Veli-Pekka Ronkainen

EFFECTS OF CHRONIC HYPOXIA ON MYOCARDIAL GENE EXPRESSION AND FUNCTION
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Oulu, Finland

**Abstract**

Molecular oxygen is a prerequisite for essential metabolic processes in multicellular organisms. However, the supply of oxygen can be disturbed and tissue aerobic metabolism becomes compromised in several pathophysiological conditions. In prolonged hypoxia, cells initiate cell type-specific adaptation processes, which are typically mediated by alterations in gene expression. Changes are mainly driven by a transcription factor called hypoxia-inducible factor 1 (HIF-1). Heart muscle is a highly oxidative tissue and HIF-1 activation turns on myocardial adaptation mechanisms for enhanced survival in oxygen-deprived conditions.

The aim of this study was to characterize myocardial gene expression changes during chronic hypoxia and couple the adaptational changes to cardiomyocyte function. The role of hypoxia and HIF-1 activation was studied by using \textit{in vitro} mouse and rat heart cell culture models, tissue perfusions and \textit{in vivo} infarction models.

In this study, apelin, sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a) and G protein-coupled receptor 35 (GPR35) were characterized as novel functionally important myocardial HIF-1 target genes. Apelin and GPR35 were induced in hypoxia, while SERCA2a expression was reduced HIF-1 dependently. HIF-1 activation also altered cardiac myocyte contractility through modulation of SERCA2a and GPR35 expression, leading to impairment of the cellular calcium metabolism. Reduced contractility was suggested to serve as an adaptive mechanism for reduced aerobic ATP production in hypoxic conditions.

This study presents novel information about the plasticity of myocardial adaptation to prolonged hypoxia. The role of a conserved transcription factor, HIF-1, was shown to be essential in the adaptation process in the myocardial cells.

**Keywords:** adaptation, cardiac myocyte, G protein-coupled receptors, heart, hypoxia, metabolism, oxygen homeostasis
Ronkainen, Veli-Pekka, Hapenpuutteen vaikutuksia sydänlihassolujen geenien ilmentymiseen ja toimintaan.

Oulun yliopiston tutkimuskeskus; Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia; Biocenter Oulu, PL 5000, 90014 Oulun yliopisto


Oulu

Tiivistelmä


Tämän tutkimuksen tavoitteena oli määrittää sydämen geenien ilmentymisen hapenpuutevasteita ja yhdistää muutokset sydänsolujen toiminnallisiin muutoksiin. Hapenpuutteen ja HIF-1:n merkitystä sopeutumisessa tutkittiin käyttämällä rotan ja hiiren sydänsoluviljelmiä, in vitro-kudosperfuusionmallia sekä in vivo-sydäninfarktimalleja.


Tämä tutkimus antaa lisätietoa sydämen soppeutumiskyvyn mukautumisesta pitkittyneeseen hapenpuutteeseen. Lisäksi tutkimus osoittaa HIF-1:n roolin olevan oleellinen myös sydänsolujen hypoksia-adaptaatioprosessissa.

Asiasanat: aineenvaihdunta, G-proteiinikytketty reseptori, hapenpuute, happitasapaino, soppeutuminen, sydän, sydänlihassolu
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Oulu, June 2012

Veli-Pekka Ronkainen
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>α-AR</td>
<td>α-adrenergic receptor</td>
</tr>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>Adm</td>
<td>adrenomedullin</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APJ</td>
<td>apelin receptor</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ATr</td>
<td>angiotensin receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>atrioventricular node</td>
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<td>β-AR</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix protein domain</td>
</tr>
<tr>
<td>BNIP3</td>
<td>pro-apoptotic Bcl-2 family member gene</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BREu</td>
<td>upstream TFIIIB recognition element</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca(^{2+})-calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3’,5’ monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>carnitine-acyltransferase</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CPT1</td>
<td>carnitine palmitoyltransferase</td>
</tr>
<tr>
<td>CTAD</td>
<td>C-terminal transactivation domain</td>
</tr>
<tr>
<td>DAG</td>
<td>diacyl glycerol</td>
</tr>
<tr>
<td>DCE</td>
<td>downstream core element</td>
</tr>
<tr>
<td>CPT1</td>
<td>carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>DFO</td>
<td>desferrioxamine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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</table>
DPE  downstream promoter element
E10.5  embryonic day 10.5
EGFP  enhanced green fluorescent protein
Em  membrane potential
EPO  erythropoietin
ER  endoplasmic reticulum
ET-1  endothelin-1
FAT  fatty acid transporter
FIH  factor inhibiting HIF
Gs  guanine nucleotide-binding protein alpha subunit, stimulatory
Gai  guanine nucleotide-binding protein alpha subunit, inhibitory
Gaq  guanine nucleotide-binding protein alpha subunit, PLC activator
GATA-4  GATA binding protein-4
GFP  green fluorescent protein
GLUT  glucose transporter
GPCR  G protein-coupled receptor
GPR35  G protein-coupled receptor 35
GTP  guanosine-5'-triphosphate
HBS  HIF-binding site
HDAC  histone deacetylase
HIF  hypoxia-inducible factor
HIF-P4H  HIF prolyl 4-hydroxylase
HL-1  heart like-1 cells
HRE  hypoxia response element
INa  Na⁺ current
Inr  initiator element
IP₃  inositol 1,4,5-triphosphate
IPAS  inhibitory Per-ARNT-Sim protein
Kₘ  Michaelis-Menten constant
KO  knock-out
KYNA  kynurenic acid
LAD  left anterior descending coronary artery
LDH  lactate dehydrogenase
LPA  lysophosphatidic acid
LTCC  L-type voltage dependent calcium channel
MCT  monocarboxylic acid transporter
Mef2C  myocyte enhancer factor 2C
10
mRNA       messenger RNA  
MTE        motif ten element  
NADH       nicotinamide adenine dinucleotide  
NFAT       Nuclear factor of activated T-cells  
NCX        Na\(^+/\)Ca\(^{2+}\) -exchanger  
NHE        Na\(^+\)-H\(^+\) exchanger  
Nkx2-5     NK2 transcription factor related 5  
NOS        nitric oxide synthase  
NTAD       N-terminal transactivation domain  
ODDD       oxygen-dependent degradation domain  
PBS        phosphate buffered saline  
PCR        polymerase chain reaction  
PDH        puruvate dehydrogenase  
PDK1       puruvate dehydrogenase kinase  
PHD        prolyl hydroxylase domain containing protein  
Per        periodic circadian protein  
PIC        pre-initiation complex  
PIP2       phosphatidylinositol 4,5-bisphosphate  
PKA        protein kinase A  
PKC        protein kinase C  
PLC        phospholipase C  
PLN        phospholamban  
POLII      RNA polymerase II  
PPAR       peroxisome proliferator activated receptor  
PTX        pertussis toxin  
pVHL       von Hippel-Lindau tumor-suppressor protein  
qPCR       real time quantitative PCR  
Rho        Ras homology gene family  
RNA        ribonucleic acid  
ROCK       Rho-associated kinase  
ROS        reactive oxygen species  
RT-PCR      reverse transcriptase PCR  
RyR        ryanodine receptor  
siRNA      small interfering RNA  
Sim        single-minded protein  
SERCA      sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase  
SL         sarcolemma  

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>Sp1</td>
<td>specificity protein 1</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TAF</td>
<td>TBP-associated factor</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box-binding protein</td>
</tr>
<tr>
<td>Tbx5</td>
<td>T-box 5 transcription factor</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TFII</td>
<td>transcription factor for RNA polymerase</td>
</tr>
<tr>
<td>TnC</td>
<td>troponin C, Ca$^{2+}$-binding subunit</td>
</tr>
<tr>
<td>TnI</td>
<td>troponin I, inhibitory subunit</td>
</tr>
<tr>
<td>TnT</td>
<td>troponin T, tropomyosin-binding subunit</td>
</tr>
<tr>
<td>Tm</td>
<td>tropomyosin</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VP16</td>
<td><em>Herpes simplex</em> virus transactivation protein</td>
</tr>
<tr>
<td>XCPE1</td>
<td>X core promoter element 1</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


Table of contents

Abstract
Tiivistelmä
Acknowledgements 7
Abbreviations 9
List of original articles 13
Table of contents 15
1 Introduction 19
2 Review of the literature 23
   2.1 General chronic hypoxia sensing pathway ................................. 23
      2.1.1 Systemic hypoxia responses ............................................. 23
      2.1.2 Local hypoxia responses ................................................... 24
      2.1.3 Hypoxia responses at the cellular level ............................ 24
   2.2 Regulation of eukaryotic transcription ...................................... 26
      2.2.1 Gene promoter types ...................................................... 26
      2.2.2 Common promoter elements .......................................... 27
      2.2.3 Common transcription factors and the general
               transcription machinery ............................................. 28
      2.2.4 Regulation of transcription ........................................... 29
      2.2.5 Enhancers .................................................................. 29
   2.3 Gene expression is regulated by hypoxia-inducible factors .......... 32
      2.3.1 Structure and different isoforms of HIFs ........................... 32
      2.3.2 Oxygen-dependent regulation of HIF ................................. 33
   2.4 Hypoxia-responsive promoters and target genes ....................... 37
      2.4.1 Hypoxia response element .............................................. 37
      2.4.2 HIF-binding promotes co-factor recruitment ..................... 37
      2.4.3 Epigenetic control of hypoxic transcription ....................... 38
      2.4.4 Hypoxic repression of promoters ................................... 39
      2.4.5 Target genes .................................................................. 39
   2.5 Hypoxia effects on heart muscle ............................................. 41
      2.5.1 Myocardial energy metabolism ...................................... 41
      2.5.2 Developmental and pathophysiological changes in cardiac
              energy metabolism ...................................................... 44
      2.5.3 The role of HIF in pathophysiological situations ............... 45
   2.6 Cardiovascular system development ....................................... 46
      2.6.1 Hypoxia drives mammalian development ......................... 46
2.6.2 The role of HIF in cardiogenesis .................................................. 46

2.7 Excitation-contraction coupling in myocardial cells ........................................ 48
  2.7.1 Electrical activity ........................................................................ 48
  2.7.2 Calcium signaling ........................................................................ 49
  2.7.3 Energy consumption ..................................................................... 51
  2.7.4 The role of SERCA2 in excitation-contraction coupling ...................... 51

2.8 Cardiac excitation-contraction coupling is regulated via G protein-coupled receptors .......................................................................................................................... 53
  2.8.1 G protein-coupled receptors .......................................................... 53
  2.8.2 Activation of a G protein ................................................................ 53
  2.8.3 GPCRs in myocardium .................................................................. 55

2.9 Apelin is an endogenous modulator of the G protein signaling ......................... 58
  2.9.1 Apelin has beneficial effects in the cardiovascular system ................. 60
  2.9.2 Apelin improves cardiomyocyte function ...................................... 61
  2.9.3 Regulation of apelin expression ..................................................... 61

2.10 A novel G protein-coupled receptor 35 ..................................................... 62
  2.10.1 GPR35 activation has diverse effects in cells ................................. 62
  2.10.2 GPR35 expression and function is also associated to the cardiovascular system .......................................................... 63

3 Aims of the research 65

4 Summary of materials and methods 67
  4.1 Rat auricle superfusion model (I) ....................................................... 67
  4.2 In vivo infarction model (I) ................................................................. 67
  4.3 Cell Isolation and Culturing (I, III, III) .............................................. 67
  4.4 Transfections and plasmid constructs (I, II, III) ........................................ 68
  4.5 Gene expression measurements (I, II, III) .......................................... 68
  4.6 Reporter analysis (I, II, III) ................................................................. 69
  4.7 Ca²⁺ imaging (II, III) .......................................................................... 69
  4.8 Immunofluorescence labeling and microscopy (I, II, III) ....................... 69
  4.9 Western blot analysis/PLB phosphorylation (II) .................................... 70
  4.10 Apelin radioimmunoassay (I) ............................................................ 70
  4.11 Chromatin immunoprecipitation (III) ............................................... 70
  4.12 Statistical analysis (I,II,III) ............................................................... 71

5 Results 73
  5.1 Hypoxia regulates cardiomyocyte apelin expression and secretion ............ 73
5.1.1 Hypoxia increases apelin gene expression in perfused rat atrium ................................................................. 73
5.1.2 Apelin expression is regulated time-dependently in cultured neonatal rat cardiomyocytes ......................... 73
5.1.3 HIF-1 is involved in apelin gene expression regulation .......... 73
5.1.4 Hypoxia increases the secretion of biologically active forms of apelin .............................................................. 74
5.1.5 Apelin gene expression is increased in vivo after myocardial infarction .......................................................... 75
5.2 Hypoxia and HIF-1 activation suppress SERCA2a expression in embryonic cardiac myocytes through two interdependent hypoxia response elements .................................................. 75
5.2.1 Cultured embryonic cardiomyocytes are hypoxia-tolerant........ 75
5.2.2 SERCA2a gene expression is regulated by oxygen levels in cardiac myocytes ................................................ 75
5.2.3 HIF-activation reduces SERCA2a expression also in normoxia................................................................. 76
5.2.4 SERCA2a protein levels are reduced and the cellular distribution is interfered in hypoxic myocytes ............... 76
5.2.5 The hypoxic loss of functional SERCA2a affects cardiomyocyte calcium cycling function negatively .......... 76
5.2.6 Characterization of SERCA2a promoter, molecular mechanism for the hypoxic down-regulation .............. 77
5.3 HIF-1-inducible G protein-coupled receptor 35 expression modulates cardiomyocyte function ........................................ 77
5.3.1 Myocardial expression of the different GPR35 isoforms ........ 77
5.3.2 Hypoxia and HIF-1 activation or inhibition modulates GPR35 gene expression in cardiac myocytes ............. 78
5.3.3 GPR35 promoter hypoxia response element is responsible for the HIF-dependent regulation ...................... 78
5.3.4 Hypoxia increases the number and density of receptor molecules on the cardiomyocyte plasma membrane .... 79
5.3.5 GPR35 agonist Zaprinast induces receptor internalization in cardiac myocytes .................................................. 80
5.3.6 Over-expression of GPR35 changes cardiac myocyte morphology .................................................................. 80
5.3.7 Activation of hypoxia-induced GPR35 affects cardiomyocyte Ca²⁺-activity ......................................................... 80

6 Discussion 83
6.1 The general role of HIF-1 in the regulation of gene expression .......... 83
6.2 The hypoxic regulation of apelin expression ........................................ 84
6.3 Hypoxia adaptation mechanisms driven by apelin ............................ 84
   6.3.1 Apelin increases blood delivery to tissues ................................. 84
   6.3.2 Apelin modulates [pH], and contractility of cardiac myocytes .......... 86
   6.3.3 Apelin prevents apoptosis ....................................................... 87
6.4 Hypoxic modulation of SERCA2 expression and excitation-contraction coupling ................................................................. 88
   6.4.1 SERCA2 gene and promoter properties .................................... 88
   6.4.2 SERCA2a expression and function is reduced in pathological situations ................................................................. 89
   6.4.3 Hypoxia down-regulates SERCA2a expression and pumping function ................................................................. 90
   6.4.4 The role of HIF in the regulation of SERCA2a promoter activity ................................................................. 90
   6.4.5 SERCA2 reduction may serve as an adaptive mechanism for reduced ATP production ................................................................. 93
   6.4.6 SERCA2a knock-out and knock-down cells resemble hypoxia-exposed phenotype ................................................................. 93
6.5 A novel cardiac hypoxia inducible gene, G protein-coupled receptor 35 ................................................................. 94
   6.5.1 Isoforms of GPR35 in cardiac myocytes .................................... 94
   6.5.2 GPR35 is up-regulated in hypoxic myocardial cells through HIF-1 pathway ................................................................. 94
   6.5.3 GPR35 promoter has a functional hypoxia response element ................................................................. 95
   6.5.4 GPR35 is also functional in cardiac myocytes .................................. 95
   6.5.5 Physiological responses caused by GPR35 activation .................. 96

7 Summary and conclusions 99
References 103
Original publications 125
1 Introduction

Molecular oxygen is a prerequisite for metazoan life and maintenance of oxygen homeostasis is critical for all aerobic organisms. In developed multicellular species, highly complex organs have evolved to maintain and control cellular oxygen balance. In mammalians, lungs have developed for oxygen uptake and blood and cardiovascular system for the transportation and tissue distribution of oxygen. In addition to central oxygen sensors, also local cellular partial oxygen pressure regulates the function of cells. This provides a high level of plasticity to the function of cells, tissues and organs in physiological processes (for review see Ward 2008). Inhaled ambient pO$_2$ is about 21 kPa (21% of the atmospheric pressure) at sea level, while arterial pO$_2$ in mammalian adults is about 13 kPa, and intracellular pO$_2$ may be only 2 kPa in physiological conditions, and even less in highly metabolically active tissues. Oxygen gradient is formed when cellular O$_2$ consumption is coupled with the constraints of diffusion through the tissues. Moreover, in the fetus arterial pO$_2$ is only about 5 kPa (for review see Ward 2008).

Hypoxia can be referred to as a physiological or pathological condition in which the whole body, tissues or cells are deprived of adequate oxygen supply. It is defined as a condition in which failure of either delivery or use of O$_2$ limits normal tissue function. Complete lack of oxygen is often referred to as anoxia and normal oxygenation as normoxia. The causes of hypoxia can be categorized into four subgroups. In hypoxemic hypoxia red blood cell oxygen content is reduced due to e.g. low atmospheric O$_2$ or reduced breathing activity in high altitudes or due to airway obstruction. Anemic hypoxia is a consequence of reduced red blood cell O$_2$ delivery capacity caused by e.g. carbon monoxide or decreased number of erythrocytes or total hemoglobin. Distributive hypoxia rises locally from low flow e.g. due to occlusion of blood-delivering artery. Histotoxic hypoxia can be inflicted by poisoning, e.g. cyanide, which prevents normal cellular O$_2$ usage causing hypoxic cellular responses.

In addition to certain types categorized by origin, hypoxia can be divided according to exposure time to acute or chronic hypoxia. In acute hypoxia, cells react in a rapid and transient way to reduced pO$_2$, which is mediated mainly through modification processes of existing proteins and metabolic pathways. In the carotid body, specialized O$_2$-sensing cells (glomus cells type I) are depolarized in response to reduced arterial pO$_2$, and send impulses to the brainstem centers that control cardiac output and breathing reflexes (Prabhakar
Other hypoxia-sensitive excitable neurosecretory cells are found in the neuroepithelial bodies of the lung and neonatal adrenal chromaffin cells, which also mediate fast cardiorespiratory adjustments to hypoxia. The exact mechanisms how the O_2 sensors interact with the ion channels are unknown. Several parallel mechanisms for detecting O_2 may exist, ranging from mitochondrial haem-containing cytochromes and ROS production to AMP-activated protein kinase (AMPK) activation during acute hypoxia (for review see Ward 2008). However, the final response to hypoxia involves inhibition of K⁺ channels, depolarization and activation of voltage-gated Ca^{2+} channels, which increases intracellular Ca^{2+} concentration and causes release of transmitters onto the sensory afferent nerves (Lopez-Barneo et al. 2004). The feedback from the carotid body is sent to the cardiorespiratory centers in the medulla oblongata via the afferent branches of the glossopharyngeal nerve. These centers integrate afferent signals and regulate breathing and cardiovascular functions which act to relieve systemic hypoxia. In addition, autoregulation of arterioles in the systemic circulation dilate in response to hypoxia to maintain tissue oxygenation.

When acute respiratory and cardiovascular responses are not able to improve body/tissue oxygenation, acute hypoxia turns into chronic. In prolonged hypoxia, cells initiate slower adaptation processes, which are typically mediated by alterations in gene expression and protein synthesis. However, exposure to O_2 must be limited to relatively narrow range, since hyperoxic environment produces reactive oxygen species (ROS) which are harmful to cellular macromolecules as they cause oxidative damage. Under these circumstances, multicellular organisms have developed a complex physiological regulation network of oxygen homeostasis to maintain the O_2 concentration to which each cell is exposed within a narrow range.

The most important function of the heart is a contractile function which ensures the continuous pumping of oxygen- and nutrient-rich blood through the cardiovascular system. Most of the energy for cardiac work comes from the aerobic metabolism. Inadequate oxygen supply, for example in myocardial infarction, compromises energy production and contractile function. Cardiac ischemia arises from partial or complete interruption of coronary blood flow by obstruction or spasm, resulting in angina pectoris or myocardial infarction. In the ischemic heart, blood perfusion is compromised, tissue becomes hypoxic and the aerobic energy production ceases. The pumping function of the heart will then decrease and finally stop with lethal consequences. Disturbances in the heart
oxygen supply are a major cause of death in Western countries. However, cardiac myocytes have a variety of means to adapt to the changing oxygenation in order to promote cell survival and cardiac pumping function. Coordination of oxygen supply and demand involves a short-term immediate adaptation that includes e.g. dilatation of the coronary vessels and a long-term adaptation which results from the changes in cardiac gene expression.

The aim of this study was to characterize cardiac gene expression changes during prolonged hypoxia and to evaluate the physiological responses caused by the long-term adaptation processes.
2 Review of the literature

2.1 General chronic hypoxia sensing pathway

The general mechanisms of cellular adaptation to hypoxia are similar in a wide variety of cells and tissues. This process includes: 1) sensing the oxygen deficiency, 2) transcriptional activation leading to 3) expression of proteins required for adaptation. Hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) are oxygen-sensitive transcription factors which function as master regulators of oxygen homeostasis in all metazoan species and have an essential role in development, physiology, and disease pathogenesis (for review see Semenza 2009). Hypoxia-inducible factors are powerful target gene regulators, which are activated when the oxygen partial pressure gets low enough in cells. All cells can sense and respond to hypoxia, but the response may differ between cell types. Some cells switch their own metabolism from aerobic to anaerobic to sustain ATP production in hypoxia, while others respond primarily in a systemic way trying to enhance the whole body oxygen delivery through secreted hypoxia-induced metabolites and hormones which increase respiratory function, angiogenesis and red blood cell production (for review see Semenza 2010).

2.1.1 Systemic hypoxia responses

Erythropoiesis, angiogenesis, and glycolysis are examples of systemic, local tissue, and cellular adaptive responses to hypoxia. Of systemic responses, a well-known example is erythropoietin: its synthesis and secretion is increased mainly in renal cortex peritubular capillary endothelial cells when circulating blood pO₂ is reduced e.g. due to acute blood loss, ascent to high altitude or pneumonia. Chronic hypoxia and activation of HIF-1 was found to be a key regulator in EPO gene regulation (Semenza & Wang 1992), but recently, the role of HIF-2 in EPO regulation has also been put forward (Rankin et al. 2007). Secreted erythropoietin increases the production of red blood cells by preventing bone marrow red blood cell precursor apoptosis and targeting CFU-E, pro-erythroblast and basophilic erythroblast subsets in the differentiation, thereby increasing O₂-carrying capacity of the blood (for review see Fisher 2003). In addition to EPO expression, HIF-1 and HIF-2 control the red cell production coordinately by regulating the expression of EPO receptor and enzymes/transporters required for erythropoietic...
iron uptake, transportation and metabolism (e.g. hepcidin, DMT1, transferrin and transferrin receptor) (for review see Semenza 2010).

2.1.2 Local hypoxia responses

Erythropoiesis is an adaptive response to systemic hypoxia, but angiogenesis serves as a local adaptation process for tissues facing decreased oxygenation. Hypoxia can arise from arterial occlusion, normal tissue growth or edema, when O₂ diffusion distances become too long. HIF-1 activation coordinates the transcription of a complex network of angiogenic growth factors and cytokines including vascular endothelial growth factor (VEGF), stromal-derived factor 1 (SDF-1), placental growth factor (PLGF), angiopoietin 1 and 2, and platelet-derived growth factor B (Forsythe et al. 1996, Yamakawa et al. 2003, Ceradini et al. 2004, Simon et al. 2008). HIF-1-orchestrated expression of these factors leads to the production of new capillaries (angiogenesis) and the remodeling of existing arteries (arteriogenesis) to shorten the O₂ diffusion distances and to increase blood flow locally (Pajusola et al. 2005, Patel et al. 2005). However, HIF-1 mediated vasculature remodeling can be harmful in pathological situations, such as in solid tumors, where central parts of the tumor become hypoxic during malignant growth. Expression of intra-tumoral HIF-1 or HIF-2 is dramatically increased in many human cancers and their metastases (Talks et al. 2000, Semenza 2003). In this pathological context, HIF-1 activation is harmful, promoting tumor angiogenesis and preventing hypoxia-conducted necrosis, which makes solid tumor growth possible.

2.1.3 Hypoxia responses at the cellular level

Individual cells can adapt to O₂ deprivation in an autonomous way by reprogramming their glucose and energy metabolism. Mitochondrial tricarboxylic acid cycle and respiratory chain (oxidative phosphorylation) generate most of the energy in normoxic conditions. In tricarboxylic acid cycle NAD⁺ molecules are reduced to NADH, which is then oxidized in the respiratory chain to produce proton gradient across the mitochondrial inner membrane. Electrochemical gradient is discharged via F₁F₀ ATP synthase, which generates ATP from ADP and Pᵢ. Because oxygen molecule is the terminal electron acceptor at complex IV of the respiratory chain, hypoxia inhibits the respiratory chain, ceasing ATP production. Therefore the heart must adapt to lower oxygen supply and intensify
the use of limited oxygen. Hypoxia and HIF-1 activation induces a switch in energy metabolism from mitochondrial oxidative phosphorylation to cytosolic glycolytic metabolism. First steps in the adaptation process involve a HIF-driven change in gene expression that induces subunit conversion of cytochrome c oxidase (COX), which optimizes the efficiency of respiration at reduced O₂ concentrations (Fukuda et al. 2007). HIF-1 induces the expression of glycolytic genes, since glycolysis is necessary to produce energy when low oxygen will not support oxidative phosphorylation. HIF-1 can also repress mitochondrial function and oxygen consumption by inducing the expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates pyruvate dehydrogenase (PDH), the enzyme that converts pyruvate to acetyl coenzyme A, which enters the mitochondrial tricarboxylic acid cycle (Papandreou et al. 2006). As a consequence, the Pasteur effect, described as decreased oxidative phosphorylation and increased anaerobic fermentation, is compensated by increased cellular glucose uptake via glucose transporters (GLUTs) and increased conversion of glucose to lactate by glycolytic enzymes and lactate dehydrogenase A (LDH-A). These genes are all HIF-1 inducible (Semenza et al. 1996, Seagroves et al. 2001, Lopaschuk & Jaswal 2010). Because anaerobic fermentation produces far less ATP than oxidative phosphorylation per molecule of glucose, increased activity of the glycolytic pathway is necessary to maintain free ATP levels in the hypoxic cell. In the presence of sufficient glucose, glycolysis can sustain ATP production due to increases in the activity of the glycolytic enzymes. When oxygen is absent, LDH catalyzes the reduction of the glycolytic final product pyruvate to lactate, but accumulating lactate and a drop of intracellular pH can eventually disrupt the function of the cells (Seagroves et al. 2001).

HIF not only regulates the energy metabolism; it also controls mitochondrial synthesis and catabolism. HIF-1 activation is reported to inhibit mitochondrial biogenesis and cellular respiration in a C-MYC dependent way (Zhang et al. 2007). In addition, mitochondrial autophagy (mitophagy) is a HIF-1-dependent adaptive metabolic response to hypoxia. HIF-1-regulated BNIP proteins drive this process where the cytoplasmic organelles are catabolized to energy or to protect the cells from reactive oxygen species under oxygen- or nutrient-deprived conditions (Zhang et al. 2008, Mazure & Pouyssegur 2010).
2.2 Regulation of eukaryotic transcription

2.2.1 Gene promoter types

Transcription of eukaryotic protein coding genes is mediated by the RNA polymerase II. Promoter regions that regulate RNA polymerase II binding and activity are heterogeneous and can span several kilobases from the RNA polymerase II binding and transcription start site (TSS). The core promoter region is a proximal promoter region containing approximately 50-100 bp surrounding the TSS and is defined to be the DNA region that directs the initiation site of transcription by RNA polymerase II. Regulated genes tend to have focused initiation promoters in which there is either a single major transcription start site or several start sites within a narrow region of several nucleotides (Figure 1). Constitutively expressed genes have typically dispersed initiation promoters in which several weak transcription start sites are present over a broad region of about 50 to 100 nucleotides. This is typical of CpG island promoters, which account for about 70% of human genes. Focused promoters are found in a small, but biologically important fraction of genes. Several sequence motifs have been characterized from focused promoters, such as TATA box, upstream TFIIB recognition element (BREu), Initiator (Inr), Motif Ten Element (MTE), Downstream Promoter Element (DPE), Downstream Core Element (DCE) and X Core Promoter Element 1 (XCPE1). Of these, the TATA, BRE, DPE and MTE motifs are lacking in dispersed promoters (for review see Baumann et al. 2010, Juven-Gershon & Kadonaga 2010).
Fig. 1. Eukaryotic genes have two main types of promoters. Promoters can be classified into focused initiation and dispersed initiation promoters on the basis of the core promoter elements and foci of the transcription initiation +1 nucleotide sites. Regulated genes and housekeeping genes have commonly dissimilar promoter properties, and distinct elements control the gene expression.

2.2.2 Common promoter elements

TATA box is one of the best-known promoter elements usually present in about 10–15% of mammalian core promoters, commonly found within strong tissue-specific promoters. TATA box has a consensus sequence, 5’-TATAWAAR-3’ (IUPAC nucleotide code), and it is usually located in close proximity of the transcription initiation site (Carninci et al. 2006). It serves as a binding site for the TATA-binding protein subunit (TBP) of the general transcription factor TFIID and specifies the transcription initiation point. The downstream promoter element (DPE) is typically, but not exclusively, found in TATA box-deficient (TATA-less) promoters. DPE has similar importance in the determination of transcription start site and it is recognized indirectly by TFIID (Burke & Kadonaga 1997). Focused transcription typically initiates within the Initiator element (Inr, in mammals
YYANWYY), which encompasses the transcription start site (A +1) in the middle and is probably the most commonly occurring core promoter motif (FitzGerald et al. 2006) (Figure 1). Due to strict spacing dependence it regulates the positions of other functional elements (MTE, DPE) in the core promoter (Juven-Gershon & Kadonaga 2010). However, there are no universal core promoter motifs and therefore element composition varies between different genes. Promoter properties are determined by the presence or absence of specific core promoter elements.

Another class of promoters are the CpG island promoters. Those are defined as CG-rich 0.5-2 kb DNA stretches, which typically lack TATA box and DPE, but contain GC box motifs and binding sites for specificity protein 1 (Sp1) (Figure 1). They are often associated with housekeeping genes or with genes having a tissue-restricted expression pattern. CG dinucleotide is underrepresented in the genome, since the majority of CpGs are methylated to 5-methyl cytosine, which has a tendency to mutate spontaneously to thymine. CpG islands possess high relative densities of un-methylated CpG positioned at the 5’-ends of many mammalian genes (for review see Bird 2002, Baumann et al. 2010). TATA box promoters presented only 10-15% of promoters while CpG island promoters are found in over 70% of promoters (Kim et al. 2005). CpG island promoters typically contain multiple transcription start sites that span a region of 100 bp, but the core promoter function is less well characterized, suggesting that instead of a single core promoter, several weaker core promoters may be present.

2.2.3 Common transcription factors and the general transcription machinery

Eukaryotic RNA polymerases do not recognize core promoter sequences. RNA polymerase II binding to promoter and the assembly of a transcription pre-initiation complex (PIC) is initiated by the assembly of the “general” or “basal” transcription factors. These include transcription factor for RNA polymerase A (TFIIA), TFIIB, TFIID, TFIIE, TFIIF and TFIIH, of which TFIID is a key basal factor that is involved in the recognition of the focused core promoters. TFIID is a multisubunit complex that associates with TATA box-binding protein (TBP) and about a dozen TBP-associated factors (TAFs). The core promoter binding elements are recognized by different parts of the TFIID complex, e.g. TBP binds to a specific TATA box, TAF 1 and 2 recognize the Inr, and TAF 6 and 9 appear to interact with the DPE. The other basal transcription factors act in an early phase
of the machinery assembly mainly by enhancing and stabilizing the interactions of polymerase, TBSs, TAFs and promoter elements. They unwind the DNA, which aids the formation of the general transcriptional machinery on the core promoter. Core promoters can regulate gene expression to some extent (for review see Smale 2001). For example, core promoter element combination can have an effect on promoter-enhancer interactions and the efficacy to respond to enhancer TF binding. Some enhancing TFs activate TATA-containing core promoter, but not DPE-containing promoters (for review see Smale 2001).

2.2.4 Regulation of transcription

Besides the core promoter, transcriptional activity is mainly regulated by the specific proximal cis-acting DNA sequences. These regions include enhancer, repressor, silencer, and insulator promoter elements, which can be scattered over distances of roughly 100 kb from TSS. Regulation of gene expression involves generally a spatial and temporal interplay of these trans-acting elements in association with basal transcription factors and RNA polymerase II. The regulation process involves ATP-dependent chromatin remodeling factors, enzymes that catalyze acetylation, deacetylation, phosphorylation, methylation, and ubiquitinylation of histones and other proteins related to the transcription process (for review see Lee & Young 2000, Baumann et al. 2010).

2.2.5 Enhancers

Diversity of enhancers

Enhancers are clusters of DNA binding sites for transcriptional regulators that influence transcription at distances as great as 100 kb from the start site (Figure 2). Enhancers are located upstream or downstream or even within the intron of the gene they regulate (for review see Arnosti & Kulkarni 2005). Enhancers are found to interact with both transcriptional activators and repressors. These regulatory elements may thus account for both activation and repression of gene expression (Figure 2). Enhancers operate as a scaffold to formation of transcription factors and co-factors containing a nucleoprotein complex called enhanceosome, which stimulates the assembly and activity of the pre-initiation complex in the core promoter. Enhanceosome contains multiple TFs and co-factors, which can act


synergistically. Thus even a small amount of binding factors can activate gene expression efficiently, and alterations in individual binding sites can have drastic effects on enhancer output (for review see Lee & Young 2000, Arnosti & Kulkarni 2005). In addition to the highly cooperative and coordinate action of enhanceosomes, less integrative, but more flexibly disposed enhancers exist as well. In these enhancers, separately acting factors or small groups of independently acting factors interact with their targets and multiple functional units are able to regulate gene expression. However, both types of enhancers described cause modification of chromatin structure to permit binding of the transcriptional machinery and recruit the components of the basal machinery to the promoter. Thus the sum of the repressing and activating factors drives the basal transcriptional machinery assembly. Several algorithms and matrices have been developed to identify putative transcription factor binding sites to further characterize enhancer sequences. Algorithms and online databases such as rVISTA and JASPAR (Loots et al. 2002, Sandelin et al. 2004) can be used to evaluate promoter regions and to characterize novel binding sites for transcriptional regulators.

Fig. 2. Simplified representation of the transcriptional regulatory elements. The promoter is typically comprised of proximal and core elements. Transcription of a gene can be regulated by multiple enhancers that are located distantly and interspersed with silencer and insulator elements, which bind regulatory proteins and transcription factors.
A characteristic feature of the enhancers is the modularity by which they regulate gene expression. The modularity of cis-acting elements has been studied in great detail, e.g. in the case of cardiac homeobox transcription factor Nkx2–5 gene regulation (Schwartz & Olson 1999). These studies revealed the modularity in cardiac transcription so that at least five distinct elements govern and direct Nkx2–5 gene expression to different compartments of the developing heart. It has been suggested that this regulatory complexity has also played an important role in the evolution of the multi-chambered mammalian heart (Schwartz & Olson 1999, Chi et al. 2005).

**Regulation mechanisms**

The connection between enhancer regions, transcription factors and pre-initiation complex connection is mediated by a poorly characterized, 26-subunit complex Mediator, which has been shown to be essential in protein coding transcription from yeast to man (for review see Conaway et al. 2005, Taatjes 2010). Mediator provides an extensive surface area that facilitates multiple protein-protein interactions serving as a central scaffold around which the transcription factors, co-factors and pre-initiation complex proteins are assembled. This can presumably cause structural shifts and activity modifications of the bound PIC units. It has also been demonstrated that the Mediator complex is involved in an activator-independent transcription, implying that it may provide a fundamental control of the formation of the initiation complex. However, all the existing evidence shows that Mediator significantly regulates RNA polymerase II recruitment and controls stabilization of transcription complexes at the promoter (for review see Taatjes 2010).

Repressors and silencers inhibit transcription through various mechanisms, including interfering with activator binding, preventing recruitment of the transcription apparatus by the activator, and modifying chromatin structure. Silencer regions typically repress promoter activity through induced histone deacetylation modifications and possibly by CpG dinucleotide methylations (Lee & Young 2000).
2.3 Gene expression is regulated by hypoxia-inducible factors

2.3.1 Structure and different isoforms of HIFs

**HIF-1**

HIF proteins (HIF-1, 2, 3) are coded by three distinct HIF-α-subunit genes including HIF1α, EPAS1 and HIF3A, respectively (Wang *et al.* 1995, Ema *et al.* 1997, Gu *et al.* 1998). HIF-1 is a ubiquitously expressed heterodimer that is composed of a 120-kDa (826 amino acids) HIF-1α and 91–94 kDa (800 amino acids) HIF-1β subunit, also known as ARNT (aryl hydrocarbon receptor nuclear translocator) (Wang & Semenza 1995). Amino-terminal parts of the subunits consist of the basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains. In bHLH-PAS superfamily proteins bHLH and PAS domains are required for α/β dimerization and DNA binding sequence recognition and major groove binding (Kewley *et al.* 2004). Carboxy-terminal parts contain N-terminal and C-terminal trans-activation domains (NTAD and CTAD) responsible for recruiting cofactors for transcription machinery assembly and regulatory domains for oxygen-dependent degradation regulation (oxygen-dependent degradation domain, ODDD) (Jiang *et al.* 1996a, Huang *et al.* 1998, Ivan *et al.* 2001). Comparison of human, mouse and rat HIF-1α cDNA sequences revealed over 90% homology in the amino acid sequences between species, showing a high degree of conservation between species (Wenger *et al.* 1996, Luo *et al.* 1997).

![HIF-1α and HIF-1β structure and domain composition](image)

Fig. 3. HIF-1 structure and domain composition. HIF-1 is composed of HIF-1α and HIF-1β subunits. Both subunits have basic helix-loop-helix (bHLH) and PAS (Per-Arnt-Sim) domains, which are required for dimerization and DNA binding. HIF-1α contains an oxygen-dependent degradation domain (ODDD) whose conserved proline residues (P402 and P564) are hydroxylated by HIF-P4Hs in normoxia. An N-terminal (N-TAD) transactivation domain is found only in HIF-1α. A C-terminal (C-TAD) domain contains Asn-803 residue which is also hydroxylated in normoxia.
**HIF-2**

HIF-2 DNA binding and dimerization domains are structurally similar to HIF-1, but the isoforms differ in their transactivation domains (Ema et al. 1997). HIF-2α is also oxygen-regulated and functionally related to HIF-1α. It can also heterodimerize with HIF-1β and regulate hypoxic expression of some genes, but HIF-2α has typically a more cell-type specific expression pattern (Ema et al. 1997, Tian et al. 1997, Hu et al. 2003, Sowter et al. 2003). It has also been suggested, that HIF-2 does not necessarily have as strong trans-activation capability as HIF-1 (Mole et al. 2009).

**HIF-3**

HIF-3α is structurally and functionally distinct from HIF-1α and HIF-2α and has several splicing variants, such as inhibitory PAS domain protein (IPAS) in mouse. IPAS lacks transactivation domains and is able to bind HIF-1α in cytosol and acts in a dominant-negative way with respect to HIF-dependent transcription (Gu et al. 1998, Makino et al. 2001, Makino et al. 2002). IPAS has been found to be strongly expressed in the corneal epithelium of the eye, where HIF-dependent angiogenesis is suppressed despite profound tissue hypoxia. This maintains a crucial avascular phenotype of the cornea (Makino et al. 2001), where blood vasculature would affect vision. Interestingly, HIF-3α was shown to be a target of HIF-1, but HIF-3α was not regulated at the level of protein stability (Tanaka et al. 2009). Increased HIF-3α expression was connected with reduced HIF-1 activity suggesting a physiological role for HIF-3α in the feedback modulation of HIF-1 signaling.

### 2.3.2 Oxygen-dependent regulation of HIF

**Degradation in normoxia**

HIF-1 activity is mainly regulated posttranslationally through the oxygen-dependent proteolysis of the HIF-1α subunit. The amount of HIF-1β (ARNT) is relatively stable in cells and it is not regulated in an oxygen-dependent manner (Kallio et al. 1997). Under normoxic conditions, HIF-1α is degraded by the ubiquitin-proteasome pathway (Figure 4). In normoxia, von Hippel-Lindau tumor suppressor protein (pVHL) E3 ubiquitin ligase complex binds to the hydroxylated
oxygen-dependent degradation domain (ODDD) of an α-subunit and polyubiquitination of lysine residues targets protein into the 26S proteosomal degradation (Huang et al. 1998, Kallio et al. 1999 Ivan et al. 2001, Kaelin & Ratcliffe 2008). Two conserved ODDD proline residues (Pro402 and Pro564 in human HIF-1α) (Figure 3) are hydroxylated under normoxic conditions by the specific HIF prolyl 4-hydroxylase (HIF-P4H) enzymes (Jaakkola et al. 2001) also known as prolyl hydroxylase domain-containing proteins (PHDs). The HIF-P4Hs are dioxygenases containing Fe²⁺ ion in catalytic site and requiring ascorbate as a cofactor. They use molecular oxygen and 2-oxoglutarate as substrates in an enzymatic reaction where one oxygen atom is inserted into HIF-1α and the other atom into 2-oxoglutarate to generate succinate and CO₂ (Myllyharju 2008). Under hypoxia or when the HIF-P4Hs are inactivated by competitive substrate analogs or Fe²⁺ is chelated or replaced, the HIF-1α ODDD prolines remain unmodified and thus HIF-1α avoids proteosomal degradation (Hirsila et al. 2003, Hirsila et al. 2005, Myllyharju 2008) (Figure 4). Degradation is a very rapid process; in a normoxic environment the half-life of hypoxia-accumulated HIF-1α is less than 5 minutes, which indicates almost instantaneous degradation of HIF-1α after O₂ exposure (Wang et al. 1995, Yu et al. 1998). HIF-1 DNA-binding activity was shown to increase exponentially from 5% O₂ (35 mm Hg) to 0.5% O₂ (3.5 mm Hg) with the half maximal response between 1.5 and 2% O₂ (10.5 mm Hg) (Jiang et al. 1996b).
Fig. 4. HIF-1 pathway. In hypoxia, HIF-1α accumulates, localizes into nucleus and forms functional HIF-1 by dimerizing with HIF-1β. HIF-1 binds to the HIF-binding sequence (5’-ACGTG-3’) in a hypoxia response element and regulates target gene expression. In normoxia, HIF-1α is hydroxylated by HIF-P4Hs and FIH, polyubiquitinylated by von Hippel-Lindau tumor suppressor (pVHL) complex and degraded in the 26S proteasomal pathway. Hydroxylation reactions and degradation pathway can be inhibited by prolyl hydroxylase inhibitors such as DFO.

The role of HIF-prolyl-hydroxylases

HIF-P4H isoform 2 (HIF-P4H-2, PHD2) seems to be the primary regulator of HIF-1α and it has the highest HIF-hydroxylation activity in most cell types. It is the critical oxygen sensor which imposes the low steady-state levels of HIF-1α in normoxia (Berra et al. 2003). Other isoforms (HIF-P4H-1/3, PHD1/3) can be more cell type-specific or HIF isoform-specific, depending on other external stimuli and the cell type-specific expression and abundance of the enzymes (Appelhoff et al. 2004). Interestingly, isoforms 2 and 3 are targets for HIF-1 (Metzen et al. 2005, Pescador et al. 2005). This creates a self-controlling feedback network which acts as a mechanism to stop hypoxia responses rapidly in
in re-oxygenated cells (D'Angelo et al. 2003, Stiehl et al. 2006). The feedback regulation also helps in transition between different developmental stages. Tissue-specific expression of HIF-P4Hs and differential regulation of HIF-responses create more plasticity to hypoxia responses and explain expressional variation in different cell types after hypoxic stimulus (Kelly et al. 2003).

**Factor Inhibiting HIF (FIH) regulates HIF-activity**

In addition to regulation of protein stability, HIF-activity can be modulated by hydroxylation, phosphorylation and redox reactions of N- and C-terminal transactivation domains (Ema et al. 1999, Carrero et al. 2000, Wenger 2002). HIF-1/2α carboxy-terminal trans-activation domain activity is modulated by Factor Inhibiting HIF-1 (FIH-1) (Mahon et al. 2001). FIH-1 is an Fe²⁺ and 2-oxoglutarate-dependent dioxygenase like HIF-P4Hs (Dann et al. 2002). FIH-1 acts as an asparaginyl hydroxylase and hydroxylates a conserved HIF-α carboxy-terminal TAD Asn803 which inhibits HIF-α interaction with coactivator CBP/p300 CH1 region, and the assembly and activity of the transcriptional machinery (Mahon et al. 2001, Freedman et al. 2002, Lando et al. 2002). FIH-1 remains active at lower O₂ concentrations than HIF-P4Hs and can still modulate HIF-1α activity even if it escapes the proteosomal degradation in moderate hypoxia (Koivunen et al. 2004). Thus, in addition to regulation of half-life of HIF-α proteins, HIF transcriptional activity is also regulated by hydroxylation reactions modulated by cellular O₂ concentrations. Hydroxylation reactions link the surrounding molecular O₂ concentration directly to transcriptional cascades and adaptational responses, serving as a modification step in hypoxic gene expression changes. In hypoxia the hydroxylation reactions are inhibited as a result of O₂ substrate deprivation.

**Oxygen-independent stability and activity regulation**

HIF-1 degradation and activity regulation can also occur more or less without O₂ participation. The receptor of activated protein kinase C (RACK1) has been shown to bind HIF-1α PAS-domain and promote polyubiquitination, thus bypassing the O₂/H-P4H/pVHL-dependent degradation pathway independently of the ODD domains (Liu et al. 2007). The oxygen-independent ubiquitination process can be enhanced by utilization of Spermidine/spermine N1-acetyltransferase-1 (SSAT1) to HIF-1-RACK1-complex which stabilizes the
interaction (Baek et al. 2007). In addition, HIF-1 stability can be modulated independent of O$_2$ by a small ubiquitin-related modifier (SUMO) mediated SUMOylation process, which promotes pVHL binding to HIF-1$\alpha$ without preceding Pro402 or Pro564 hydroxylation and predisposes HIF-1$\alpha$ to proteosomal degradation (Cheng et al. 2007). The physiological importance of the process is still controversial and there are contradictory reports on the SUMOylation effect on HIF-1 function (Carbia-Nagashima et al. 2007).

2.4 Hypoxia-responsive promoters and target genes

2.4.1 Hypoxia response element

HIF-1$\alpha$ translocates into the nucleus due to nuclear localization signal (NLS) within the C-terminal end of the subunit (Kallio et al. 1998). NLS tags proteins for nuclear transport through the nuclear envelope pores. HIF-1$\alpha$ and HIF-1$\beta$ subunits have to heterodimerize in the nucleus to form a functional HIF-1 dimer and regulate the target genes (Kallio et al. 1998). The target gene promoter region contains a so-called cis-acting Hypoxia Response Element (HRE), which contains a five base pair long conserved HIF-1 Binding Sequence (HBS). HBS consists of an asymmetric E-box motif (5´-(G)/ACGTG-3´) which was first characterized from an EPO gene enhancer (Semenza & Wang 1992) and later e.g. from aldolase-1, endolase-1, lactate dehydrogenase A (Semenza et al. 1996) and vascular endothelial growth factor A (Forsythe et al. 1996) enhancers. Recently, new HIF-1 binding site properties were also characterized by using genome-wide computational analysis (Mole et al. 2009). The HBS region is necessary for HIF-1 binding and hypoxic regulation of HIF target genes. To confer full hypoxia responsiveness, some other surrounding transcription factor binding sites like ATF-1/CREB-1 and activator protein-1 (AP-1) may be crucial (Firth et al. 1995, Damert et al. 1997, Ebert & Bunn 1998). These regions are not necessarily hypoxia-regulated, but binding of the specific factors can enhance the HIF-1-dependent response and generate tissue specificity for the HREs.

2.4.2 HIF-binding promotes co-factor recruitment

Binding of HIF-1 to target gene promoter usually enhances and occasionally represses the general transcription machinery assembly and transcription (Wang
& Semenza 1993, Camenisch et al. 2001, Semenza 2010). Assembly is mainly orchestrated by adapter proteins CREB-binding protein (CBP) and p300. CBP possesses strong histone acetyltransferase (HAT) activity and it also recruits other HATs which promote remodeling and opening of local chromatin structures and increase DNA accessibility to transcription factors (Bannister & Kouzarides 1996); CBP/p300 has been shown to have an essential role in the cellular response to hypoxia (Arany et al. 1996). HIF-1 CTAD has been shown to interact with cysteine/histidine-rich region 1 (CH1) of the coactivator CBP/p300 in a strictly hypoxia-dependent manner regulated by FIH (Ruas et al. 2002), but recently NTAD was found to associate with region 3 (CH3) facilitating transactivation function of NTAD as well (Ruas et al. 2010). When bound to HIF-1, CBP/p300 stabilizes HIF-1α/ARNT dimerization, serving as a platform and recruiting additional co-factors including p160/SRC-1 family of proteins (SRC1), TIF-2 and Ref-1 and (Carrero et al. 2000, Ruas et al. 2005).

2.4.3 Epigenetic control of hypoxic transcription

Conserved HBS (5′-CGTG-3′) core sequence contains CpG dinucleotide, which is known to be prone to cytosine methylation outside of the so-called CpG islands, which are almost entirely methylation-free (for review see Deaton & Bird 2011). DNA methyltransferases (DNMT1 and DNMT3) catalyze the transfer of methyl group to cytosine and conversion to 5-methyl-cytosine, which interferes with transcription factor binding to DNA major groove. Methylation provides steric hindrance or promotes binding of repressor methyl-CpG-binding domain proteins (MBDs) and other repressional factors and probably links methylation to histone deacetylase activation (Adams 1990, Boyes & Bird 1991, Hendrich & Bird 1998, Wenger et al. 1998, Wade 2001). EPO enhancer HBS cytosine methylation was shown to repress HIF-1 DNA binding in cell culture, and the degree of CpG methylation was inversely proportional to hypoxic induction of the EPO gene (Wenger et al. 1998). This suggests an essential role of methylation-free core HIF-1 binding sequence for hypoxic EPO expression and HIF-1-dependent induction (Wenger et al. 1998). Epigenetic control of HIF-1 target gene expression may thus also contribute to a tissue-specific and developmental stage-specific hypoxic gene regulation. Ubiquitously expressed genes typically contain housekeeping-type CpG island promoters (Zhu et al. 2008), which are mostly methylation free (Deaton & Bird 2011). Methylation can therefore control only a portion of promoters containing HBS, as several HIF-1 target promoters are CpG
island type promoters (e.g. glycolytic enzymes) and are most likely methylation free in all tissues (Zhu et al. 2008). It has been proposed that tissue and developmental stage-specific HIF-1 activation can arise from normoxic HBS binding of other tissue-specific transcription factors, which mask and protect CpG sites from methylation. This seems to vary between tissues and developmental stages during normoxia at least in EPO promoter (Kvietikova et al. 1995, Hu et al. 1997, Wenger et al. 1998).

2.4.4 Hypoxic repression of promoters

HIF-activation usually leads to increased target gene expression, but there is accumulating evidence that HIF-1 is also capable of repressing target gene expression by various mechanisms (Narravula & Colgan 2001, Mazure et al. 2002, Erler et al. 2004, Chen et al. 2005, Eltzschig et al. 2005, Morote-Garcia et al. 2008, Lee et al. 2010, Zheng et al. 2009, Wen et al. 2010). The mechanism of the HIF-1-dependent down-regulation varies a lot between different genes and cell types, and it is likely that a single universal repression mechanism does not exist. It has been postulated for example that HIF-1 will act as a repressor when the HRE strand is in a reversed orientation (Narravula & Colgan 2001, Eltzschig et al. 2005), or that HIF-1 acts as a competitive inhibitor by establishing a physical barrier which blocks the binding of other transcription factors (Mazure et al. 2002). Others have suggested that repression involves and is mediated by histone deacetylases, since HDAC inhibitor trichostatin A was shown to block HIF-1-mediated repression especially by HDAC1 (Lee et al. 2010) and HDAC7 (Wen et al. 2010). HIF-dependent recruitment of co-repressors, possibly in concert with repressors, should be taken into consideration as well (Semenza 2010).

2.4.5 Target genes

More than a hundred HIF-1 target genes have been characterized by functional assays verifying HIF binding to hypoxia-response elements (Schofield & Ratcliffe 2004, Lendahl et al. 2009, Schoedel et al. 2011). Recently, genome-wide chromatin immunoprecipitation studies linked to high throughput sequencing have produced a massive amount of data showing transcriptomic changes caused by HIF-1/2 (Mole et al. 2009, Schoedel et al. 2011). HIF-1 mediates cell autonomous, tissue-restricted and systemic homeostatic responses depending on
the nature of hypoxia, which can occur locally, e.g. after occlusion of oxygen-delivering artery, or systematically after restricted oxygen availability in hypobaric circumstances.

Classic HIF-1-driven adaptation processes include angiogenesis, erythropoiesis, glycolysis, changes in iron metabolism and cell survival. Although HIF-1 is the predominant mediator of transcriptional responses to hypoxia in all cell types, other factors have been shown to contribute to the regulation of specific genes in specific cell types (for review see Semenza 2010). In addition to direct target gene regulation, HIF-1 regulates a wide variety of genes indirectly by regulating the expression of other transcription factors, which then indirectly modulate the expression of their target genes (Krishnamachary et al. 2006). Interestingly, transcription factors were observed to be one of the largest groups of HIF-1-regulated genes in endothelial cells in hypoxia and in normoxia when the cells were expressing constitutively active HIF-1α (Manalo et al. 2005).

Germline homozygous HIF-1α and HIF-2α knock-out mice are embryonic lethal, and in Hif1a null allele mice development arrests at day E8.5 and embryos die around midgestation at E10.5 (Iyer et al. 1998, Ryan et al. 1998, Kotch et al. 1999). HIF-1α is required for mesenchymal cell survival during embryonic development and HIF-1α knockout embryos show open neural tube defects and major cardiac malformations, vascular regression, and reduced erythropoiesis, indicating that HIF-1 is required for development of the circulatory system (Iyer et al. 1998, Ryan et al. 1998, Kotch et al. 1999, Yoon et al. 2006). Mice having null allele at Epas1 locus encoding HIF-2α have various lethal phenotypes and they die due to various defects in the vasculature (Peng et al. 2000), catecholamine production (Tian et al. 1998), respiratory organ development (Compernolle et al. 2002) and multi-organ failure with mitochondrial dysfunction (Scortegagna et al. 2003). Various tissue-specific conditional Hif1a knock-out studies show that HIF-1α is essential for proper development, survival and physiological functions in numerous cell types (for review see Semenza 2010). For example, mice with cardiac myocyte-specific HIF-1α knock-out are viable, but the deletion causes a drastic change in the heart function leading to systolic and diastolic dysfunction, reduced vascularization as well as reduced high-energy phosphate and lactate production (Huang et al. 2004). Phenotypic change is a result of reduced expression of genes involved in calcium handling (SERCA2), angiogenesis (VEGF) and glucose and lactate metabolism (Glut-1, LDH) which cause a defect in cytosolic calcium removal during cell relaxation, reduction in
the left ventricle vessel count, and reduction in high-energy phosphate content (Huang et al. 2004).

In the heart, HIF-1-activated genes adapt the tissue to reduced oxygenation by increasing blood flow in the hypoxic areas by vasodilatation or by angiogenesis and neo-vascularization. These effects are mediated by adrenomedullin (Adm) (Nguyen & Claycomb 1999), atrial natriuretic peptide (ANP) (Chun et al. 2003) and vascular endothelial growth factor (VEGF) (Forsythe et al. 1996). In addition, HIF-1-driven gene expression changes enhance glucose metabolism by inducing the expression of Glut-1 and several glycolytic genes. More adaptation responses are occasionally characterized, e.g. cardiotrophin-1 was very recently characterized to be HIF-inducible and to have a cardioprotective effect in hypoxia by preventing apoptosis (Robador et al. 2011).

2.5 Hypoxia effects on heart muscle

2.5.1 Myocardial energy metabolism

Heart muscle is highly oxidative tissue that produces more than 90% of its energy from mitochondrial oxidative metabolism and has very limited anaerobic capacity. The coronary vascularization and capillary network ensure myocardial blood and oxygen supply in physiological situations, and high oxygen demand is fulfilled. Metabolic autoregulation of the coronary vasculature serves as an acute adaptation response for the increased metabolic needs due to increased cardiac output stimulated by e.g. exercise and sympathetic activation. Reduced tissue pO2 and accumulating metabolites decrease vascular smooth muscle cell tone, and vasodilation increases myocardial perfusion (Rubio & Berne 1975, Dole 1987, Tune et al. 2004).

Mitochondria occupy ~30% of cardiomyocyte space and the direct correlation between cardiac workload and oxygen consumption is well documented (for review see Jafri et al. 2001, Ventura-Clapier et al. 2004). In oxidative striated muscle, myoglobin ensures a relatively constant oxygen supply for mitochondria. Myoglobin acts as an oxygen storage/buffer protein which binds O2 reversibly in cytosol and facilitates O2 diffusion. Bound O2 is released to sustain aerobic metabolism in active heart muscle and to stabilize the O2 fluctuations due to capillary circulation changes, and to enhance O2 flux from the extracellular space to mitochondria (for review see Ordway & Garry 2004).
The heart produces energy from the oxidation of fatty acids, glucose, lactate, ketones and amino acids (Figure 5). Fatty acids are activated to fatty acyl-CoA in the cytosol and transported into mitochondria via carnitine palmitoyltransferase I (CPT1) and carnitine-acyltranslocase (CAT). Acyl-CoA enters β-oxidation, with acetyl-coenzyme A, NADH and FADH$_2$ as the main products. Glucose oxidation is a minor but important component of the total cardiac energy production. Under physiological conditions, glucose is transformed to pyruvate, which enters mitochondria and is decarboxylated to acetyl-coenzyme A by pyruvate dehydrogenase (PDH). Acetyl-CoA from both β-oxidation and glycolysis enters the tricarboxylic acid cycle, resulting in the formation of NADH and FADH$_2$, which feed electrons into the respiratory chain driving oxidative phosphorylation (Figure 5). Electrons that travel down the respiratory chain are eventually transferred to O$_2$ via cytochrome oxidase forming H$_2$O (for review see Jafri et al. 2001, Maack & O'Rourke 2007). The established large electrochemical proton gradient is discharged through F$_1$F$_0$-ATPase which couples the flow of protons through its central pore to the phosphorylation of ADP to ATP generating energy stores that can be used to perform mechanical work. In normoxia, the main part of cardiac energy comes from the oxidation of fatty acids so that 60–90% of the acetyl-CoA comes from β-oxidation, and 10–40% from the oxidation of pyruvate. Pyruvate is derived in approximately equal amounts from glycolysis and lactate oxidation (for review see Stanley et al. 2005).
Fig. 5. The main myocardial energy metabolism pathways. The primary energy sources are fatty acids, glucose and lactate. Substrates are transported to cytosol and mitochondria by specific transporters such as glucose transporters (GLUT) for glucose, monocarboxylic acid transporters (MCT) for lactate and fatty acid transporters (FAT)/carnitine palmitoyltransferases (CPT1) for fatty acids. The specific enzymes (LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase) catalyze the formation of reducing equivalents and substrates to the tricarboxylic acid (TCA) cycle. Produced NADH donates its electrons to the respiratory chain which uses energy to pump protons to the mitochondrial intermembrane space, generating an electrochemical H\(^+\) gradient across the mitochondrial inner membrane. O\(_2\) functions as the final electron acceptor in the electron transport chain. Discharging the H\(^+\) gradient through F\(_1\)F\(_0\) ATPase (F\(_1\)F\(_0\)) drives the ATP synthesis. Myoglobin buffers intracellular O\(_2\) and facilitates diffusion, augmenting constant mitochondrial oxygen supply.

The two most important factors regulating the cardiac energy production are cellular concentrations of ADP and Ca\(^{2+}\). The rate of cellular respiration is regulated by the availability of ADP to the F\(_1\)F\(_0\)-ATPase, while Ca\(^{2+}\) regulates the activity of the key enzymes of the tricarboxylic acid cycle (for review see Maack & O'Rourke 2007). In the healthy heart the rate of oxidative phosphorylation is linked to the rate of ATP hydrolysis so that ATP content remains constant even
during large increases in the cardiac power demand during exercise and acute catecholamine stress (for review see Stanley et al. 2005).

2.5.2 Developmental and pathophysiological changes in cardiac energy metabolism

Metabolism during development

Cardiac energy metabolism evolves during prenatal development, and the primary sources of energy change during cardiogenesis. In the early embryonic stage, the proliferating cardiomyocyte precursor cells are more dependent on glycolysis as a source of energy. Mitochondrial organization and oxidative metabolism is poorly developed (for review see Lopaschuk & Jaswal 2010). The heart develops in a low oxygen environment, and high rates of glycolysis and lactate production are typical, even in the presence of adequate oxygen. The fetal heart is able to oxidize lactate, the level of which is high in fetal circulation. Lactate oxidation accounts for the majority of myocardial oxygen consumption. On the contrary, fatty acid oxidation contributes only a minor fraction of ATP production (~15%) in fetal myocardium (Lopaschuk et al. 1991). A high glycolytic rate and the ability to use lactate oxidation for ATP production help cardiomyocytes to survive in a relatively hypoxic fetal environment. As cardiomyocytes differentiate more towards the adult phenotype, there is a switch from glycolytic metabolism towards mitochondrial oxidative phosphorylation in order to meet increases in workload and energy demand (for review see Lopaschuk & Jaswal 2010). During the neonatal period, β-oxidation increases (a ten-fold increase), accompanied by a parallel decrease in the glycolytic rates. Metabolic switch is mainly regulated by the expression and regulation of factors such as acetyl-coenzyme A carboxylase (ACC), whose product malonyl-CoA modifies the carnitine palmitoyltransferase I (CPT1) driven fatty acid uptake and increases mitochondrial β-oxidation (Saddik et al. 1993). As a consequence, soon after birth β-oxidation approaches levels observed in the adult heart (for review see Lopaschuk & Jaswal 2010).

Metabolism in pathological processes

Cardiac muscle cannot sustain anaerobic metabolism. When oxygen supply does not match myocardial demand, cardiac contractile dysfunction occurs, and
prolongation of this mismatch leads to apoptosis and necrosis. In pathological situations, cardiac energy substrate metabolism can revert to “fetal” type so that in failing heart the chief myocardial energy substrate switches from fatty acids to glucose. The switch is accompanied by a down-regulation of the enzymes involved in fatty acid oxidation (Sack et al. 1996, Razeghi et al. 2001). As a consequence, glycolysis is increased and fatty acid oxidation is decreased. The activity and expression of enzymes involved in these metabolic pathways is drastically changed in pressure and volume overloaded hypertrophied and failing hearts (Elalaouitalibi et al. 1992, Allard et al. 1994, Sack et al. 1996, van der Vusse et al. 2000, Morissette et al. 2003, Ingwall 2009). In the presence of glucose, metabolically active cardiac tissue can to some extent adapt to the hypoxic environment by increased glucose and lactate metabolism. Due to anaerobic metabolism, the heart becomes acidic in a number of pathological conditions, most dramatically during myocardial ischemia. Because of the accumulation of lactic acid, pH drops in the myocardium, lowering intracellular pH as well. This decreases the contraction force due to reduced sensitivity and binding of Ca\(^{2+}\) to contractile proteins. As a secondary effect, increased [Ca\(^{2+}\)] predisposes the heart to arrhythmias (Orchard & Kentish 1990, Tavi et al. 1999, for review see Crampin et al. 2006).

2.5.3 The role of HIF in pathophysiological situations

HIF-1 has been shown to be an important mediator in Pasteur effect in hypoxic cells, when the cells are transferring their metabolism from oxidative phosphorylation towards anaerobic metabolism and increased activity of the glycolytic pathway is necessary to maintain free ATP levels (Seagroves et al. 2001). It has been shown that hypertrophied heart can become hypoxic and up-regulation of HIF-1 expression is seen in pressure-overload hypertrophy (Choi et al. 2008). In pathologic cardiac hypertrophy, activation of HIF-1 and peroxisome proliferator-activated receptors (PPARs) has been shown to determine the switch of glycolytic and lipid metabolism pathways (for review see Stanley et al. 2005, Lopaschuk & Jaswal 2010). Hypoxia and HIF-1 pathway is also shown to increase glycolytic enzyme and transporter synthesis and isoform transitions in cardiac cells. This further links HIF-1 to the metabolic shift.

HIF-1α expression is increased in the myocardium of patients with ischemic heart disease, and HIF-1 has been proposed to have an important role in cardiac metabolic adaptation to chronic ischemia (Lee et al. 2000). Adenoviral delivery of
constitutively active HIF-1α improved myocardial perfusion due to enhanced collateral vessel formation and left ventricular function in infarct models (Shyu et al. 2002). In addition, cardiac HIF-1α overexpression attenuated myocardial infarction size and promoted post-ischemic function and peri-infarct capillarization after myocardial infarction in mice. HIF-induced gene expression was shown to protect the heart from ischemia-related damage as overexpression of HIF-1α reduced infarct size and limited the progression of infarct-induced cardiac failure in mice (Kido et al. 2005). Changes were mainly mediated by an increased expression of vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) in peri-infarct and infarct regions. Ischemic myocardium goes necrotic unless reperfused, but ischemia-reperfusion injury can in turn cause serious damage to the reperfused tissue (for review see Yellon & Hausenloy 2007). Recently, HIF-1 activation has been addressed in protection of reperfusion injuries as well (Cai et al. 2008, Eckle et al. 2008).

2.6 Cardiovascular system development

2.6.1 Hypoxia drives mammalian development

Mammalian placental circulation leads to lower oxygen tension in the developing embryo and fetus as compared with the air-breathing adult, suggesting that normal fetal oxygen is close to physiological hypoxia in adult tissues (for review see Patterson & Zhang 2010). Development of the mammalian cardiac and circulatory system is driven by this intra-embryonic “physiological” hypoxia during embryogenesis. Hypoxia often serves as an important, HIF-mediated developmental signal for tissues. Intrauterine oxygen concentrations range approximately from 1 to 5% (pO₂ 0.5-30 mmHg) before the development of placenta and vascularization (Fischer & Bavister 1993). During placenta development, hypoxia maintains cytotrophoblast proliferation and prevents differentiation toward an invasive phenotype, thereby regulating placental growth and organization (Genbacev et al. 1997).

2.6.2 The role of HIF in cardiogenesis

ARNT knockout studies revealed that HIF-1 is essential for mammalian placentation (Adelman et al. 2000, for review see Dunwoodie 2009). As the
embryo size increases during early post-implanted development, diffusion alone is not adequate to deliver oxygen into the inner layers of the embryo. Regional hypoxia drives mesodermal germ-layer cells to start forming cardiac precursor cells. Cardiac development proceeds in hypoxic environment, and dedicated cardiac precursor cells form a heart tube structure at embryonic day 8.25 (E8.25) in mouse. The heart tube undergoes looping morphogenesis and chamber formation between E8.5 and E12. During the remodeling stage (E12.5-E15), division of the heart chambers by septation is completed and valves ensure unidirectional blood flow. Oxygen delivery system starts to build up to relieve the hypoxic stress. In the rat, the heart starts to beat on embryonic day 10, circulating red blood cells on embryonic day 11 (for review see Dunwoodie 2009). Upon formation of the four-chambered heart and especially due to coronary vessel formation in the developing heart, hypoxic areas are restricted to smaller regions. The number of hypoxic cells is reduced in the hearts between E13.5 and E15.5 in mice, and this reduction correlates with the perfusion of the coronary vasculature following its connection to the aorta at E14.5 (Xu et al. 2007). In the developing heart, HIF-1 specifically regulates the expression of transcription factors promoting cardiomyocyte differentiation, and lack of HIF-1α results in aborted heart development and early embryonic lethality (Krishnan et al. 2008, Dunwoodie 2009). Several of these transcription factors play a crucial role in cardiogenesis having interactions between signaling networks and pathways. Cardiogenesis is governed by a network of core cardiac transcription factors such as Nkx2–5, Tbx5, Mef2C and GATA4, which work in a coordinated manner during different steps of heart organogenesis (Schlesinger et al. 2011), and it seems that HIF-1 functions as a master regulator of the core cardiac transcription factor network in hypoxia. It has been shown that HIF-1 regulates expression of looping morphogenesis and chamber formation linked Mef2C and Tbx5 transcription factors directly, and Nkx2–5-levels are decreased in HIF-1α-deficient hearts as well (Krishnan et al. 2008). In addition, HIF-1 interacts with other pathways that are active during cardiac development, and e.g. Notch signaling is modulated by HIF-1 (Poellinger & Lendahl 2008).
2.7 Excitation-contraction coupling in myocardial cells

2.7.1 Electrical activity

Cardiac excitation-contraction (EC) coupling is an electromechanical coupling process, where electrical excitation of the cardiac myocytes leads to contraction of the heart. The initial trigger to ventricular contraction is the spontaneous depolarization and generation of action potential in the sinoatrial node pacemaker cells. Electrical conduction system and cellular gap junctions allow the rapid propagation of the depolarization and repolarization wave through the heart and the sarcolemmal (SL) membranes of cardiac myocytes. The normal resting membrane potential in the ventricular myocardium is about -85 mV to -95 mV due to selective permeability of the cell membrane especially to potassium and to a lesser degree to sodium, calcium, and chloride ions. The phospholipid bilayer has a low intrinsic permeability to ions, and permeability depends on the properties of the ion channels embedded in the plasma membrane. In myocyte, the intracellular [Na⁺] is lower and intracellular [K⁺] is higher than extracellular due to the ion transporter Na⁺-K⁺-ATPase activity. The pump exchanges three Na⁺ ions from the intracellular space for two K⁺ ions from the extracellular space on each cycle. In the resting myocyte, there is a low open probability for Na⁺ channel, but a moderate one for K⁺ channels. The negative membrane potential forms due to K⁺ diffusion down the concentration gradient to the outside of the cell through potassium-selective leak-channels leaving an anion surplus (from e.g. proteins) and negative charge inside the cell. In addition to Na⁺-K⁺-ATPase this electrical gradient is maintained by ion channels, pumps and exchanger mechanisms, including Na⁺-Ca²⁺ exchanger and Iₖ₁ inward rectifying K⁺ channels. In cardiac myocytes, resting membrane potential is associated with the diastolic phase of the cardiac cycle (Bers 2001, Bers 2002, Nerbonne & Kass 2005, Chen et al. 2007).

During action potential, the opening of the fast Na⁺ channels causes a rapid increase in the membrane conductance to Na⁺, and extracellular Na⁺ enters the cell following its electrochemical gradient. This depolarizes cell membrane potential (E_m) towards +70 mV. Depolarization leads to rapid inactivation and closure of Na⁺ channels. Sarcolemmal Ca²⁺ channels are important in sustaining an action potential during the plateau phase in adult ventricular myocytes. During the action potential plateau phase in long action potential species (e.g. human, rabbit, guinea pig), there is a balance between the inward current of Ca²⁺ through
L-type calcium channels and the outward current of K\(^+\) through the slow delayed rectifier potassium channels. After a delay the Ca\(^{2+}\) channels close, leaving the slow delayed rectifier channel open. Development of negative membrane potential allows the rapid delayed rectifier and inward rectifier potassium channels to reopen, which results in more K\(^+\) flow out of the cell and repolarization of the membrane to the resting state (Bers 2002, Nerbonne & Kass 2005, Chen et al. 2007).

### 2.7.2 Calcium signaling

Depolarization causes a change in conduction of sarcolemmal voltage-gated calcium channels and Ca\(^{2+}\) entry to cell. There are two classes of membrane potential (Em)-dependent Ca\(^{2+}\) channels in cardiac myocytes, L-type (long-lasting) and T-type (transient) channels. T-type channels are not present in most adult ventricular myocytes; they are mainly expressed in conducting and pacemaker cells as well as immature or developing ventricular myocytes. L-type channels are major sarcolemmal Ca\(^{2+}\) channels and respond to higher membrane potentials, open more slowly, and remain open longer than T-type channels. L-type channels are important in the regulation of the cardiac cell electrical activity described in the previous chapter, but they also have a distinct role in the cardiac electromechanical coupling and contractile function (Fabiato & Fabiato 1975, Kirby et al. 1992, Lamb 2000, Bers 2001, Geeves et al. 2005, Chen et al. 2007).

During the plateau phase of the action potential, a small amount of extracellular calcium enters the cell, generating an L-type Ca\(^{2+}\) current. Increased sub-sarcolemmal [Ca\(^{2+}\)] leads to calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR) through Ca\(^{2+}\)-sensitive ryanodine receptors (RyR) (Figure 6). Cardiac RyR2 isoform receptors detect Ca\(^{2+}\) entering the cellular SL/SR junctional space, which increases the open probability of RyRs and triggers Ca\(^{2+}\) release from the SR in the CICR process. Sarcoplasmic reticulum is the major source for Ca\(^{2+}\) and the release through RyRs increases the cytosolic free Ca\(^{2+}\) concentration massively from diastolic ~0.1 µM to systolic 1-2 µM. Local increment of [Ca\(^{2+}\)] and SR Ca\(^{2+}\) depletion inactivates RyRs and closes the channels. Released Ca\(^{2+}\) diffuses into the cytosol and binds to the actin myofilament-associated protein troponin C. Ca\(^{2+}\) binding to the troponin complex moves the tropomyosin away from the actin binding site uncovering an actin-myosin binding region and allowing the myosin head to bind to the actin filament. This switches on the contractile movement where the thin actin and thick myosin
filaments form ATP-consuming cross-bridges followed by filament sliding and contraction of the muscle. Thus Ca\(^{2+}\) ion is the activator of the myofilaments and contractility depends largely on intracellular Ca\(^{2+}\) concentration. Larger [Ca\(^{2+}\)]\(_i\) allows more actin-myosin interactions and results in stronger contraction at a cost of increased ATP consumption (Fabiato & Fabiato 1975, Kirby et al. 1992, Lamb 2000, Bers 2002, Geeves et al. 2005).

Fig. 6. Excitation-contraction coupling in ventricular cardiac myocytes. After depolarization Ca\(^{2+}\) enters the cells through L-type calcium channels (LTCC). A small amount of extracellular Ca\(^{2+}\) opens sarcoplasmic reticulum ryanodine receptors (RyR) and leads to calcium-induced calcium release. Ca\(^{2+}\) binds to myofilament troponin C (TnC) releasing troponin I (TnI), troponin T (TnT) and tropomyosin (Tm) inhibitory effect from actin filament, which allows actin-myosin cross-bridge formation and ATP-consuming filament sliding. Ca\(^{2+}\) is pumped back to SR during relaxation by SERCA2 and outside the cell by Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) and SL Ca\(^{2+}\)-ATPase (ATP).

The main part of Ca\(^{2+}\) is pumped back into the SR by sarcoplasmic reticulum calcium ATPase2a (SERCA2a) (Figure 6). This enzyme catalyzes the hydrolysis of ATP coupled with the translocation of calcium from the cytosol into the sarcoplasmic reticulum lumen. Intracellular calcium concentration drops to resting values, leading to tissue relaxation and diastolic phase of the cardiac cycle.
Two parallel sarcolemmal Ca\(^{2+}\) extrusion mechanisms exist as well. Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) changes extracellular Na\(^+\) to Ca\(^{2+}\) and reduces [Ca\(^{2+}\)], and a minor part of the calcium is pumped out by SL Ca\(^{2+}\)-ATPase and by mitochondrial uptake (Figure 6). In physiological conditions other extrusion mechanisms have far less capacity to lower cytosolic Ca\(^{2+}\) than SERCA2, which is thus an important regulator of cytosolic Ca\(^{2+}\) concentration, SR calcium load and the relaxation of the heart. Because the amount of Ca\(^{2+}\) stored in the SR determines the amount of Ca\(^{2+}\) released into the cytosol during systole, the SERCA2a pump is also involved in the regulation of myocardial contractility (Kirby et al. 1992, Bers 2001, Bers 2002).

### 2.7.3 Energy consumption

Cardiac excitation contraction-coupling consumes large amounts of energy and it has been estimated that 2% of the cellular ATP is consumed during each contraction cycle, primarily for contraction but also for ionic homeostasis (for review see Jafri et al. 2001, Maack & O'Rourke 2007). The main cardiomyocyte energy consumers are the myosin ATPase of the contractile filaments and the calcium-pumping plasmalemmal Na\(^+\)/K\(^-\)-ATPase and sarcoplasmic reticulum SERCA2. Approximately 60–70% of ATP goes to contractile shortening, and the remaining 30–40% is primarily used for SERCA2 and other ion pumps so that as much as 15% of cardiac energy expenditure is due to SERCA2 ATPase activity (Schramm et al. 1994, Stanley et al. 2005).

### 2.7.4 The role of SERCA2 in excitation-contraction coupling

From the early embryonic development, SERCA2a is required in maintaining the activity of cardiomyocytes (Sasse et al. 2007, Rapila et al. 2008). During a single contraction cycle the main part (~92% in adult mouse) of the released Ca\(^{2+}\) is pumped back into the sarcoplasmic reticulum by SERCA2 (Bers 2002). Homozygous SERCA2a knock-out mice die before birth (Periasamy et al. 1999). Heterozygous SERCA2a mice are viable, but ~35% reduction in SERCA2a protein slows down the SR Ca\(^{2+}\) uptake and results in impaired contractility and relaxation of the isolated cardiomyocytes (Periasamy et al. 1999, Ji et al. 2000). Stable SERCA2a expression is vital also in adult hearts. This was demonstrated in an inducible cardiac-specific mouse SERCA2a knock-out model where SERCA2a deletion reduced cardiac contractility significantly in vivo (Andersson et al. 2009,
On the other hand, SERCA2 overexpression in cultured myocytes improves intracellular calcium uptake during relaxation (Hajjar et al. 1997). A positive correlation between SERCA2a levels and hemodynamic function has also been reported (Aoyagi et al. 1999, Hasenfuss & Pieske 2002, Piacentino et al. 2003), and SERCA2 overexpression in transgenic mice accelerates calcium transients and cardiac relaxation (He et al. 1997, Baker et al. 1998). Consequently, SERCA2 is one of the main regulators of cardiac contraction and relaxation, and altered SERCA activity has great impact on the contractile function of the heart (Diaz et al. 2005). Reduced SERCA2a activity is deleterious for the heart and has been associated with various animal models of pressure or volume overload as well as in patients with heart failure (Hasenfuss & Pieske 2002, Piacentino et al. 2003, Periasamy et al. 2008). SERCA2a down-regulation and subsequent disturbances in calcium metabolism lead to contractile dysfunction observed in failing hearts by increasing the time course of Ca\(^{2+}\) transient decay, decreasing SR Ca\(^{2+}\) release and reducing peak systolic [Ca\(^{2+}\)]\(_i\) (Hasenfuss & Pieske 2002, Piacentino et al. 2003). In addition, it has been demonstrated that reduced SERCA2a mRNA level and function correlate with an impaired relaxation of hypertrophied and failing hearts (Gwathmey et al. 1987, Mercadier et al. 1990, Arai et al. 1993, Piacentino et al. 2003). SERCA2a expression is suppressed in various ischemic cardiac diseases as well as in early response to hypobaric hypoxia (Sharma et al. 2004b). Supporting the causal role of reduced SERCA activity and impaired function in cardiac failure, adenoviral- and lentiviral-mediated SERCA2 over-expression studies have shown that compensation of SERCA2 loss after various heart failure situations improves cardiac and isolated cardiomyocyte function and survival (del Monte et al. 1999, Miyamoto et al. 2000, Niwano et al. 2008). However, these beneficial effects of SERCA2 over-expression are gained only if adequate amounts of O\(_2\) are available for the increased ATP production needed for increased SERCA2 activity.

The most important regulator of SERCA2 activity is a 22-kDa cardiac SR and SERCA2 associated homopentameric protein, phospholamban (PLN) (for review see MacLennan & Kranias 2003). PLN binds to and regulates the activity of SERCA2 depending on its phosphorylation status. Dephosphorylated PLN associates with SERCA2 and reduces the SERCA2 Ca\(^{2+}\) affinity, thus inhibiting the pumping function (Hughes et al. 1996). Phosphorylation of PLN Serine 16 and Threonine 17 residues relieves the inhibition by dissociating the PLN from SERCA2. SERCA2 activity is therefore increased two- to threefold by e.g. during β-stimulation evoked G-protein signaling and cAMP-dependent protein kinase
(=protein kinase A, PKA) or Ca\textsuperscript{2+/}\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMKII) activation. PLN phosphorylation enhances SERCA2 pumping capacity and leads to the inotropic and lusitropic responses elicited in the heart by beta-agonists (for review see MacLennan & Kranias 2003, Bhupathy et al. 2007). The cost of increased PLN phosphorylation and reduced SERCA2 inhibition is increased myocardial ATP consumption and oxygen demand.

2.8 Cardiac excitation-contraction coupling is regulated via G protein-coupled receptors

2.8.1 G protein-coupled receptors

The superfamily of G protein-coupled receptors (GPCRs) belongs to one of the largest and most studied gene families in the genome and constitutes the largest family of the cell surface receptors (Fredriksson & Schioth 2005). Seven-transmembrane domain GPCRs are activated by a wide variety of stimuli from electromagnetic radiation (light) to ions, peptides, lipids and metabolic intermediates. They mediate most cellular responses to hormones and neurotransmitters, in addition to being responsible for vision, olfaction and taste. During recent years, these receptors, especially orphan receptors without a known endogenous ligand, have been under increasing research interest, since many of them may play an important role in cellular and physiological responses and could also serve as novel targets for drug development.

2.8.2 Activation of a G protein

GPCR signaling is activated by ligand binding to an extracellular/transmembrane active site of the receptor. Depending on the ligand and the type of GPCR, the active site is located on the N-terminal tail, extracellular loops, or exofacial transmembrane helices of the receptor protein. Upon activation by its endogenous ligand, GPCRs undergo conformational changes that allow specific coupling of activated receptor with intracellular heterotrimeric guanine-nucleotide binding proteins (G-proteins). The heterotrimeric G protein consists of a G\textsubscript{\alpha}-subunit that binds and hydrolyzes GTP and G\textsubscript{\beta}- and G\textsubscript{\gamma}-subunits that form an un-dissociable G\textsubscript{\beta\gamma}-complex (Figure 7). In the basal state, the \beta\gamma-complex and the GDP-bound \alpha-subunit are associated. Receptor agonist binding results in a conformational
change in the receptor that allows the receptor to function as a guanine nucleotide exchange factor (GEF) that exchanges GDP for GTP on the Gα subunit. The conversion of GDP to GTP dissociates the Gα from the Gβγ subunits, which mediates the downstream signaling and second messenger pathways. Dissociated Gα subunits can regulate downstream effectors, enzymes such as adenylate cyclase (AC) and phospholipase C β (PLCβ), or ion channels. Dissociated Gβγ subunits target a range of signaling pathways involved in desensitization, down-regulation, apoptosis, and ion channel activity. Signaling is self-limited and is terminated by the spontaneous hydrolysis of GTP to GDP due to the intrinsic α-subunit GTPase activity. The resulting GDP-bound α-subunit re-associates with the βγ-complex and can be activated again by GPCR (Wettschureck & Offermanns 2005, Salazar et al. 2007, Rosenbaum et al. 2009). GPCR activity is regulated by different mechanisms. Most activated GPCRs are rapidly desensitized at the cell surface by phosphorylation by G protein-coupled receptor kinases (GRKs) and by β-arrestin binding, which mediates receptor desensitization, internalization, and trafficking. GPCRs are internalized, but some receptors can continue to signal independently of G proteins from intracellular compartments. Within endosomes, GPCRs are dephosphorylated and efficiently recycled back to the cell surface in a re-sensitized state in which the receptors are able to start the signaling cascade again. Thus, GPCR trafficking has critical functions in signal termination and propagation as well as in receptor re-sensitization (Patel et al. 2009).
Fig. 7. Overview of the main types of the cardiac G protein-coupled receptor signaling pathways. Agonist binding to receptor results in the GDP for GTP exchange in the $G\alpha$ subunit, dissociation of the $G\alpha$ and $G\beta\gamma$ subunits, and activation of receptor associated G-protein. $G\alpha$ activates a membrane-bound effector molecule, such as adenylyl cyclase (AC) or phospholipase C $\beta$ (PLC$\beta$) to produce secondary messengers such as cyclic adenosine 3',5' monophosphate (cAMP) from ATP or diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$) from phosphatidylinositol 4,5-bisphosphate (PIP2). These activate a variety of downstream effectors such as protein kinase A (PKA) with a range of physiological responses in the heart. G$\alpha_i$ signaling opposes G$\alpha_s$ signaling by inhibition of AC and cAMP production. SERCA2a = sarcoplasmic reticulum calcium ATPase2a, PLN = phospholamban, RyR2 = ryanodine receptor, LTCC = L-type Ca$^{2+}$ channel, TnI = Troponin I.

### 2.8.3 GPCRs in myocardium

Among an estimated 200 cardiac GPCRs, GPCRs comprise the largest cell-surface receptor superfamily and are also the largest class of drug targets to which agonists and antagonists are currently directed (Tang & Insel 2004). There are numerous subtypes of G proteins, and several $\alpha$-, $\beta$-, and $\gamma$-subunits have been described. G$\alpha$ proteins can be divided into four functionally distinct subtypes: G$\alpha_s$ (stimulatory), G$\alpha_i$ (inhibitory), G$\alpha_q$ and G$\alpha_{12/13}$. GPCR/G protein-coupling diverge at this point, so that the activation of a particular $\alpha$-subtype causes a
typical downstream signaling pathway activation and consequential physiological response (Figure 7).

**Gas-coupled signaling**

The Gαs protein stimulates adenylate cyclase which is responsible for the intracellular production of the secondary messenger 3’–5’-cyclic adenosine monophosphate (cAMP) (Figure 7). In cardiac myocytes, cAMP activates PKA which phosphorylates a wide variety of targets that modify cardiac excitation-contraction coupling (Wettschureck & Offermanns 2005, Salazar et al. 2007). PKA phosphorylates e.g. voltage-gated ion channels, L-type Ca²⁺ channel, SERCA2a, phospholamban, troponin I and RyR2. Generally, PKA phosphorylation enhances myocyte Ca²⁺ release and extrusion, increases Ca²⁺ sensitivity of calcium handling proteins and affects SR loading. These changes allow larger [Ca²⁺], transients and increased contractility (Dai et al. 2009). E.g. phospholamban phosphorylation is directly associated with an increase in SERCA2Ca²⁺ affinity and enhanced cytosolic sequestration of Ca²⁺ back to SR. This can improve diastolic function substantially, as discussed before. Adrenergic receptors belong to the GPCR family. β-adrenergic receptor 1 (β1AR) is the main cardiac subtype. It couples to Gαs protein and stimulates heart rate and contractility in the presence of catecholamine (noradrenaline, adrenaline) agonist. Noradrenaline binds to β1-adrenergic receptors in the sinoatrial node leading to increased pacemaker spontaneous firing rate and inotrophy (Kang et al. 2007). Although the β1-adrenoceptor is the predominant subtype expressed in cardiomyocytes, β2-adrenergic receptors are expressed in the heart as well. Less abundant β2AR are also coupled to the Gαs protein, but interestingly, can switch the target to Gαi after PKA phosphorylation as a delayed response to prolonged activation (Daaka et al. 1997, Salazar et al. 2007).

There are many short-term benefits of increased β-adrenergic stimulation on cardiac function. Slow heart rate and submaximal contractility at rest can be rapidly increased through the sympathetic nervous system. Cardiac reserve is large and can rapidly be mobilized to meet the physiological demands. On the other hand, sympathetic activation leads to energetically costly adjustments since releasing channels, pumps and myosin motors are cycling faster and consume more ATP per time unit. Prolonged chronic stimulation of ventricular β-receptors promotes detrimental cardiac remodeling. Several reports show that disturbances in βAR/Gas signaling can lead to myocardial damage and pathological conditions.
such as cellular hypertrophy, fibrosis, necrosis, arrhythmias, reduced contractility and sudden cardiac death (for review see Kang et al. 2007, Salazar et al. 2007).

**Ga\textsubscript{i} protein coupled signaling**

Ga\textsubscript{i} signaling opposes Ga\textsubscript{s} signaling by a receptor-dependent inhibition of AC and a reduction of cAMP production. This leads to reduced Ga\textsubscript{s} agonist-stimulated PKA activity and cardiac contractility. Declined PKA activity leads to reduction in e.g. L-type Ca\textsuperscript{2+} currents, RyR2 and SERCA2a activity and reduction of contraction force and heart rate. In the heart, Ga\textsubscript{i} protein couples with A1 adenosine receptors (A1Rs), M2 muscarinic receptors (M2Rs), and \(\alpha\text{-2-}\)adrenergic receptors (\(\alpha\text{2ARs}\)) (Figure 7). Adenosine receptor 1 and 3 couples with Ga\textsubscript{i} protein inhibiting cAMP production, but A2R is coupled primarily with the Ga\textsubscript{s} pathway. In heart failure, A1R agonists have shown cardioprotective effects and improved function and recovery after ischemia-reperfusion (Salazar et al. 2007). Up-regulation of Ga\textsubscript{i} signaling in human heart failure may be a mechanism to counteract deleterious Ga\textsubscript{s}-mediated signaling (Wettschureck & Offermanns 2005).

The predominant muscarinic isoform in the heart is M2 which couples with the Gi protein (Figure 7). M2 muscarinic acetylcholine receptors mediate the parasympathetic negative chronotropic and inotropic effects resulting from the Ga\textsubscript{i}-mediated inhibition of AC and downstream effectors. In addition, the activation of G-protein-regulated inward rectifier potassium channels is regulated by \(\beta\gamma\)-subunits released from the activated Ga\textsubscript{i} (Logothetis et al. 1987, Stanfield et al. 2002).

**Ga\textsubscript{q} protein-coupled signaling**

Ga\textsubscript{q}-mediated signaling is initiated by the membrane-recruitment and activation of \(\beta\)-isoforms of phospholipase C (PLC\(\beta\)) by a Gq protein. PLC\(\beta\) is responsible for hydrolyzation of phosphatidylinositol 4,5 biphosphate (PIP2) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP\textsubscript{3}) second messengers (Figure 7). DAG activates protein kinase C isoforms (PKC\(\alpha,\beta,\delta,\epsilon\)) that phosphorylate multiple downstream effectors modulating e.g. phospholamban and SERCA2 phosphorylation, myocyte apoptosis, necrosis and hypertrophic responses (Braz et al. 2004, Salazar et al. 2007). IP\textsubscript{3} induces SR Ca\textsuperscript{2+} release channel opening and sustained rise of intracellular [Ca\textsuperscript{2+}], affecting e.g.
calcineurin and nuclear factor of activated T-cells (NFAT) activation, which triggers the complex cardiac gene expression program in hypertrophied hearts (Molkentin et al. 1998, Berry et al. 2011). There are several known Gq-coupled receptors in the heart. Angiotensin receptors (ATRs) bind circulating angiotensin II (AngII) peptide and mediate hypertrophic remodeling of the heart. AngII signals primarily through the Gq-coupled AT1 receptor (AT1R) and is a well-known factor involved in pathological hypertrophy and heart failure. Angiotensin receptor blockers and angiotensin-converting enzyme (ACE) inhibitors have been shown to reverse cardiac remodeling, reduce cardiac hypertrophy, slow the progression to heart failure, and improve survival in heart failure patients (Billet et al. 2008, for review see Salazar et al. 2007).

Endothelin (ET-1) is a strong vasoconstrictor, causing Gq-dependent and IP3 mediated arterial smooth muscle [Ca2+]i increase and contraction. ET-1 is expressed in the heart and it has direct influence on cardiac myocyte function and survival. Despite having primary targets in the peripheral cardiovascular system, ET-1 promotes hypertrophic remodeling in the heart through endothelin-1 receptors (ET1Rs), which also mediate ET’s chronotropic and inotropic effects. ET-1 binding to ETR stimulates signaling cascades that include activation of protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K), with subsequent effects on intracellular calcium that can stimulate calmodulin-dependent pathways. In cardiomyocytes, the ETα receptors are abundant (90%) and considered to be more important for the cardiac effects of ET-1. ETβ receptors are primarily located on the endothelial cells and are primarily involved in NO release and vasodilatation. It is known that ET-1 signaling is activated in human heart failure and multiple pathophysiological stimuli, including hypertrophy and cardiac damage, can lead to enhanced endothelin expression (Kang et al. 2007, Salazar et al. 2007, Schorlemmer et al. 2008).

Sympathetic stimulus can also be coupled with Gq proteins through α1A, α1B or α1D-adrenergic receptors. In the heart, α1-adrenergic (α1AR) subtypes interact with each other in conjunction with Gs-coupled β1ARs, thus regulating cardiac Ca2+ dynamics, intracellular Ca2+ concentration, contractile function and hypertrophic responses (Salazar et al. 2007).

2.9 Apelin is an endogenous modulator of the G protein signaling

Apelin has been identified as an endogenous ligand of the orphan G-protein-coupled receptor, APJ (O’Dowd et al. 1998, Tatemoto et al. 1998). They are both
widely expressed in the central nervous system (CNS) and periphery (Kleinz & Davenport 2005). APJ is suggested to couple with inhibitory Gi-proteins (Habata et al. 1999, Masri et al. 2006) and apelin actions have shown to be pertussis toxin (PTX) sensitive (Hosoya et al. 2000, Masri et al. 2002). The function of the Gi proteins has often been studied using a toxin from Bordetella pertussis, which ADP ribosylates COOH-terminus of Gαi protein ad prevents G protein receptor binding and activation (Wetschureck & Offermanns 2005). Apelin-APJ system has a role in the regulation of fluid and glucose homeostasis, feeding behavior, vessel formation, cell proliferation and immunity (Masri et al. 2005, Winzell et al. 2005). The apelin gene encodes the 77-amino acid preproapelin (Lee et al. 2000). Preproapelin contains a number of paired basic amino acids residues (Arg-Arg and Arg-Lys) that are possible cleavage sites for endopeptidases. Cleavage at these sites would produce a predicted family of C-terminal fragments, including apelin-36, apelin-17 and apelin-13 (Pitkin et al. 2010). A 36-amino acid C-terminal fragment (apelin-36) of preproapelin was the first endogenous ligand characterized for APJ, but several smaller C-terminal peptides have been shown to be even more potent APJ agonists (Tatemoto et al. 1998) (Figure 8). Different apelin peptides vary in their potency to mediate different biological effects of apelin. Although apelin-36 is the most potent APJ receptor-dependent blocker of HIV infection, the smaller peptides, like apelin-13, are more potent in mediating cardiovascular effects (Kleinz & Davenport 2005). Apelin has been proposed to act mainly as a paracrine modulator of cell functions. Circulating apelin levels are low and vary little in various pathological situations (Kleinz & Davenport 2005, Miettinen et al. 2007).
2.9.1 Apelin has beneficial effects in the cardiovascular system

A line of evidence indicates that the cardiovascular system is one of the main targets of apelin. High levels of mRNA of both APJ and apelin are found in cardiac myocytes, vascular smooth muscle and endothelial cells (O’Carroll et al. 2000, Kawamata et al. 2001, Kleinz & Davenport 2004, Kleinz et al. 2005). It was shown that apelin increases cardiac contractility in a dose-dependent manner in isolated rat hearts (Szokodi et al. 2002). Apelin induces acute positive inotrophy in vivo in normal and failing post-infarction rat hearts (Berry et al. 2004). A long-term improvement of cardiac contractile function was also observed after apelin administration without evidence of hypertrophic heart growth (Ashley et al. 2005). In addition to the inotropic effects, several mechanisms have been described whereby apelin regulates vascular tone and blood pressure. Intravenous injection of apelin lowers blood pressure by triggering the release of nitric oxide from endothelial cells (Tatemoto et al. 2001), and reduces water and sodium uptake in the kidneys by inhibiting vasopressin release (De Mota et al. 2004). In addition, apelin shares a degradation pathway with angiotensin II (AngII). Both are substrates for angiotensin-converting enzyme 2, an important enzyme of the renin-angiotensin system. Mice deficient in the APJ receptor have normal blood pressure but increased vasopressor
response to angiotensin II (Ishida et al. 2004), suggesting a role for apelin in counteracting angiotensin-induced vasoconstriction.

2.9.2 Apelin improves cardiomyocyte function

In cardiac myocytes, apelin was proposed to induce inotropy by activating the sarcolemmal Na\(^+\)-H\(^+\) exchanger (NHE). NHE regulates intracellular pH when it drops to acidic values by exchanging intracellular H\(^+\) to extracellular Na\(^+\) (Leem et al. 1999). Secondarily, NHE activation may result in an increase in the intracellular calcium via reverse mode of the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX). NHE activation increases [Na\(^+\)], which can launch the reversal mode of NCX due to diminishing the Na\(^+\) transmembrane concentration gradient. In reversal mode, Ca\(^{2+}\) is imported into cytosol and Na\(^+\) is exported outside the cell. In this scheme, increased activity of the NHE would also lead to a rise in intracellular pH, which further increases the contraction force. Interestingly, the alkalinization induced by this mechanism is likely to be greatest in cardiac tissue suffering from acidosis (Leem et al. 1999), e.g. during ischemia and hypoxia. It was recently observed that the apelin-APJ system is an intrinsic protective pathway in ischemia-reperfusion injury in perfused hearts and cultured cells, and that administration of apelin-13 limited infarct size and improved post-ischemic mechanical recovery (Zeng et al. 2009, Rastaldo et al. 2011). This effect can be mediated, at least in part, through the inhibition of ER-dependent apoptotic pathways, since apelin was shown to attenuate ER stress-induced apoptosis and to protect the heart against IR injury in a time-dependent manner (Tao et al. 2011).

2.9.3 Regulation of apelin expression

The molecular mechanisms influencing apelin expression are still largely unknown. Specific stimuli for cardiomyocyte apelin production had not been characterized before our experiments. Apelin is present in human plasma and myocardium, and left ventricle apelin mRNA levels increase in chronic heart failure due to coronary heart disease and dilated cardiomyopathy (Foldes et al. 2003). A relationship between changes in cardiac apelin expression and myocardial ischemia has also been postulated (Chen et al. 2003, Foldes et al. 2003). In addition, apelin plasma levels are reported to increase especially in the early stage of left ventricular dysfunction despite the fact that the apelin levels
found in plasma from healthy and diseased patients were questionable high (Chen et al. 2003) compared to other studies (Foldes et al. 2003).

2.10 A novel G protein-coupled receptor 35

One GPCR family member is a poorly characterized G protein-coupled receptor 35 (GPR35) which is a 309-amino acid long seven-transmembrane domain receptor characterized originally as an orphan receptor without a known endogenous ligand (O’Dowd et al. 1998). Ligand screening studies have revealed that GPR35 can be activated by a tryptophan metabolite, kynurenic acid (KYNA), which can be found in plasma in nano to micromolar concentrations. It was suggested that KYNA could be an endogenous ligand for GPR35 (Forrest et al. 2006, Wang et al. 2006). However, some challenging results show that a very high concentration of KYNA is required for the receptor activation (Jenkins et al. 2011). By now some other candidates exist as well. Endogenous lysophosphatidic acids (LPAs), especially 2-acyl LPA, have also been suggested to be an endogenous ligand for GPR35 and activate the receptor at lower nanomolar concentrations (Oka et al. 2010). In addition to putative endogenous ligands, several synthetic GPR35 activators have been found, for example the GPR35 agonist zaprinast (2-(2-propyloxyphenyl)-8-azapurin-6-one) (Taniguchi et al. 2006).

2.10.1 GPR35 activation has diverse effects in cells

Receptor activation by agonists or over-expression of GPR35 alters cellular responses in a cell type-specific manner. G protein-coupling profile studies have suggested that GPR35 is coupled with pertussis toxin (PTX)-sensitive Gi-protein, and activation of GPR35 was shown to inhibit forskolin-stimulated cAMP production in dorsal root ganglion neurons (Wang et al. 2006, Ohshiro et al. 2008). It has been shown that Gi-protein activation also recruits β-arrestin-2, which was suggested to be crucial in mediating the downstream effects of GPR35 (Zhao et al. 2010). In addition, GPR35 over-expression was shown to inhibit neuronal N-type calcium channels while repressor-effect could be blocked by pretreating the cells with pertussis toxin (Guo et al. 2008). Recently, GPR35 activation was also connected to alternative β-arrestin-2 and Ga13 pathways, and it was also shown that at least part of the GPR35 signaling involves PTX-sensitive ERK1/2 activation (Jenkins et al. 2010, Zhao et al. 2010, Jenkins et al. 2010).
2011). It has been deliberated that GPR35 activation response may be biased and vary in agonist-, species- and cell type-specific manner so that either G\textsubscript{i} or β-arrestin and G\textsubscript{α}13 pathways prevail (Milligan 2011), and that it may be possible to identify ligands that differ in their ability to activate either G protein or β-arrestin signaling pathways (Zhao \textit{et al.} 2010).

\subsection*{2.10.2 GPR35 expression and function is also associated to the cardiovascular system}

The cardiovascular role of GPR35 is still mainly uncharacterized. Knock-out mice lacking GPR35 expression were reported to have increased systemic blood pressure, but the mechanism was not studied further (Min \textit{et al.} 2010). It was shown that a high amount of GPR35 is expressed in the heart and myocardial GPR35 gene expression is increased in heart failure. This suggested GPR35 to be a novel heart failure-associated gene. At the cellular level, adenoviral over-expression of GPR35 caused hypertrophic-like morphology changes and reduced cell viability (Min \textit{et al.} 2010). Interestingly, a genome-wide association study suggested GPR35 single nucleotide polymorphism (SNP) causing C-terminal mutation from Ser294 to Arg 294 to be linked to coronary artery calcification and coronary artery disease. Mutation was a risk factor and replicable predictor for the development of coronary atherosclerosis in the individuals studied (Sun \textit{et al.} 2008). These novel findings suggest that GPR35 may also have a role in the cardiovascular system and in the maintenance of circulation homeostasis. So far, very little is known about GPR35 gene expression and regulation. Tissue distribution of GPR35 has been characterized (for review see Milligan 2011), but the regulation of gene expression is not known.
3 Aims of the research

Cardiovascular adaptation to hypoxia is an essential process in metazoan development and in life sustaining energy metabolism processes. Myocardial gene expression is changed in many pathological conditions, in part due to lack of proper myocardial oxygenation. As a highly differentiated organ, the heart has unique ways to maintain sufficient energy production even in hypoxic environment. This is due to adaptive changes in myocardial gene and protein expression. Despite extensive research, the role of HIF-1 in cardiac hypoxia adaptation is incompletely known. This study aims to clarify the role of HIF in the regulation of cardiac gene expression. The physiological responses to the adaptation processes are also evaluated. The transcription factor network is tissue-specific and unique. Cardiac transcription factors such as Nkx2–5, Gata4 and Mef2a form an integrative response network in the developing and adult heart (Schlesinger et al. 2011). Cardiomyocyte responses to hypoxia may vary in a plethora of genes because of collaborative and interdependent binding of HIF and core cardiac transcription factors to gene promoters and enhancers. To study this, the specific aims were:

1. To study hypoxia-driven gene expression changes in heart muscle and myocardial cells in vitro and in vivo. To establish novel HIF-dependent adaptation pathways to chronic hypoxia in cardiac myocytes. To characterize at the cellular level the molecular mechanisms behind the regulation of gene expression.

2. To elucidate the integration of HIF-regulated gene expression and functional changes seen in hypoxic cardiomyocytes, and to study whether reduced Ca\(^{2+}\) activity during hypoxic excitation-contraction coupling is connected to down-regulation of Ca\(^{2+}\) handling proteins.

3. To examine whether there are some novel cardiac-specific, yet undefined hypoxia-regulated genes in heart cells and the possible role of HIF-1 in induction of these genes. To characterize what degree and length of oxygen deprivation is required to change the expression pattern and what is the time scale for that. To characterize how the changes in gene expression are coupled with the cardiac hypoxia adaptation process and myocyte function.
4 Summary of materials and methods

The materials and methods used in this thesis are summarized in below. Detailed descriptions with references can be found in the original articles indicated as I-III. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu.

4.1 Rat auricle superfusion model (I)

Male Sprague-Dawley rats weighing 330g–380g from the Center for Experimental Animals at the University of Oulu were used in the perfusion. The experimental model used in this study was the isolated perfused rat atrium, prepared as previously described (Tavi et al. 1999). The partial oxygen pressure of the atrium was manipulated using three different gas mixtures to bubble the Krebs-Henseleit perfusion buffer: 1) 95% O2 + 5% CO2 (normoxia), 2) 15% O2 + 80% N2 + 5% CO2 (mild hypoxia), 3) 95% N2 + 5% CO2 (raw hypoxia).

4.2 In vivo infarction model (I)

Myocardial infarction was produced by ligation of the left anterior descending coronary (LAD) artery. A left thoracotomy and pericardial incision was performed and the LAD was ligated. The sham-operated rats underwent the same surgical procedure without the ligation of LAD.

4.3 Cell Isolation and Culturing (I, III, III)

Described methods were used to isolate and culture Heart-Like-1 atrial myocytes (HL-1), neonatal rat and mouse ventricular myocytes and mouse embryonic day 12.5 (E12.5) cardiomyocytes. To induce hypoxia cells were cultured in a Biospherix C-Chamber (model C-274, Biospherix Ltd., USA) inside a standard culture chamber. Oxygen concentration was set to 0.5–5% and carbon dioxide concentration was held at 5% inside the C-Chamber by injecting N2 and CO2 into the chamber with a ProOx 110 Oxygen controller and a ProCO2 CO2-controller (Biospherix Ltd, USA). To chemically mimic hypoxia responses, cells were incubated in the presence of a HIF prolyl-4-hydroxylase (HIF-P4H) inhibitor, desferrioxamine (DFO) (100 μmol/L, Sigma).
4.4 Transfections and plasmid constructs (I, II, III)

HL-1 and neonatal cells were transiently transfected with Lipofectamine 2000 (Invitrogen, USA) (I, III). E12.5 cells were transfected by electroporation (BTX ECM-830, Harvard Apparatus, USA) immediately after the isolation process (II). To measure the transcriptional activity of HIF cells were transfected with the pT81/HRE-luc reporter plasmid and the luciferase signal was measured and normalized with the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) (I). Inhibitory PAS (Per/Arnt/Sim) domain protein (IPAS) expression vector (pCMV-Flag-IPAS) was used to suppress endogenous HIF activity (I, III). To activate HIF-1 target genes, cells were transfected with the pFlag-HIF-1α(1-390)-VP16 plasmid, which produces a constitutively active, stable form of HIF-1α (I,II,III).

SERCA2a-luc reporter plasmids were a generous gift from Dr. WS Simonides (Vlasblom et al. 2004). SERCA2a-luc HIF-1 binding core sequences were mutated by using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, USA) (II).

GPR35 over-expression plasmids were generous gifts from Mr. Stephen R. Ikeda and have been described earlier (Guo et al. 2008). Shortly, human GPR35 cDNA (GPR35a) was cloned into CMV-driven mammalian expression vector pCI. To provide a visual indicator for GPR35 expression cDNA was also cloned into plasmid Venus-N1, which adds an improved variant of enhanced yellow fluorescent protein to expressed GPR35 (III).

GPR35 promoter constructs were generated from mouse genomic DNA. Full-length GPR35-Luc reporter plasmids and truncated/mutated promoter constructs were generated by nested PCR and restriction reactions as well as site-directed mutagenesis by QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, USA) (III).

4.5 Gene expression measurements (I, II, III)

Total RNA from cultured cells was isolated using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, USA). After cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Fermentas), quantitative PCR reactions were performed with the ABI 7300 Sequence Detection System (Applied Biosystems, USA) using the TaqMan chemistry.
4.6 Reporter analysis (I, II, III)

Twenty-four hours after transfection and plating, cells were grown for another 24 h at normoxia (21% O2) or hypoxia (1% O2) or in the presence of DFO (100 µM). To measure the transcriptional activity of the pT81/HRE-luc, SERCA2a-luc and GPR35-luc reporter plasmids, the luciferase signal was measured and normalized with the renilla-luciferase signal derived from the co-transfected pRL-TK vector by using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA).

4.7 Ca2+ imaging (II, III)

E12.5 and neonatal mouse cardiomyocytes were loaded with Fluo-4-acetoxyethyl (AM)-ester. After this, the culturing dishes were placed in a custom-made perfusion system built into a confocal inverted microscope (FluoView 1000; Olympus). To measure myocyte calcium signals, Fluo-4-loaded myocytes were excited at 488 nm and the emitted light was collected with a spectral detector from 520 to 620 nm. At the time of electrical stimulation or spontaneous activity, 4–8 cells/plate were line scanned at around 100 Hz depending on the length of the scanning line, with a fixed pixel time of 10 µs. The line scan images were analyzed with the Origin 7.5 (OriginLab Co., USA) program. Fluo-4 fluorescence intensity is expressed as an F/F0-ratio, where F is the background subtracted fluorescence intensity and F0 is background subtracted minimum fluorescence value measured from each cell at rest.

4.8 Immunofluorescence labeling and microscopy (I, II, III)

Cells were fixed with 3% paraformaldehyde and usually permeabilized with 0.5% Triton X-100. The primary antibodies were incubated for 1–24 h in RT or at +4°C. The secondary antibodies were incubated for 1 h in RT. Dilutions for primary antibodies were 1:250 (apelin), 1:2000 (HIF-1α), 1:250 (ANP), 1:100 (SERCA2a), 1:100 (GPR35). Secondary antibody dilution was 1:750 in all groups. Actin fibers were labeled with Alexa Fluor 568 phalloidin (Molecular Probes, USA) or with Phalloidin-TRITC (Sigma Aldrich, USA) and nuclei with DAPI (Molecular Probes, USA). After labeling, images were taken freshly with an Olympus FV1000 confocal microscope.

GPR35 immunolabeling was performed without preliminary permeabilization before the primary antibody incubation to stain only plasmalemmal GPR35.
without significant SR originating background from immature non-functional receptors. Cells were depth-scanned at 0.41 µm intervals and generated stack of z-layers were combined to maximum Z-intensity projection images by using Olympus FluoView 10.0 software. The number, density and coverage of neonatal mouse cardiomyocyte plasma membrane immunoreactive receptor spots were calculated from confocal microscope maximum intensity z-projection images by using ImageJ software’s threshold and analyze particles-based algorithms.

4.9 Western blot analysis/PLB phosphorylation (II)

Total protein was extracted from embryonic cardiomyocytes. Equal amounts of protein (15 µg PLB and 40 µg SERCA2a) were electrophoresed on SDS-PAGE (15% PLN and 8% SERCA2a) gels and semi-dry blotted onto nitrocellulose or PVDF membranes. Membranes were labeled with antibodies against phospho-(Ser 16)-phospholamban, phospho-(Thr 17)-phospholamban, actin, SERCA2a (Santa Cruz Biotechnology, USA), phospholamban (Badrilla, UK) and α-tubulin (Sigma-Aldrich). Signals were developed by ECL.

4.10 Apelin radioimmunoassay (I)

Apelin was measured using a goat antiserum raised against a carbodiimide conjugate of human preproapelin66-77 (apelin-12) and horseshoe crab hemocyanin. Preproapelin62-77-Tyr77 (Apelin-16 with tyrosine added to the carboxy terminus) was radioiodinated using chloramine-T and purified by gel filtration followed by reverse-phase HPLC as described previously (Ala-Kopsala et al. 2004). Synthetic human preproapelin42-77 (apelin-36, Phoenix) was used as the standard. Synthetic peptides (apelin-10, apelin-12, apelin-13 and apelin-16) were assembled using Fmoc chemistry with an ABI 433A Peptide Synthesizer (Applied Biosystems) and purified by reverse-phase HPLC, as described previously (Ala-Kopsala et al. 2004). Apelin-36, used as the assay standard, was obtained from the human apelin-36 radioimmunoassay kit purchased from Phoenix Europe GmbH (Karlsruhe, Germany).

4.11 Chromatin immunoprecipitation (III)

Magna Protein A Magnetic Beads (Millipore) were coupled with Hif1α rabbit antiserum (ab2185 Abcam) or normal rabbit serum (NRS) at +4°C overnight.
Cells were cross-linked in 1% formaldehyde and harvested into Farmham lysis buffer. The chromatin was sonicated into 150–300 bp fragments using Bioruptor UCD-300-TO (Diagenode s.a. Belgium) and used for immunocomplex forming with the coupled beads at +4°C overnight. Precipitate was eluted from the beads and cross-linking was reversed with Proteinase K at +65°C overnight. DNA was isolated using QIAquick PCR purification system (Qiagen GmbH, Hilden, Germany). Input samples were treated similarly except that immunoprecipitation was not performed. Genomic regions were examined with real-time PCR primers specific for the GPR35 HIF-1 site or for the negative control area. Relative HIF-1 binding was calculated and normalized to the amount of chromatin used for the preparation of output and input samples. NRS precipitated templates served as specificity controls.

4.12 Statistical analysis (I,II,III)

The values are presented as mean ± SEM. Statistical significance was evaluated using unpaired Student’s t-test. Multiple comparisons involving more than two groups were performed using ANOVA and Bonferroni post-hoc tests. Statistical tests were performed with Origin 7.5 and 8.5 software (OriginLab Co., USA). P-values less than 0.05 were considered statistically significant.
5 Results

5.1 Hypoxia regulates cardiomyocyte apelin expression and secretion

5.1.1 Hypoxia increases apelin gene expression in perfused rat atrium

In the first set of experiments, we characterized how isolated rat left atrial appendage gene expression levels respond to reduced levels of oxygen and different degrees of hypoxia. Perfusion buffer oxygenation was reduced from normoxic (58% O₂) to mild (18% O₂) and severe (8% O₂) hypoxia by altering perfusion medium N₂ and O₂ content. Severe hypoxia increased apelin (Apln) gene expression after a 3-h perfusion significantly, so that apelin mRNA levels increased 2.5-fold compared to normoxic samples.

5.1.2 Apelin expression is regulated time-dependently in cultured neonatal rat cardiomyocytes

Next we examined the time course of apelin expression in hypoxia. Cultured neonatal rat cardiomyocytes were exposed to 1, 2, 8 and 24 h of hypoxia (2% O₂). Apelin gene expression was induced rapidly, as after 2 h the expression of apelin was 3.7-fold (p<0.001) and after 8 h 5.6-fold (p<0.001) higher than in normoxic cultures. Incubation under hypoxic conditions for up to 24 h sustained, but did not result in a further increase in apelin gene expression (5.6-fold, p<0.001).

5.1.3 HIF-1 is involved in apelin gene expression regulation

We characterized the role of HIF in the hypoxic induction of Apln gene expression. In HL-1 cells, hypoxia (2% O₂) or the addition of the prolyl hydroxylase inhibitor desferrioxamine (DFO, 100 µmol/L) resulted in HIF-1α stabilization and nuclear accumulation which was accompanied by an increase in apelin gene expression. Nuclear HIF-1α immunofluorescence increased significantly in both hypoxic (14.0-fold, p<0.001) and DFO-treated groups (7.4-fold, p<0.001) after 24-h exposure; HIF-1 activation was accompanied by a 27-
fold (p<0.001) and 12.8-fold (p<0.001) increase in the apelin mRNA levels, respectively.

In addition to chemical manipulation of the HIF-pathway, we saw in HL-1 cells that the expression of dominant-negative and constitutively active forms of HIF-1α affected cardiomyocyte apelin expression. To inhibit or activate HIF-dependent transcription, HL-1 cells were transfected with plasmids producing IPAS or HIF-1α(1-390)-VP16. Expression of the specific HIF inhibitor IPAS decreased the hypoxia responses and reduced significantly the induction of apelin in hypoxia (p<0.01). Expression of HIF-1α(1–390)-VP16 increased apelin (4.2-fold, p<0.05) mRNA levels after 48 h in normoxia, further confirming that HIF-1 activates apelin gene expression.

5.1.4 Hypoxia increases the secretion of biologically active forms of apelin

To measure apelin protein levels and secretion, we developed a new radioimmunoassay for the detection of different forms of apelin. Hypoxia (2% O₂) and DFO (100 µmol/L) increased immunoreactive apelin (p< 0.001 and p<0.001) in the neonatal cardiac myocyte culture medium. Elucidation of the processes involved in apelin secretion revealed that during hypoxia, cardiomyocytes process the storage form, proapelin, into smaller, biologically active, secreted forms of apelin. Secreted apelin forms were shorter than apelin-36, and surprisingly, apelin-36, a common molecular form of apelin with biological activity, was not found in cardiomyocyte culture medium. Secreted forms appear to be between apelin-16 and apelin-12 in size, but the molecular form of apelin in cellular fractions was almost exclusively a peptide that was significantly longer than apelin-36.

On the basis of apelin RIA and immunostaining, cardiomyocytes contain rather low amounts of apelin peptide, suggesting that cells do not have large stores of apelin, but that secretion relies mostly on the expression and synthesis of de novo apelin. Immunostained apelin was shown to localize in the perinuclear area of the myocytes, demonstrating that synthesized pro-apelin is secreted via a sarco-/endoplasmic network probably by means of vesicular exocytosis.
5.1.5 Apelin gene expression is increased in vivo after myocardial infarction

In addition to cell culture and perfusion models, we characterized the regulation of apelin gene expression also in rat myocardial infarction model in vivo. Apelin expression increased in infarcted ventricles, where the lack of oxygen is likely to be one of the major stimuli affecting gene expression. The ligation of the left anterior descending artery (LAD) resulted after 24 h in impaired myocardial function, dilative changes in ventricular morphology and significant up-regulation of apelin expression (p<0.05). However, the expression of apelin returns to basal levels after 2 weeks of the infarction, and the expression levels are comparable with the sham-operated hearts.

5.2 Hypoxia and HIF-1 activation suppress SERCA2a expression in embryonic cardiac myocytes through two interdependent hypoxia response elements

5.2.1 Cultured embryonic cardiomyocytes are hypoxia-tolerant

Primary isolated mouse embryonic cardiomyocytes at E12.5 retain their spontaneous rhythmic beating in 1% O₂ and are thus a good model for investigating heart calcium cycling in hypoxic conditions. Determination of histone-complexed DNA fragments in cytoplasm showed that hypoxia (1% O₂, 24 h) causes only a small increase in the apoptosis rate of embryonic cardiomyocytes and that neonatal myocytes are more prone to hypoxia-induced apoptosis.

5.2.2 SERCA2a gene expression is regulated by oxygen levels in cardiac myocytes

Exposure to hypoxia causes a rapid decrease in SERCA2a mRNA expression in cultured E12.5 cardiac myocytes. Down-regulation was significant already after 2 h (-21%, p<0.01), being more pronounced after 12-h (-46%, p<0.001) and 24-h (-43%, p<0.001) hypoxia exposure.
5.2.3 HIF-activation reduces SERCA2a expression also in normoxia

The HIF-1-activating prolyl-4-hydroxylase inhibitor DFO (100 μM, 24 h) decreased SERCA2a mRNA levels significantly (p<0.001) in E12.5 cardiomyocytes cultured in normoxia. This supported the role of HIF in the regulation process. Next we over-expressed normoxia-stable, constitutively active HIF-1 (HIF1/VP16) chimera in embryonic cardiomyocytes and saw a significant HIF-1-specific down-regulation of the SERCA2a gene (p<0.001). This genetic modification further emphasizes the specific role of HIF-1 in the negative regulation of myocardial SERCA2 gene expression.

5.2.4 SERCA2a protein levels are reduced and the cellular distribution is interfered in hypoxic myocytes

HIF-1 activation by hypoxia or DFO led to disruption of the normal SERCA2a striation-like immunofluorescence staining pattern. In addition to gradual disappearance of the cytosolic SERCA2a structures seen in confocal microscopy, the SERCA2a protein quantitation with Western blot showed significant reduction of SERCA2a protein in cell cultures exposed to hypoxia (p<0.001). The reduction of the protein was of the same magnitude (-50%) with the mRNA reduction seen earlier.

5.2.5 The hypoxic loss of functional SERCA2a affects cardiomyocyte calcium cycling function negatively

Reduced SERCA2a expression leads to reduced SERCA2a activity, which distinctly changes cardiomyocyte Ca^{2+}-signaling. At all tested beating frequencies (spontaneous, 2 Hz, and 3 Hz), Ca^{2+}-transient amplitudes were significantly (p<0.01) smaller in hypoxic (24 h, 1% O_2) than in normoxic cells. Ca^{2+}-transient decay times, which indicate the efficiency of cytosolic calcium removal, were prolonged as well (p<0.001), showing reduced SERCA2a pumping capacity after chronic hypoxia. Sarcoplasmic reticulum Ca^{2+} content was measured by depleting the stores with a rapid application of caffeine (=caffeine pulse), which induces a large release of Ca^{2+} from SR. Caffeine pulse Ca^{2+}-release amplitudes were lower (p<0.01) and fractional releases were smaller (p<0.05) in hypoxic cells indicating lower SR calcium content due to reduced SERCA2a pumping function and SR loading capacity. The effect was not derived from the altered activity of the main
SERCA2a activity regulator protein phospholamban (PLN), since the phosphorylation status of PLN remained constant during hypoxia. This excludes the possibility that reduction of SERCA2a pumping activity was a secondary effect induced by an altered amount of PLN expression or phosphorylation.

5.2.6 Characterization of SERCA2a promoter, molecular mechanism for the hypoxic down-regulation

Rat and mouse promoter analysis revealed two conserved HIF-1 binding core sequences (5’-ACGTG-3’) on the SERCA2 (Atp2a2) promoter. Multi-Vista analysis (mVISTA) showed 86.9% homology and a high degree of conservation in rat and mouse SERCA2 promoters up to -1828bp from the transcription start site (TSS). Hypoxia response elements (HRE) were located between -777 bp to -781 bp (HRE1) and -1073bp to -1077 bp (HRE2) relative to TSS in rat genomic sequence.

To study the role of the specific HREs in the HIF-mediated trans-inhibition process, we generated SERCA2a-promoter-luciferase constructs and measured the activity. Full-length SERCA2a promoter (-3263 bp from TSS) activity was reduced after hypoxia (p<0.001), DFO (p<0.01) and HIF1/VP16 (p<0.05) in line with the changes in mRNA expression. The truncated promoter construct (-550 bp) lacks the HIF-1 binding 5′-ACGTG-3′ sequences and it was shown to be insensitive to hypoxia (p>0.05). In addition, mutation of HIF-1 binding core sequence 5′-ACGTG-3′ to 5′-AATGT-3′ diminished the hypoxia, chemical (DFO) and HIF1/VP16-driven SERCA2a repression seen in non-mutated full-length promoter. Both interdependent HIF-1 binding sites were essential for the down-regulation process, since only one intact binding site was not capable of repressing the expression.

5.3 HIF-1-inducible G protein-coupled receptor 35 expression modulates cardiomyocyte function

5.3.1 Myocardial expression of the different GPR35 isoforms

Gastric cancer cells have been reported to express GPR35 mRNA in two different alternatively spliced isoforms (Okumura et al. 2004). In mouse, the variants encode the same protein but differ in the 5′ UTR so that the variant
NM_022320.3 represents the longer transcript and NM_001104529.1 the shorter one. We used splicing variant-specific quantitative real-time PCR primers and probes, designed according to mouse GPR35 sequences NM_022320.3 and NM_001104529.1, to test if the two reported isoforms are expressed in neonatal mouse ventricular myocytes. Both reported NM_022320.3 and shorter NM_001104529.1 mRNAs were present. Splicing variant mRNA levels were at similar level in normal conditions. In addition, hypoxia regulated the expression of both splicing variants in a similar way. These findings suggest that in mouse cardiac myocytes, the expression of both isoforms is driven by the same promoter.

5.3.2 Hypoxia and HIF-1 activation or inhibition modulates GPR35 gene expression in cardiac myocytes

GPR35 mRNA levels increased in hypoxic (1% O₂) embryonic and neonatal mouse cardiac myocytes as a function of the duration of hypoxia. GPR35 mRNA increased significantly after 12 h (p<0.001) and further up to 48 h (p<0.001) in neonatal cells. In addition, the degree of hypoxia affected GPR35 expression with half-maximal activation at 2.9% O₂ and maximum induction at 0.5% O₂. A moderate induction was detected at 5% atmospheric oxygen, but only 2% O₂ was sufficient to cause a significant increase in GPR35 (p<0.001) and 1% and 0.5% O₂ was required for the maximal up-regulation (p<0.001). Also chemical HIF-activation in normoxia by DFO increased GPR35 expression in neonatal cells (p<0.001) and in immortalized adult atrial HL-1 cardiomyocytes (p<0.001). In genetic modification studies, over-expression of constitutively active HIF-1/VP16 increased GPR35 gene expression in normoxic HL-1 cells (p<0.001) and in immortalized adult atrial HL-1 cardiomyocytes (p<0.001). In addition, hypoxic induction of GPR35 was abolished when transfected HL-1 cells were expressing the specific HIF inhibitor IPAS (p<0.001). These responses together link hypoxia-sensitive regulation of GPR35 to HIF-1 activation.

5.3.3 GPR35 promoter hypoxia response element is responsible for the HIF-dependent regulation

We found one putative HIF-binding sequence (HBS) 5′-ACGTG-3′ from mouse GPR35 promoter. HBS is located at -372 bp 5′-direction from the transcription start site relative to NM_022320.3 mRNA sequence. HL-1 cells were transiently transfected with the GPR35 promoter-luciferase reporter construct containing a GPR35 promoter fragment from -2435 to +192 bp, and luciferase activity was
measured after the modulation of HIF-activity. Hypoxia (p<0.001), DFO (p<0.001) and HIF-1/VP16 (p<0.001) increased promoter activity as seen at the mRNA level. In addition, HIF-inhibition by IPAS reduced GPR35 promoter activity in hypoxia (p<0.001).

Promoter 5´-end deletion studies showed that HIF-dependent activation is retained in a 5´-truncated shorter promoter construct (-881 bp to +192) still containing the intact HBS, whereas induction by hypoxia and DFO is lost (p>0.05) in a -361 to +192 bp fragment construct, which lacks the HBS. We confirmed the essential role of the specific -372 bp 5´-ACGTG-3´ in the regulation by site-directed mutagenesis. Hypoxia-responsive construct (-881 to +192) HBS core nucleotide (5´-ACGTG-3´ to 5´-CATGT-3´) mutations aborted HIF-dependent induction of the GPR35 promoter completely. Hypoxia, DFO and HIF-1/VP16 were all unable (p>0.05) to up-regulate the hypoxia-inducible luciferase expression seen in the non-mutated version of the construct.

In addition, HIF-1 binding to the endogenous GPR35 promoter HBS was confirmed with chromatin immunoprecipitation assay (ChIP). ChIP analysis shows that HIF-1 binding to GPR35 promoter increased in hypoxia; altogether these findings clearly confirmed a prerequisite role of the functional -372 bp HBS to HIF-1-mediated activation of the GPR35 gene in hypoxic cardiac myocytes.

5.3.4 Hypoxia increases the number and density of receptor molecules on the cardiomyocyte plasma membrane

Induced GPR35 gene expression can be detected as an increased amount of GPR35 proteins, labeled as ~0.2 µm² spots on plasma membranes of non-permeabilized cardiomyocytes. Immunofluorescence labeling showed that hypoxia (1% O₂, 48 h) increased the number of plasma membrane-bound GPR35 receptors from normoxic average count of 142 to hypoxic 196 per cell (p<0.01). Receptor coverage area/cell was increased from 34 to 44 µm²/cell (p<0.01) without change in the total myocyte cellular surface area (p>0.05). Thus plasma membrane receptor covered area was increased from normoxic 4.2% to hypoxic 5.2% (p<0.05). These findings indicate that increased gene expression is translated into increased GPR35 protein on the functional cellular compartment of G protein-coupled receptors.
5.3.5 GPR35 agonist Zaprinast induces receptor internalization in cardiac myocytes

Time-lapse live cell confocal microscopy experiments showed that GPR35 agonist Zaprinast (Taniguchi et al. 2006) induced internalization of over-expressed GPR35-Venus chimera protein in cardiac HL-1 myocytes which were transiently transfected with GPR35a(S294)-Venus. Venus-derived fluorescence signal was mainly located on the plasma membranes of the transfected myocytes. Zaprinast (10 µM) induced time-dependent GPR35a(S294)-Venus internalization and subcellular accumulation during the 60-minute imaging period. Consequently, over-expressed receptor was functional and active in cardiac myocytes and responded to the ligand stimulation.

5.3.6 Over-expression of GPR35 changes cardiac myocyte morphology

Severe morphological changes were observed in neonatal mouse ventricular myocytes over-expressing GPR35. Live cell confocal microscopy showed pronounced cellular membrane ruffling-type morphology changes and formation of retraction fibers in cells over-expressing GPR35a(S294)-Venus. Morphology changes were associated with a disruption of cellular actin fiber network. Control cells expressing GFP retained their morphology without a disruption of actin filament network and cellular shape remained similar compared to non-transfected myocytes. These morphological changes suggest that GPR35 triggers pathways that mitigate the phenotypes of cardiac myocytes.

5.3.7 Activation of hypoxia-induced GPR35 affects cardiomyocyte Ca²⁺-activity

GPR35 ligand Zaprinast affects the calcium signaling of cardiomyocytes pre-exposed to 24-h hypoxia. The effect of GPR35 agonist on normoxic myocytes was negligible, but the cells pre-exposed to hypoxia expressing more GPR35 responded to Zaprinast drastically due to increased amount of GPR35, which sensitizes cardiomyocytes to the agonist. GPR35 activation altered hypoxic cells excitation-contraction coupling by slowing down the cytosolic calcium removal time constant (p<0.001) during relaxation, resulting in substantial diastolic calcium accumulation (p<0.001) and a decrease in the calcium transient.
amplitude (p<0.001) acutely after 3–6 minutes of Zaprinast application. These results suggest that activation of hypoxia-induced GPR35 interferes with cardiomyocyte calcium balance by slowing down the mechanisms involved in calcium removal from the cytosol, possibly including SERCA2a and NCX function.
6 Discussion

The role of HIF-1 in cardiac adaptation to hypoxia is incompletely understood. This study focused on the role of HIF-1 in the regulation of cardiac gene expression and in the functional adaptation processes at the cellular level. We characterized new cardiac-specific, previously unidentified hypoxia-regulated genes and the role of HIF-1 in the regulation processes. The tissue-specific cardiac transcription factors such as Nkx2-5, Gata4 and Mef2a form an interdependent response network modulating cardiac gene expression and function (Schlesinger et al. 2011). Hypoxia responses may vary in a plethora of genes expressed in the heart cells due to combinatorial regulation by multiple transcription factors with HIF-1, depending heavily on the collaborative binding of the core cardiac transcription factors and HIF to promoter and enhancer elements. The aim of this study was not only to characterize molecular mechanisms controlling the cardiac gene expression and adaptation, but also to measure how the expressional changes are coupled with the functional changes seen in hypoxic cardiac myocytes.

6.1 The general role of HIF-1 in the regulation of gene expression

(Semenza 2010) summarized generally accepted criteria to demonstrate that a gene is a direct target of regulation by HIF-1: “1) Gain or loss of HIF-1 activity leads to corresponding changes in target gene transcription under hypoxic conditions. 2) A \textit{cis}-acting HRE must be identified in the gene. The presence of a HIF-1 binding site matching the core sequence 5-RCGTG-3 is necessary but not sufficient: HREs are defined functionally as sequences that when inserted into a heterologous reporter gene render its expression \textit{O}_2-regulated. 3) Mutations that disrupt the binding of HIF-1 must also result in a loss of \textit{O}_2-regulated reporter gene expression.” In this research, the role of HIF was shown to be essential in the regulation of three novel hypoxia-regulated genes expressed in cardiac myocytes. The genes met the abovementioned criteria and HIF-activation led to distinguished changes in the gene expression compared to normal expression profile. These novel HIF-regulated genes are involved in the long-term cardiac hypoxia adaptation processes through the modulation of e.g. excitation-contraction coupling, contractility and survival.
6.2 The hypoxic regulation of apelin expression

Since apelin has been implicated in ischemic heart disease (Chen et al. 2003, Foldes et al. 2003), we asked whether apelin gene expression would be controlled by oxygen levels in cardiac myocytes. We were able to show that apelin, a potent inotropic hormone, is regulated by the hypoxia-HIF-1 pathway in myocardial cells (Ronkainen et al. 2007). Our results imply that hypoxic induction of apelin is part of the response to an impaired oxygen supply in the heart and up-regulation is also detected during acute myocardial infarction. We were not able to characterize the exact location of the functional HRE, but later experiments located HRE within the first intron of the human apelin gene (Eyries et al. 2008). We found that hypoxia is a specific stimulus not only for the gene expression but also for augmented processing and secretion of biologically active forms of apelin. Our findings establish a novel regulatory pathway that might improve cardiac function during compromised oxygen supply. In myocardium with a limited oxygen supply, increased apelin expression and secretion could serve as a paracrine adaptive mechanism to maintain contractile function, increase angiogenesis, prevent apoptosis and protect the heart from reperfusion injury. Apelin has been proposed to act mainly as a paracrine modulator of cell functions, because circulating apelin levels are very low and fluctuate little in various pathological situations (Kleinz & Davenport 2005, Miettinen et al. 2007). It is possible that endogenous apelin secretion is targeted to acutely compensate for the decreased contractility and oxygen supply of the myocardium suffering from hypoxic insult. Apelin may protect the heart against hypoxia-induced damage and it is therefore possible that apelin could serve as therapeutic target for ischemic heart failure.

6.3 Hypoxia adaptation mechanisms driven by apelin

6.3.1 Apelin increases blood delivery to tissues

Apelin was shown to be mitogenic for endothelial cells (EC) and to act as a paracrine angiogenic factor in tumors promoting the formation of new vessels necessary for tumor growth (Masri et al. 2004, Sorli et al. 2007). Apelin was shown to be an angiogenic factor also in retinal endothelial cells in vitro, and apelin enhanced migration, proliferation, and capillary-like tube formation of cultured cells (Kasai et al. 2004). Apelin-induced, endothelium-derived
vasodilatory effects have also been reported, and apelin has been shown to lower blood pressure through increased endothelial NO release (Tatemoto et al. 2001). It was recently shown in a hind limb ischemia model that apelin together with VEGF induced effectively the formation of functional vessels larger than with VEGF alone, and prevented the formation of leaky blood vessels typical for VEGF-induced angiogenesis (Kidoya et al. 2010). Apelin induces larger cords of ECs mediating mainly cell-cell aggregation, which results in enlarged caliber size of blood vessels (Kidoya et al. 2008). It has been reported that apelin expression is induced by HIF-1 also in endothelial cells where apelin induces EC proliferation and regenerative angiogenesis in hypoxia and acts by both autocrine and paracrine mechanisms (Eyries et al. 2008). We saw an increased apelin expression and secretion in hypoxic myocardial cells. Apelin was processed from the longer storage forms to the secreted shorter peptides, which were accumulating to the culture medium of the hypoxic cardiac myocytes (Figure 8). Increased apelin secretion from the large myocardial myocyte pool may increase local tissue concentration of apelin abundantly and act as a paracrine factor enhancing hypoxic region EC proliferation and angiogenesis, as also seen in tumor and cell culture models (Figure 9). Hypoxia-induced apelin and VEGF-driven collaborative angiogenesis would then relieve regional hypoxia and serve as a formerly uncharacterized adaptation mechanism for reduced myocardial oxygenation in ischemic and hypoxic hearts.
6.3.2 Apelin modulates [pH]i and contractility of cardiac myocytes

Apelin-APJ receptor stimulation activates the sarcolemmal \( \text{Na}^+-\text{H}^+ \) exchanger (NHE) and increases intracellular pH (Farkasfalvi et al. 2007) resulting in an increase in the intracellular calcium levels via reverse mode of the \( \text{Na}^+-\text{Ca}^{2+} \) exchanger (NCX) (Figure 9). Alkalization could serve as a useful compensatory mechanism for reduced TnC Ca\(^{2+}\) sensitivity due to an increased number of protons, which are released to the cytosol during anaerobic energy production in hypoxic conditions (Figure 9). The extent of alkalinization induced by this mechanism is likely to be the greatest in the cardiac tissue suffering from acidosis (Leem et al. 1999), such as during ischemia and hypoxia. Chronic hypoxia in the presence of high glucose leads to progressive acidosis in cultured cardiac myocytes (Graham et al. 2004). To remain viable under hypoxia, the cells must be able to maintain glycolysis at a level that is sufficient to sustain ATP. In addition, the cell must be able to clear excess acid produced by anaerobic glycolysis (Graham et al. 2004).

Apelin enhances the contractility of Langendorff-perfused hearts (Szokodi et al. 2002) and increases sarcomere shortening also in isolated normal and failing cardiomyocytes without significant increase in [\( \text{Ca}^{2+} \)], transient amplitude (Farkasfalvi et al. 2007). However, controversial results exist and the increased...
force development was also shown to be the result of increased calcium transient amplitudes rather than changes in myofilament calcium sensitivity (Dai et al. 2006, Wang et al. 2008). It is therefore unclear whether apelin directly increases [Ca²⁺]i or sensitizes myofilaments to calcium. However, in myocardium with a limited oxygen supply, increased apelin expression could serve as an adaptive mechanism to maintain the contractile function of the heart under hypoxic acidosis. In addition to calcium metabolism, apelin affects other myocardial ion fluctuations as well. It was shown in isolated cardiomyocyte patch clamp experiments that apelin also has an effect on the cardiac sodium current (I_{Na}) (Chamberland et al. 2010). Protein kinase C (PKC) linked phosphorylation of sodium channels and modulation of I_{Na} gating and amplitude may thus contribute to the increased cardiac conduction, excitability and inotropic effects seen in earlier studies and have a beneficial anti-arrhythmic effect, especially in diseases where I_{Na} amplitude is reduced.

6.3.3 Apelin prevents apoptosis

It has been shown that apelin inhibits human osteoblast apoptosis during serum deprivation and suppression of APJ with small-interfering RNA (siRNA) abolishes the anti-apoptotic activity (Xie et al. 2007). It has been suggested that apelin induces the expression of the anti-apoptotic factor Bcl-2 and reduces pro-apoptotic Bax. More recent results have shown the same also with human vascular smooth muscle cells (VSMCs), where apelin reduced serum deprivation induced apoptosis (Cui et al. 2010). Apelin/APJ system is an intrinsic protective pathway in ischemia/reperfusion injury in perfused hearts, and cultured cells and administered apelin-13 limited infarct size and improved cardiac post-ischemic mechanical recovery (Zeng et al. 2009, Rastaldo et al. 2011). Thus increased apelin expression and secretion in hypoxia could serve as an adaptive anti-apoptotic mechanism to prevent myocardial damage during hypoxia-reperfusion (Figure 9).

Hypoxia and acidosis cause a strong induction of the gene encoding the pro-apoptotic Bcl-2 family member BNIP3. Cell fractionation experiments also indicated that BNIP3 was loosely bound to mitochondria under conditions of neutral hypoxia but was translocated into the membrane and activated when the myocytes were acidotic (Graham et al. 2004). In this study it was seen that hypoxia up-regulates the expression of pro-apoptotic Bcl-2 family member BNIP3 in isolated cardiac cells; similar results have been obtained earlier in
immortalized cell lines (Bruick 2000). BNIP3 is a well-known HIF-1 target gene (Greijer & van der Wall 2004) and it was up-regulated in neonatal mouse ventricular cells but far less in embryonic cardiomyocytes during hypoxia. The finding was supported by the difference in the cell culture apoptosis rate in embryonic versus neonatal cells (Ronkainen et al. 2011). The ability of embryonic cells to resist apoptosis could thus be one of the mechanisms underlying the better adaptation capability of these “naturally” hypoxic cells. One important function for the up-regulated apelin could be the blocking of BNIP3 induction and translocation and lowering the rate of apoptosis. This mechanism would be driven by the prevention of cellular acidosis through apelin-induced NHE activity (Farkasfalvi et al. 2007) (Figure 9). It remains to be seen if the cells are more sensitive to hypoxia after apelin receptor blockade or apelin/APJ knock-down e.g. by siRNA.

6.4 Hypoxic modulation of SERCA2 expression and excitation-contraction coupling

6.4.1 SERCA2 gene and promoter properties

SERCA2 isoforms

The SERCA2 gene encodes SERCA2a and SERCA2b isoforms which are produced by alternative splicing in a tissue- or cell-type-specific fashion. Alternative splicing of the last two exons of the pre-mRNA produce the cardiac/slow-twitch muscle-specific SERCA2a isoform and the ubiquitous SERCA2b isoform (Lytton et al. 1989). SERCA2a transcript is found almost exclusively in cardiac and slow-twitch skeletal muscle, whereas the SERCA2b mRNA is present in a large variety of cells (Lytton et al. 1989).

Promoter

Previous SERCA2 promoter analyses have shown that the 5’-regulatory sequence of the proximal -225bp promoter of the SERCA2 gene is 80% GC-rich CpG island-type promoter (see Introduction), and is conserved among human, rabbit, rat, and mouse species (Wankerl et al. 1996). It contains a TATA-like-box, an E-box/USF sequence, a CAAT-box and four Sp1 binding sites, which are typical of
regulated genes. In addition, the promoter contains a thyroid hormone-responsive element (TRE). There are two other conserved regulatory regions located between positions -410 to -661 bp and from -919 to -1410 bp (Zarain-Herzberg & Alvarez-Fernandez 2002). Among the DNA cis-acting elements present in these two regulatory regions, there are potential binding sites for GATA-4, -5, -6, Nkx2-5/Csx, OTF-1, USF, MEF-2, SRF, PPAR/RXR, AP-2, and TREs (Zarain-Herzberg & Alvarez-Fernandez 2002). Upstream from position -1.5 kb, there is no significant homology among the SERCA2 genes (Zarain-Herzberg & Alvarez-Fernandez 2002).

**Promoter regulation**

It has been shown that proximal promoter region is sufficient for the SERCA2a expression in cardiac myocytes, and the region from -225 to -1232 bp contains regulatory DNA elements which down-regulate expression in neonatal rat cardiomyocytes (Zarain-Herzberg & Alvarez-Fernandez 2002). Promoter activity has been studied *in vivo* and in primary cell cultures by using SERCA2a-luciferase constructs (Aoyagi *et al.* 1999, Brady *et al.* 2003, Vlasblom *et al.* 2004). Promoter element deletion studies have shown that some unidentified cis-acting repressive element is located at the distal promoter (-1110 to -658 bp) of rabbit SERCA2 (Aoyagi *et al.* 1999). The region was suggested to be the reason for down-regulation of SERCA2 promoter in failing heart model *in vivo*. A negative regulatory element has been identified earlier in human SERCA2 promoter in the same region (-1741 to -412 bp) (Wankerl *et al.* 1996). Proximal promoter Sp1 and Sp3 trans-activation dependency has also been shown (Brady *et al.* 2003). Calcium-dependent signaling and the synergistic effect of NFATc4/MEF2c on SERCA2a promoter-luc activity was studied in cultured neonatal rat ventricular myocytes, where the relationship of these core cardiac transcription factors and SERCA2 expression was characterized (Vlasblom *et al.* 2004).

### 6.4.2 SERCA2a expression and function is reduced in pathological situations

SERCA2a activity and expression is reduced in various animal models of pressure or volume overload and in patients with heart failure (Hasenfuss & Pieske 2002, Piacentino *et al.* 2003, Periasamy *et al.* 2008). Reduced SERCA2a
mRNA expression and function correlate with a prolonged diastolic phase in hypertrophied and failing hearts and are associated with a slower reduction of cytosolic Ca\textsuperscript{2+} (Gwathmey et al. 1987, Mercadier et al. 1990, Arai et al. 1993, Piacentino et al. 2003). Reduction of SERCA2a protein expression is probably the main cause for the decreased SERCA2a activity in pathological developments. Ventricular SERCA2a expression is down-regulated during early response to hypobaric hypoxia suggesting possible direct oxygen-dependent regulation of the gene (Sharma et al. 2004b). Expressional regulation of SERCA2a has been studied in various animal and cell models, but the basic cellular mechanisms underlying SERCA2a expressional repression during diverse heart conditions are still largely unclear.

6.4.3 Hypoxia down-regulates SERCA2a expression and pumping function

In this study, we showed that hypoxia causes a direct down-regulation of SERCA2a gene and protein expression through the HIF-1 pathway in mouse embryonic cardiac myocytes. We further demonstrated that this effect is due to cooperative HIF-1 binding to two hypoxia response elements on the SERCA2 promoter. We also measured the effect of SERCA2a reduction on cardiac myocyte function. The results indicate that even a slight hypoxia-induced reduction in the expression of SERCA2a results in distinct changes in cardiomyocyte Ca\textsuperscript{2+}-signaling, which were linked to hypoxic impairment of cardiac myocyte Ca\textsuperscript{2+}-metabolism and contractile function. We characterized hypoxic SERCA2a down-regulation especially in embryonic cardiomyocytes. If hypoxia and HIF regulate SERCA2a expression also in adult myocytes, our results could offer one explanation for the reduced SERCA2a activity and disturbances in calcium pumping seen in congestive, ischemic and post-infarcted failing hearts. Our results are novel, showing that the expression of the SERCA2a gene is affected by the endogenous HIF-signaling pathway and may work in conjunction with other cardiac transcription factors.

6.4.4 The role of HIF in the regulation of SERCA2a promoter activity

While HIF-1 is usually associated with the induction of hypoxia-sensitive genes, there is accumulating evidence that HIF-1 is also capable of repressing gene expression in various ways. It has been postulated that HIF-1 acts as a repressor
when the HRE strand is in reversed orientation on the gene promoter (Narravula & Colgan 2001, Eltzschig et al. 2005). It has also been suggested that HIF-1 acts as a competitive inhibitor by establishing a physical barrier that blocks the binding of other transcription factors on SERCA2a promoter (Mazure et al. 2002). Although SERCA2a promoter analysis has revealed several transcription regulatory sites, hypoxia response elements had not been characterized before our experiments. We found two relatively close HIF-1 binding sequences from mouse and rat SERCA2a promoters. Both of these interdependent binding sites are required for the HIF-1-mediated inhibition. In addition, we found several putative cardiac-associated transcription factor binding sites in close proximity of the HRE regions. We suggest that HIF-1 establishes a physical barrier on SERCA2a promoter in hypoxia. HIF-1 blocks the binding of core cardiac transcription factors like Nkx2–5, Gata-4 and Mef2c, NFAT and Tbx5 to proximal promoter, thus silencing the SERCA2a expression in hypoxia (Figure 10). The combinatorial binding of core cardiac transcription factors to chromatin is essential to transcriptome involved in the cardiac differentiation, maturation and homeostasis (Schlesinger et al. 2011). For example, Nkx2–5 was shown to regulate SERCA2a expression in mice and knock-out mice with a loss of Nkx2–5 had decreased SERCA2a levels in the heart (Briggs et al. 2008). Since conserved SERCA2a promoter has only two putative Nkx2–5 binding sites, their functionality is presumably essential for the normal SERCA2a gene expression. The amount of HIF-1 is increased massively during hypoxia and the probability of trans-inhibitory HIF-1 promoter binding is increased, interfering with the assembly of the general transcription machinery. Therefore HIF-1 acts as an inhibitory factor competing for the same DNA binding regions with the factors essential to cardiac gene program and SERCA2a expression (Figure 10). In normoxia, the core factor binding predominates and SERCA2a expression remains at normal level.
Fig. 10. Schematic presentation of HIF-1-dependent inhibition of the SERCA2 promoter in hypoxia. In hypoxia, HIF-1 and co-factor (e.g. CBP/p300) binding masks core cardiac transcription factor binding sites on the SERCA2 promoter and inhibits transcription. In normoxia, HIF-1α is rapidly degraded and core TFs can bind to clustered cis-acting regulatory elements and promote SERCA2 transcription.
6.4.5 SERCA2 reduction may serve as an adaptive mechanism for reduced ATP production

Generally ATP is in excess of the SERCA2a and Ca\(^{2+}\) is the limiting substrate for the pumping reaction. When cellular ATP levels fall during oxygen starvation during hypoxia, there may be some decline in SERCA2a pumping due to the allosteric effect, but [ATP] would have to be extremely low to prevent ATP binding to the SERCA2a substrate site. This makes [ATP] a poor regulator of SERCA2 activity. It has been reported that [ATP] does not drop (~25%) below the ATPase Km values in failing hearts (Bers 2001, Ingwall 2009). Therefore, instead of the regulation of calcium pumping activity by ATP, other adaptational changes must occur in hypoxia. The hypoxic down-regulation of SERCA2a seems to be involved in these adaptation processes. Down-regulation of SERCA2a leads primarily to reduced SR Ca\(^{2+}\)-pumping and smaller Ca\(^{2+}\)-transients. Secondarily, when less calcium is available for triggering the contraction, the ATP consumption of the contractile element is reduced. Therefore, hypoxia-induced SERCA2a repression results in blunted calcium signals and contractility, which could be a compensatory mechanism by which the myocardial cells tend to prevent excessive energy loss while maintaining at least some contractile activity. It can also be that in the developing heart HIF-1 and hypoxia-induced SERCA2a down-regulation helps to adjust contractile activity to oxygen availability, preventing exhaustive activity of embryonic cardiomyocytes in hypoxic environment.

6.4.6 SERCA2a knock-out and knock-down cells resemble hypoxia-exposed phenotype

In recent studies, cardiac contractility was reduced in inducible cardiac specific SERCA2a knock-out mice (Andersson et al. 2009, Louch et al. 2010). SERCA2a KO cardiomyocytes showed reduced magnitude of contraction and the maximal rate of relaxation was slowed as well. Ca\(^{2+}\) transient amplitudes were smaller and the decay of the Ca\(^{2+}\) transient was prolonged. Caffeine pulse-stimulated SR Ca\(^{2+}\) release was shown to be smaller after the deletion of SERCA2a gene indicating a reduction in SR calcium content. Knock-down experiments with SERCA2a siRNA led to similar dysfunction in cultured rat neonatal cardiomyocytes (Seth et al. 2004). Lower calcium transient amplitude and decline of cytosolic Ca\(^{2+}\) during CICR indicated reduced SR calcium content due to reduced SERCA2a activity.
These effects are similar to the effects of hypoxia. SERCA2a reduction seems to cause a similar Ca\(^{2+}\)-handling phenotype in genetically modified myocytes and in myocytes exposed to pathophysiological hypoxia stimulus. Interestingly, transgenic conditional HIF-1α over-expression in normoxia results in the down-regulation of SERCA2a and calcium reuptake in mouse hearts, suggesting a direct role for HIF in the down-regulation of SERCA2a, even without hypoxia-derived effects (Bekeredjian et al. 2010).

6.5 A novel cardiac hypoxia inducible gene, G protein-coupled receptor 35

6.5.1 Isoforms of GPR35 in cardiac myocytes

In gastric cancer cells GPR35 is expressed in two different alternatively spliced isoforms and significant up-regulation of their expression was observed in cancer tissue versus surrounding non-cancerous regions (Okumura et al. 2004). The longer transcript (GPR35b) contains the 31-amino acid N-terminal extension compared to the shorter (GPR35a) one, but the functional differences and the significance of the isoforms is still not clear (Okumura et al. 2004). We used splicing variant-specific quantitative real-time PCR primers and probes to test if the expression of the two reported isoforms differs from each other in myocardial cells under normoxia or hypoxia. The messenger RNA encoding the extended variant was present at higher levels in gastric cancer cells (Okumura et al. 2004), but in cardiac myocytes GPR35a and GPR35b mRNA levels were similar in normoxia. The hypoxic induction of both variants was similar. These findings let us assume that in mouse cardiac myocytes, expression of both isoforms is driven by the same promoter and enhancers. However, the possible functional difference of the two isoforms remains to be characterized.

6.5.2 GPR35 is up-regulated in hypoxic myocardial cells through HIF-1 pathway

There are no published studies on the transcriptional regulation of the GPR35 gene to date. There are a few reports describing the alteration of GPR35 expression pattern in pathological conditions, but the molecular mechanisms have not been elucidated. In this study, GPR35 gene expression appeared to be
hypoxia-sensitive and the hypoxic regulation of GPR35 was characterized comprehensively. Hypoxia induced GPR35 mRNA synthesis strongly in a rapid time-dependent and dose-dependent manner. Interestingly, 5% O₂ was not sufficient to induce GPR35 expression and the threshold for the hypoxic response was somewhere between 2% O₂ and 5% O₂. Oxygen percentage from 1 to 0.5 was required for the maximal up-regulation. It can therefore be proposed that HIF degradation pathway and HIF-P4Hs are still active in the cells cultured in 5% environmental oxygen. This is in line with the earlier reports showing that HIF-1 DNA binding activity increases exponentially from 5% O₂ to 0.5% O₂ with the half maximal response between 1.5 and 2% O₂ (10.5 mm Hg) (Jiang et al. 1996b). Chemical and genetic HIF-1 activation/repression confirmed the involvement of HIF-1 in the regulation process. In addition to changes in mRNA, GPR35 protein was increased on the cell surfaces of the myocytes by hypoxia. These findings indicate that increased amount of gene expression is translated into an increased amount of GPR35 protein, which is efficiently translocated to the functional cellular compartment of the G protein-coupled receptor signaling. Increased amount of GPR35 on plasma membrane can emphasize the G-protein-mediated cellular responses even at constant concentration of the surrounding receptor ligand.

6.5.3 GPR35 promoter has a functional hypoxia response element

The results show that mouse GPR35 promoter contains an important hypoxia response element at -372 bp upstream from the transcription start site. All HIF-pathway interfering responses show the absolute requirement of this particular HRE site for GPR35 gene induction and the endogenous site was also shown to increasingly bind HIF-1 in hypoxic conditions. Interestingly, GPR35 was not up-regulated in lung epithelial cell line after hypoxia treatment (data not shown). This suggests a cardiac-specific regulation mechanism, but the possible tissue-specific regulation still needs further clarification. The core cardiac transcription factors may again have an interdependent role with HIF-1 in the regulation of GPR35 in cardiac myocytes.

6.5.4 GPR35 is also functional in cardiac myocytes

Our experiments showed that a known GPR35 agonist, Zaprinast, caused receptor internalization in cardiac cells. This had not been characterized in cardiomyocytes
before. Internalization was earlier characterized by kynurenic acid, an endogenous agonist, in HeLa cells (Wang et al. 2006). The ligand-induced cellular redistribution of GPR35 has also been shown to occur with 2-acyl LPA and pamoic acid in U2OS and HEK293 cells, where the relationship between receptor internalization and β-arrestin activation was studied (Zhao et al. 2010). Most activated G protein-coupled receptors are desensitized and internalized after activation. Internalization is promoted by β-arrestins in order to desensitize or down-regulate the amount of GPCR after prolonged activation and eventually recycle some re-sensitized receptors back to the cell membrane (Marchese et al. 2008). Consequently, over-expressed receptor was functional and active in cardiac myocytes and responded to the ligand stimulation in the way reported earlier.

6.5.5 Physiological responses caused by GPR35 activation

Morphology changes

In this study we showed that over-expressed GPR35 causes distinct morphological changes in cardiac muscle cells, possibly due to tonic receptor activation (Taniguchi et al. 2008). GPR35 may induce morphological changes through activation of Ga_{12/13} (Jenkins et al. 2010), which is reported to launch receptor-mediated stress fiber formation with the regulation of the actin cytoskeleton (Gohla et al. 1999). We saw a prominent stress and retraction fiber formation in GPR35 over-expressing cells suggesting involvement of tonic GPR35 mediated activation of Ga_{12/13}. Following this logic GPR35 would induce constitutive Ga_{12/13} activation which drives Rho-dependent remodeling of actin cytoskeleton and formation of stress fibers (Buhl et al. 1995). Activation of β-arrestin signaling has also been linked to membrane ruffling and retraction fiber formation (Scott et al. 2006), and GPR35 activation was recently linked to β-arrestin activation (Jenkins et al. 2010). According to previous and our results it can be hypothesized that in the GPR35 over-expressing cells, tonic receptor activation could drive constitutive β-arrestin activity which then launches membrane ruffling and retraction fiber formation seen by microscopy.

On the other hand, activation of endogenous GPR35 by KYNA has been reported to increase integrin-mediated firm adhesion of leukocytes to extracellular matrix and endothelial cells (Barth et al. 2009). According to those results it can be postulated that tonic activation of over-expressed GPR35 would increase the
maturation of focal complexes and generate larger and stable focal adhesions on cardiac myocytes cultured on fibronectin-coated culture plates. Abnormal cell-extracellular matrix adhesions could explain the characteristics of the GPR35 over-expressing cells, since the synthesis and degradation of focal adhesions is abnormal. It could inflict cellular extracellular matrix anchorage that is too prominent as well as two-dimensional spreading of the cells on the top of the matrix. It has been suggested that adhesion is triggered by G\textsubscript{i} -mediated processes (Barth \textit{et al.} 2009), but focal adhesion assembly could be mediated by GPR35 activated G\textsubscript{a12/13} as well (Buhl \textit{et al.} 1995, Jenkins \textit{et al.} 2010).

However, the characterization of molecular mechanisms and pathways causing drastic cellular phenotype changes was not the main focus of this study and those processes still need to be further characterized. The involvement of \textbeta-arrestin and G\textsubscript{a12/13} -pathways in the regulation of the cellular morphology via activation of GPR35 should be taken under further observation.

\textit{Endogenous activator in cell culture}

Earlier studies suggested that over-expressed GPR35 is presumably constitutively active in the serum-rich cell culture conditions without addition of any external receptor agonist, suggesting that high cell surface receptor density causes a tonic activation of GPR35 (Guo \textit{et al.} 2008). However, it is possible that an endogenous agonist secreted by the cells affects GPR35 activity as well. For example, lysosphosphatic acids (LPAs) were recently characterized as possible endogenous ligands for GPR35 (Oka \textit{et al.} 2010). LPAs were capable of activating the receptor at the nanomolar range. Interestingly, lysosphatidic acids are reported to be one major constituent in serum, and in humans LPA concentration in serum is in the micromolar range (Baker \textit{et al.} 2001). LPAs also accumulate in conditioned media from cultured cells (Moolenaar 1999). According to those results, it is highly possible that tonic activation of over-expressed GPR35 originates from the serum-derived or cell-secreted LPAs. It has been shown that plasma membrane G protein-coupled receptors mediate the diverse cellular effects of LPA stimulating e.g. proliferation, cell motility and migration through Rho-mediated signaling pathways (Moolenaar 1999). LPA was shown to stimulate cytoskeletal rearrangement in breast cancer cells cultured in serum-free conditions causing cellular retraction and forming of retraction fibers, followed by membrane ruffling (Li \textit{et al.} 2009). Similar changes were seen in our
live cell confocal microscopy imaging of mouse cardiac myocytes over-expressing GPR35.

**Acute effects**

The cells pre-exposed to hypoxia responded to GPR35 agonist fast by slowing down the cytosolic Ca^{2+}-removal leading to diastolic calcium accumulation and decreased Ca^{2+}-transient amplitude. In normoxic cells with a lower level of GPR35 expression, the response for GPR35 activation was minute. The results indicate that the GPR35 pathway is most likely linked to SERCA2a and/or Na^{+}-Ca^{2+} exchanger in cardiac myocytes, but the exact mechanisms still remain to be characterized. However, GPR35 activation has been linked to activation of the Gi-pathway (Wang *et al.* 2006, Ohshiro *et al.* 2008) which inhibits the function of adenylate cyclase and reduces the production of cAMP (for review see Salazar *et al.* 2007) suppressing the activity of PKA (for review see Hammond & Balligand 2012). PKA is one of the master regulators of SERCA2a activity, and the L-type calcium channels and suppression of this pathway, if stimulated, might explain the findings seen with GPR35 stimulation in cells pre-exposed to hypoxia. This might counteract some prolonged deleterious effects of Gs signaling seen e.g. in heart failure and equalize βAR/Gs signaling disturbances which expose the heart to various pathological symptoms discussed in the Introduction. Reduced contractility would protect the heart in hypoxic conditions in the same way as proposed in the case of SERCA2a, since reduced cardiac work consumes less ATP in energy-starved conditions. On the other hand, the substantial Ca^{2+}-accumulation and cytosolic overload can lead to a so-called ER stress response and apoptosis (Ferri & Kroemer 2001), which in conjunction with autophagy may serve as an adaptive survival mechanism for oxygen-deprived conditions (for review see Zhang *et al.* 2008, Dorn 2009).
7 Summary and conclusions

HIF-activation has been shown to have a crucial role in several situations ranging from tumorigenesis to physiological high altitude adaptation. The role of cardiac tissue-specific HIF-dependent gene expression regulation is still incompletely characterized. Heart tissue relies heavily on aerobic metabolism, and cardiac energy production is sensitive to reduced oxygen availability. Unlike skeletal muscle, cardiac muscle cannot resist the drop of [ATP], by resting and ceasing the contractile function. Therefore, myocardial fatigue resistance and hypoxia adaptation mechanisms have to be well developed.

This study provides novel information about the plasticity of myocardial adaptation for prolonged hypoxia. The main questions of this study concerned the hypoxia-regulated gene expression at the cellular and molecular level. Expressional changes were connected to physiological responses of hypoxic cardiomyocytes. The role of a conserved transcription factor, HIF-1, was shown to be essential in the hypoxia adaptation process in myocardial cells as well.

Our study models included three different types of cultured cardiac cells, in vitro atrial appendix tissue perfusions and in vivo acute myocardial infarction models. HIF-1 pathway interferences with genetic or chemical modifications are easier, faster and more reproducible in cell cultures than in complex tissue or whole animal models. That is the main reason why most of the experiments were performed in a cell culture environment. Isolated and cultured cardiac myocytes show clear similarities with in vivo myocardial cells. Excitation-contraction coupling is functional also in cells grown as a monolayer on hard plastic or glass surfaces. Intracellular calcium metabolism follows more or less in vivo situation in the intact heart, and calcium-modulated gene expression is controlled as well (Ronkainen et al. 2011b). Cultured cells beat in a continuous regular way, which is one of the most unique features of cardiac tissue, making the characterization of contractility-related phenotype possible. However, there are some striking differences between cultured cells and cells living in 3D environment in vivo. It has to be realized that myocardium consists of many different cell types, and the lack of non-myocyte cells (e.g. cardiac fibroblasts and endothelial cells) may affect the behavior and phenotype of the cells in culture conditions. In addition, isolated neonatal and embryonic myocytes are not terminally differentiated cardiac cells; unlike adult cells they can divide and survive in cell culture conditions. Being aware of adult myocyte special characteristics, the findings
made by using cardiac cell culture models cannot necessarily be directly converted to living whole organs and organisms.

The main findings of this study were the identification of three novel myocardial HIF-1 regulated genes. Apelin expression, synthesis, processing and secretion were found to increase in prolonged hypoxia. In this study, pathological processes were also linked to the HIF-1-regulated gene expression. Our studies show that apelin expression is increased during acute myocardial infarction, when the tissue suffers from a lack of oxygen due to ischemia originating from the ligation of coronary artery. The exact role of apelin in the myocardium is still not totally clear, and the specific intracellular signaling pathways modified by apelin-APJ are not well characterized. Several studies show that administrated apelin has varying responses in the heart and the cardiovascular system, but the role of endogenous, paracrinaely secreted apelin is still poorly characterized. However, our results were the first to identify myocardial apelin processing and secretion, and the results clearly distinguish the isoforms of secreted and stored apelin. However, the role of endogenous apelin in the hypoxic myocardium is not known, and the physiological significance of the increased apelin expression and secretion in hypoxia remains to be characterized.

On rare occasions, HIF-1 is also capable of repressing gene expression. In this study, HIF-1-driven SERCA2a repression was observed to be an adaptive mechanism to hypoxia in cultured embryonic cardiac myocytes. HIF-driven suppression of SERCA2a expression was directly linked to cardiomyocyte EC-coupling and reduced cytosolic calcium removal capacity. The reduction of SERCA2a alters cellular calcium cycling towards a less energy consuming and less contractive phenotype. This spares energy in oxygen- and ATP-deprived conditions and could serve an adaptive mechanism preventing complete myocardial exhaustion.

In this study, HIF-1 was also shown to regulate the expression of a novel G-protein coupled receptor, GPR35, in cardiac myocytes. GPR35 has been under increasing research interest in recent years, but the studies have focused mainly on endogenous or chemical receptor ligand characterization. Just a minor part of the studies has described the physiological responses caused by the receptor activation, while the regulation of GPR35 gene expression is even less characterized. Hypoxia is involved in the majority of cardiac pathophysiological conditions. Consequently, hypoxic GPR35 up-regulation may also have a role in heart failure, and the earlier reported induction of GPR35 in failing hearts may be driven by the myocardial HIF-activation. Hypoxia-dependent regulation of
GPR35 can connect the descriptive expression data obtained earlier from heart failure models to integrative hypoxia adaptation network in the heart. The results from this study can serve as a starting point for deeper appreciation of the adaptive processes in which GPR35 may be involved in hypoxia. Our observation of the role of GPR35 in the heart suggests the heart as one of the major organs influenced by administrated GPR35 agonists.
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