate the function of NGAL in coronary artery disease, recombinant NGAL could be administered via intracardiac injections or adenoviral transfection to the heart. The NGAL gene has been targeted in embryonic stem cells but since NGAL is an important protein in embryo development the foetuses were not viable.

● Although NGAL may possess important functions *per se* it is as a complex with MMP-9 that it may contribute to plaque vulnerability. Therefore, efforts should be made to further investigate the amount of NGAL/MMP-9 complex in the mouse model.

● Last but not least, it should be emphasised that this murine model has served as an experimental model for a human disease. Therefore, results obtained from this model should be verified in patients with coronary artery disease.

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Notes: