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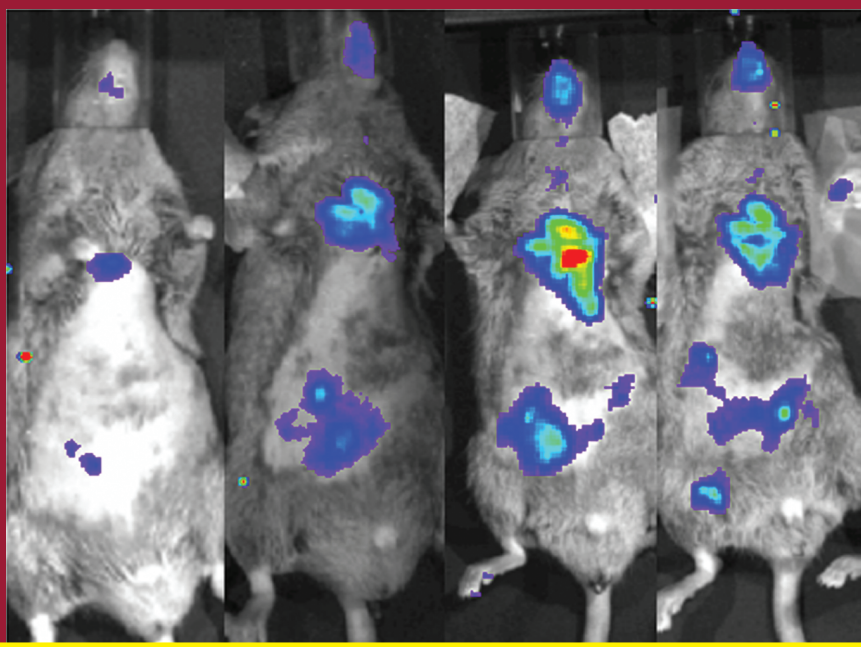
Cardiovascular Healing – Focus on Inflammation

Leopoldina Symposium Wuerzburg, June 23 to 24, 2006

Johann Bauersachs / Stefan Frantz / Georg Ertl (Eds.)



Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 2008
In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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NOVA ACTA LEOPOLDINA

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Vizepräsident der Akademie

NEUE FOLGE NUMMER 351 BAND 95

Cardiovascular Healing – Focus on Inflammation

Leopoldina Symposium

Wuerzburg
June 23 to 24, 2006

Organized by

Medical Clinic and Polyclinic I
Julius Maximilians University Wuerzburg
German Academy of Sciences Leopoldina

Editors:

Johann BAUERSACHS (Wuerzburg)
Stefan FRANTZ (Wuerzburg)
Georg ERTL (Wuerzburg)
Member of the Leopoldina

With 11 Figures

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Einbandbild:

Das Immunsystem höherer Vertebraten besteht aus zwei Komponenten: dem System der angeborenen und dem der adaptiven Immunität. Bei kardialen Erkrankungen kommt es zu einer Aktivierung von Komponenten des Systems der angeborenen Immunität, die ihrerseits wieder eine stimulierende Wirkung z. B. auf den Transkriptionsfaktor *Nuclear Factor kappa B* (NF-κB) entfaltet. Das Coverbild zeigt den experimentellen Verlauf der NF-κB-abhängigen Lumineszenz im Herz einer transgenen Maus nach Eintritt des Herzinfarktes sowie die NF-κB-Aktivität ein, drei bzw. sieben Tage nach dem Ereignis (von links nach rechts; siehe Beitrag FRANTZ et al. auf den Seiten 17ff.).

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Pro and Contra: Stem Cell Therapy in the Heart

Introduction

Johann Bauersachs, Stefan Frantz and Georg Ertl ML (Wuerzburg)

The III. international Cardiovascular Healing Symposium was organized by the *Medizinische Klinik* and *Poliklinik I, Herz-Kreislaufzentrum der Universität Würzburg* and the *Deutsche Akademie der Naturforscher Leopoldina*. The purpose of the symposium was to gather an interdisciplinary panel to discuss cardiovascular healing with a special focus on inflammatory reactions. The response to injury involves inflammation and an activation of the immune system. These inflammatory reactions are important for healing, however, they need to be restricted with regard to time and space to avoid harm of un-injured tissue. Novel results suggest that in cardiovascular diseases such as heart failure or atherosclerosis, inflammation has an important pathophysiological role. The "infarct wound" and the arteriosclerotic plaque are characterized by infiltration of inflammatory cells that are necessary for infarct healing but may stimulate progressive left ventricular remodeling or atherosclerosis.

During the III. Cardiovascular Healing Symposium the role of the immune system, reactive oxygen species, nitric oxide and the nitric oxide synthases for healing and cardiovascular diseases was discussed. One of the highlights of the seminar included critical reviews of the role of stem cells in different diseases. In a direct pro and contra session, the importance of stem cells in myocardial regeneration after myocardial infarction was discussed.

In the current monograph, the presenters contribute an abstract or a short review article. Stefan Frantz (Wuerzburg) describes the central role of the transcription factor NF-κB for heart failure and ischemia-reperfusion injury. NF-κB induces numerous genes such as cytokines und chemokines, adhesion molecules as well as regulators of cell survival, proliferation and apoptosis. A mouse model deficient for the NF-κB subunit p50 was protected against cardiac ischemia-reperfusion injury and chronic left ventricular remodeling. Using cardiomyocyte specific inhibition of NF-κB, Martin Bergmann (Berlin) demonstrates improved cardiac remodeling and attenuated cardiomyocyte hypertrophy after in vivo infusion of angiotensin II.

Roberto Latini (Milano) reviews the role of cytokines and chemokines in heart failure. It is still unclear which cytokines at which time point may induce cardiovascular protection in addition to their well-described negative effects. Increased concentrations of cytokines such as TNF-alpha, Interleukin 6, CRP are proven predictors of a bad prognosis, however, to date there is no proof for a therapeutic effect by specific inhibition of single cytokines in heart failure. For most patients with heart failure, the inhibition of neurohumoral systems using beta-blockers, inhibitors of the angiotensin conversion enzyme, angiotensin II receptor blockers as well as aldosterone-antagonists remains the cornerstone for reducing mortality and morbidity. Johann Bauersachs (Wuerzburg) reviews recent experimental evidence that a selective aldosterone receptor blockade immediately after myocardial infarction improves early

healing processes. As early as seven days after myocardial infarction left ventricular function was improved and dilatation was reduced in rats treated with the aldosterone blocker.

Dworakowski et al. (London) review the role of NADPH oxidases in cardiac disease. Out of the five known Nox subunits, Nox 1, Nox 2, and Nox 4 are expressed in cardiovascular cells. They catalyze the electron transfer from NADPH to molecular oxygen resulting in superoxide anion formation. Ingrid Fleming (Frankfurt/Main) identified cytochrome P450 2C as a relevant source of superoxide anions in the vascular system. Inhibition of CYP 2C9 using sulfaphenazole reduced superoxide formation and improved acetylcholine-induced endothelium-dependent vasodilatation in patients with coronary artery disease. The importance of chemokines for the different steps of cell invasion in vascular lesions is reviewed by Christian Weber (Aachen). Especially platelet-derived chemokines are necessary for monocyte adhesion. Raffaele De Caterina (Pisa) focuses on vascular inflammation in diabetes. He shows that during hyperglycemia, stimulation of the receptor for advanced glycation endproducts (RAGE) induces local vascular inflammation. Unfortunately, insulin, the widely used treatment for patients with diabetes mellitus, stimulates the expression of adhesion molecules in the vasculature which may enhance macrovascular atherosclerotic disease.

Stefan Wiese at al. (Wuerzburg) review the role of neurotrophic factors for survival and differentiation of neural stem cells. With regard to heart disease, cardiotrophin and insulin-like growth factor I (IGF-I) appear to be important, as mutant mice deficient for these factors display degeneration of motoneurons as well as reduced heart function. Despite the encouraging results of the Repair-AMI-Trial (presented by Andreas Zeiher, Frankfurt), a randomized, double-blind placebo controlled trial showing improved left ventricular function in patients with acute myocardial infarction by bone marrow transplantation Anthony D. Ho (Heidelberg), disputes that there is any evidence for differentiation of adult stem cells from the bone marrow to relevant numbers of mature cardiomyocytes. He advocates for generation of more experimental evidence to answer numerous basic question before larger clinical trials are performed.

Taken together, the III. Leopoldina Symposium on Cardiovascular Healing covered a broad spectrum from basic principles of wounding and healing in the cardiovascular system to recent advances in the treatment of patients with myocardial infarction and heart failure. The fruitful collaboration of basic scientists and clinicians will help to identify novel therapeutic approaches to stimulate inflammatory reactions where necessary for healing processes, but to inhibit inflammation where it is detrimental to the cardiovascular system.

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Nova Acta Leopoldina NF *95*, Nr. 351, 11–13 (2008)

Welcome Address

Hubert E. Blum ML (Freiburg)

Member of the Presidium of the German Academy of Sciences Leopoldina

Esteemed Rector,
Esteemed colleagues Bauersachs, Frantz, Ertl, Kochsiek, Lohse and Walter,
Ladies and Gentlemen,

On behalf of the German Academy of Sciences Leopoldina and its President, professor Volker ter Meulen, I would like to welcome you most cordially today to the beginning of the symposium "Cardiovascular Healing – Focus on Inflammation", organized by the Medical Clinic and Polyclinic I of the Julius Maximilians University Wuerzburg and the German Academy of Sciences Leopoldina. I would like to take this opportunity to make you briefly familiar with the history and objectives of the Leopoldina and discuss its connections with cardiovascular diseases.

The German Academy of Sciences Leopoldina was founded in Schweinfurt in 1652 and shifted headquarters to Halle (Saale) in 1878. It is a supra-regional society of scholars and pursues non-profit tasks and objectives, such as interdisciplinary and transdisciplinary discussions in public symposia, meetings and talks, the setting up of working parties, the dissemination of scientific findings and the issuing of statements on issues of social relevance as advisor to the public and to political decision-makers. Another objective of the Leopoldina is to assist the professional careers of young researchers and to study the history of science. The Leopoldina is financed by the German government (Federal Ministry of Education and Research, BMBF, 80 %) and the State of Saxony-Anhalt (20 %).

The German Academy of Sciences Leopoldina has about 1200 members in all parts of the world. About three in four members are from Germany, Austria and Switzerland, and one in four come from over 30 other countries all over the world. Members of the Leopoldina are researchers with outstanding achievements in the natural sciences, medicine, the engineering sciences, cultural sciences, empirical humanities as well as behavioral and social sciences. The Leopoldina has 28 sections, of which the sections 14 "Human Genetics and Molecular Medicine", 15 "Physiology and Pharmacology/Toxicology" and 16 "Internal Medicine and Dermatology" are represented here.

The connection of the German Academy of Sciences Leopoldina with the symposium "Cardiovascular Healing – Focus on Inflammation" is based, for one, on the members of the Academy some of whom, through their work, have left indelible imprints in these disciplines and, for another, on the activities of the Leopoldina in this subject in papers, publications, biennial assemblies and symposia.

Well-known and outstanding members of the German Academy of Sciences Leopoldina who focused their work on the physiology and pathophysiology and the diseases of the heart and circulation included Giovanni Battista Morgagni (1682–1771, elected in 1708), who was the first to describe tricuspid valve diseases (stenosis) and the atrioventricular block of the heart; Antonio Scarpa (1752–1832, elected in 1780), who made very important contributions to the macroscopic and microscopic anatomy of the coronary arteriosclerosis; Jan Evangelista Purkinje (Purkyně) (1787–1869, elected in 1829), who, in 1846, discovered the Purkinje's fibers as peripheral fibers of the impulse conduction system of the heart; Carl Frhr. von Rokitansky (1804–1878, elected in 1856), pathologist, who proved in 1846 that cholesterol in the atheromatous plaques and the fat metabolism and nutritional habits contributes to the development of cardiovascular disease; Carl Friedrich Wilhelm Ludwig (1816–1895, elected in 1867), physiologist in Leipzig, who studied the cardiovascular system, mechanocardiography, the recording of blood pressure curves and the nervous control of cardiac activity; Wilhelm His junior (1863–1934, elected in 1922) discovered in 1893 the His bundle named after him as part of the atrioventricular impulse conduction system of the heart; Willem Einthoven (1860–1927, elected in 1925), physiologist, studied electro- and phonocardiography, coined the term "electrocardiogram" (ECG) and won the Nobel Prize for Physiology or Medicine in 1924; Karel Frederik Wenckebach (1864–1940, elected in 1925) dealt with cardiac arrhythmia and in 1927 authored the first book on arrhythmia; Ludwig Aschoff (1866–1942, elected in 1926) described the rheumatic nodule in rheumatic heart valve diseases in 1924 and was author of many papers on the histological characterization of endocarditis, the theory of normal and pathological heart function and the development of atherosclerosis and discovered the Aschoff-Tawara node in the impulse conduction system of the heart; Otto Loewi (1873–1961, elected 1932) discovered the chemical transfer of nerve impulses at cardiac nerves, and Walter Frey (1884–1972, elected in 1941) introduced quinidine as antiarrhythmic agent in the treatment of cardiac arrhythmia.

"Heart and Circulation" was the topic of several biennial assemblies and symposia. For example, the biennial assembly of 1963 "Neurophysiology as Seen Today" with a contribution by Bernd Lueken (1908–1978, elected in 1957) on "The Neurophysiology of Heart Regulation", the biennial assembly of 1967 "Biological Models" with a paper by Wilhelm Doerr (1914–1996, elected in 1965) on "The Physical Heart Model", the biennial assembly of 1985 "Singularities" with a paper by Gotthard Schettler (1917–1996, elected in 1971) on "The Myocardial Infarction" and the biennial assembly of 1989 "Anomalies" with a paper by Wilhelm Doerr on "Fundamental Laws of Pathogenesis" (with the Heart as model organ).

"Heart and Circulation" were also discussed at several meetings and symposia of the Leopoldina. For example, "Medicine in the Area of Conflict of Modern Methods" in Halle in 1996, with papers by Kurt Kochsiek (*1930, elected in 1989) on "Acquisition and Therapy of Electrophysiological Disorders of the Heart", by Udo Sechtem on "New Imaging Methods in Cardiac Diagnostics", by Wolfram Knapp (*1945, elected in 1998) on "Positron Emission Tomography of the Heart" and by Friedrich-Wilhelm Mohr (*1951, elected in 1999) on "New Techniques and Outlooks in Cardiac Surgery".

The III. Symposium on "Cardiovascular Healing – Focus on Inflammation" beginning today continues the tradition of the two extremely successful earlier symposia in Wuerzburg in 2004 on "Cardiovascular Healing" and in 2005 on "Cardiovascular Imaging". Following the example of earlier Leopoldina events, this symposium again addresses a very wide range of

topics, from the biomedical basics to pathophysiology and clinical applications. Very special thanks for the excellent preparation and organization of this symposium go to the Academy members Georg Ertl (*1950, elected in 2002), Kurt Kochsiek (*1930, elected in 1989), Martin J. Lohse (*1956, elected in 2000) and Ulrich Walter (*1949, elected in 2004) as well as Mr. Bauersachs and Mr. Frantz.

On behalf of the German Academy of Sciences Leopoldina and by its President, professor Volker ter Meulen, I wish you now an interesting and successful conference, with new information and lively discussions as well as opportunities for personal meetings and talks.

Acknowledgement

I am deeply indebted to Dr. Michael Kaasch, editorial staff of Nova Acta Leopoldina, German Academy of Sciences Leopoldina, for his search of data from the history of medicine.

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Role of Immunity in Heart Failure

Nova Acta Leopoldina NF *95*, Nr. 351, 17–20 (2008)

Innate Immunity in Heart Failure

Stefan Frantz, Georg Ertl ML, and Johann Bauersachs (Wuerzburg)

With 2 Figures

Abstract

The immune system of higher vertebrates consists of two components: the innate and adaptive immunity. While the adaptive immune system relies on somatically generated and clonally selected antigen receptors, the innate immune system detects the presence of pathogens by their evolutionarily highly conserved, relatively invariant structural motifs. Various components of the innate immune system are activated in cardiac diseases without direct involvement of infectious pathogens. For example, a number of inflammatory cytokines, including TNF (tumor necrosis factor), IL (interleukin)-1β, IL-6 and IL-8, as well as iNOS (inducible nitric oxide synthase), all components of innate immunity, are increased after cardiac injury. Moreover, they are all functionally implicated in ischemia/reperfusion injury, and in the abnormal myocardial remodeling characteristic of advanced congestive heart failure. Additionally, downstream targets of these proteins, the transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), are activated in cardiac injury. Thus, an understanding of the regulation and activation of the innate immune system in diseases not obviously related to an immune response to specific pathogens could provide new therapeutic targets for cardiovascular diseases.

Zusammenfassung

Das Immunsystem höherer Vertebraten besteht aus zwei Komponenten, dem angeborenen und dem adaptiven Immunsystem. Während die adaptive Immunität die Neuproduktion und klonale Selektion von Antigen-Rezeptoren beinhaltet, detektiert die angeborene Immunität invariable Strukturen, die nicht nur Bestandteil eines Pathogens sind, sondern einer ganzen Pathogenklasse zugeordnet werden können. Verschiedene Komponenten des Systems der angeborenen Immunität sind bei kardialen Erkrankungen aktiviert, auch ohne dass direkt eine Infektion nachgewiesen werden kann. Zum Beispiel werden eine ganze Reihe von Zytokinen, wie Tumor-Nekrose-Faktor (TNF) oder Interleukin (IL) 1β, aber auch die induzierbare NO-Synthase (iNOS) nicht nur vermehrt exprimiert, sondern haben auch funktionelle Relevanz nach kardialer Schädigung. Ein Großteil der Zytokine und Effektorproteine der angeborenen Immunität führt zu einer Aktivierung der Transkriptionsfaktoren *Nuclear Factor kappa B* (NF-κB) und Aktivatorprotein 1 (AP-1), die ebenfalls nach kardialer Ischämie aktiviert sind. Somit könnte ein besseres Verständnis der Aktivierung der angeborenen Immunität in Krankheiten, die primär nicht mit einer Infektion in Verbindung gebracht werden, zur Generierung neuer therapeutischer Möglichkeiten beitragen.

1. Introduction

Recent data implicate a number of inflammatory stimuli, including cytokines like tumor necrosis factor (TNF) and interleukin (IL)-1β in myocardial depression and the abnormal myocardial remodeling characteristic of congestive heart failure. Since there is no evidence for an infectious pathogen in most pathophysiologic states associated with heart failure except infectious myocarditis, the proximal events triggering cytokine expression are not well understood. However, all of these proinflammatory proteins are part of the "innate immune" response.

2. Innate Immunity

Immunity to infections is mediated by two distinct pathways: the innate and the acquired immune response. Whereas acquired immunity recognizes specific antigens by distinct antigen receptors based on clonal selection of B and T cells, innate immunity recognizes specific invariant patterns shared by groups of microorganisms, but not by the host. For example all different lipopolysaccharides (LPS), components of the outer bacterial wall, can be recognized by a single innate immune receptor, toll like receptor 4 (TLR). By consecutive production of costimulatory molecules (e.g. B7.1, B7.2) as well as effector cytokines, the innate immune system can then activate the adaptive immune response (Frantz et al. 2005).

We have hypothesized that the innate immune system in cardiac diseases is activated analogous to the immune system (see Fig. 1). Upon a cardiac injury, ligands are generated, like collagen, uric acid, heat shock proteins, etc. Those ligands can bind to the specific innate immune receptors and then activate innate immune specific transcription factors, like NF-κB or AP-1. This leads then to the production of cytokines, matrix-metalloproteinases, etc., all factors implicated in cardiac remodeling.

Toll like receptors (TLRs) have recently been recognized as the most important innate immune receptors (Medzhitov and Janeway 1997). TLR 2 and 4 are critical components of the bacterial lipoprotein and lipopolysaccharide signaling pathway, respectively. TLRs induce

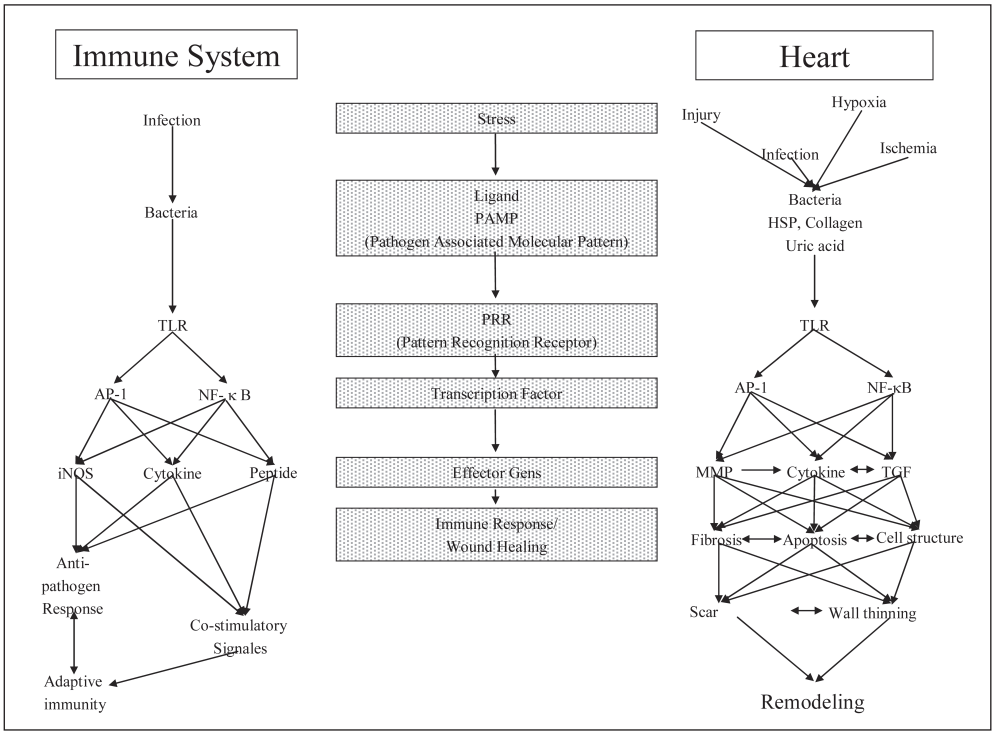

Fig. 1 Parallel activation of the innate immune system in the heart and in the immune system. Reprinted from *Current Pharmaceutical Design* (Frantz et al. 2005); Copyright 2005, with permission from Bentham Science Publishers Ltd.

NF-κB, IL-1β, IL-6, IL-8, and the costimulatory molecule B7.1 (Medzhitov et al. 1997). Indeed, these important immune receptors are expressed in the heart (Frantz et al. 1999). TLR4 KO mice have reduced ischemia reperfusion injury (Oyama et al. 2002), indicating that TLRs could play an important role in the response to cardiac injury.

The signal transduction cascade of TLRs is similar to that employed by IL-1 and IL-18 with a final activation of NF-κB. Thus, should IL and TLR signaling be important in myocardial ischemia, also the transcription factor NF-κB should be activated. Indeed, we could demonstrate an activation of NF-κB in rats with chronic myocardial infarction (Frantz et al. 2003, Tillmanns et al. 2006) (see Fig. 2.). This has also functional importance, since prevention of NF-κB activation in mice with targeted deletion of the NF-κB subunit p50, was

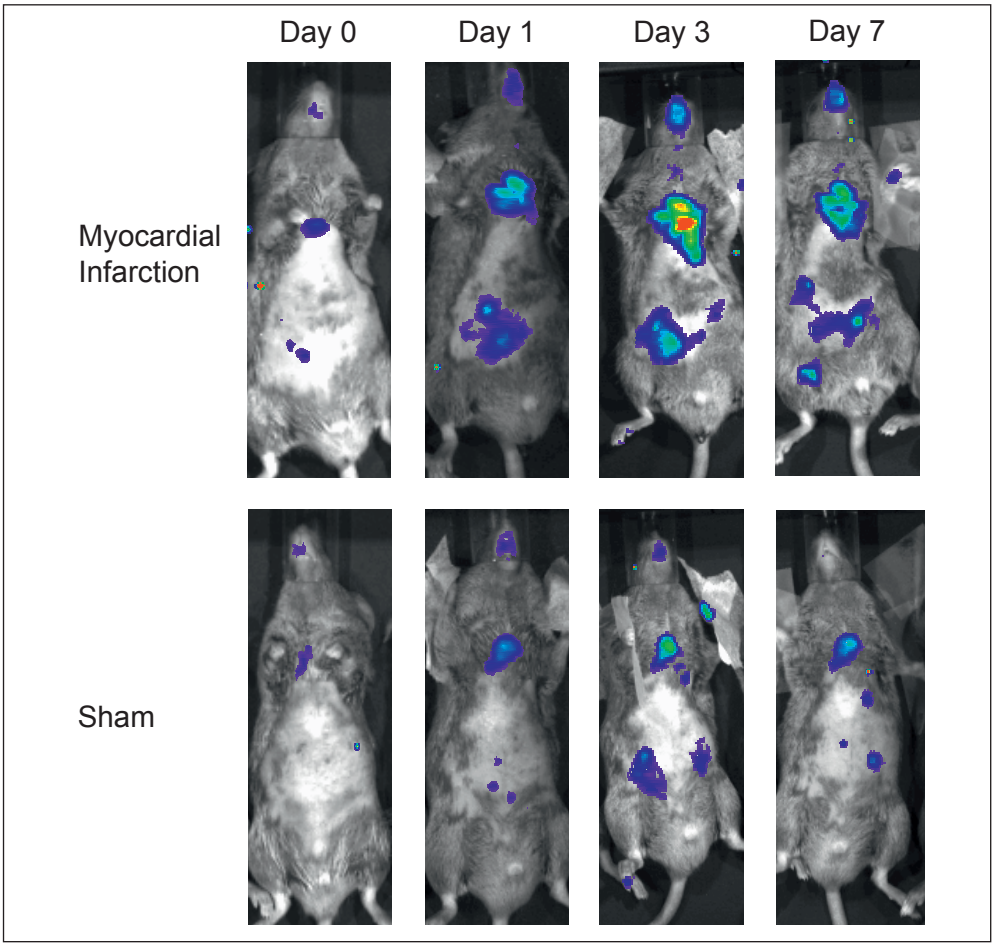

Fig. 2 Myocardial infarction induces NF-κB dependent luminescence in the heart of transgenic mice when compared to sham-operated mice. Maximal NF-κB activity was observed 3 days after myocardial infarction by serial molecular imaging. Reprinted from *Biochemical and Biophysical Research Communications* (Tillmanns et al. 2006); Copyright 2006, with permission from Elsevier.

associated with significantly improved left ventricular remodeling after myocardial infarction (Frantz et al. 2006).

In conclusion, cardiac injury activates pro-inflammatory proteins that are part of the innate immune response. The components of the innate immune system are expressed in the heart and have functional importance. Thus, a better understanding of the regulation and activation of the innate immune system in the heart could provide new therapeutic targets for cardiovascular diseases.

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Insights into Molecular Mechanisms of Cardiac Remodeling Gained by Heart-specific Inhibition of Transcription Factor NF-κB

Martin W. Bergmann and Rainer Dietz (Berlin)

With 1 Figure

Abstract

On a cellular basis, left ventricular (LV) remodeling leading to heart failure occurs by hypertrophy and apoptosis of cardiomyocytes. NF-κB is a ubiquitous transcription factor known to control gene expression of a plethora of inflammatory cytokines, adhesion molecules and chemokines. In addition, *in vitro* studies have suggested NF-κB to be implicated in cardiomyocyte hypertrophy and protection from apoptosis. In order to study this factor *in vivo*, we generated a mouse model with cardiomyocyte-restricted NF-κB inhibition employing the Cre/lox technique.

No basal phenotype was observed in mice with inhibition of NF-κB in cells expressing α-myosin heavy chain (MHC). Infusion of angiotensin II for 14 days induced cardiac hypertrophy and focal perivascular inflammation in control mice. Echocardiography, histology and gene expression analysis revealed AngII-induced hypertrophy to be abrogated in mice with cardiomyocyte-restricted NF-κB inhibition. However, perivascular inflammation was unchanged. This finding indicates that NF-κB is involved in cardiomyocyte hypertrophy upon AngII challenge but that cardiomyocyte-secreted cytokines are not involved in AngII-induced perivascular inflammation. Furthermore, no increased apoptosis was observed in mice with NF-κB inhibition. In order to identify the signaling pathways upstream of NF-κB, isolated adult rat cardiomyocytes were analyzed. AngII lead to a modest increase in NF-κB DNA binding much less than the positive control, TNF-α stimulation. However, AngII was found to stimulate IL-6 cytokine release from cardiac fibroblasts through MAPK ERK and transcription factor CREB activation. IL-6 cytokines, which are also implicated in the overall inflammatory response, potently induced NF-κB DNA binding in cardiomyocytes. Furthermore, gene array expression analysis revealed the IL-6-receptor gp130 to be regulated by NF-κB possibly giving insights into the molecular mechanism responsible for the effect of NF-κB on cardiac hypertrophy. In summary, NF-κB inhibition in cardiomyocytes exerts beneficial effects on cardiac remodeling upon AngII challenge: (*i*) inhibition of cardiomyocyte hypertrophy without increased apoptosis despite the documented *in vitro* role for NF-κB as a survival factor; (*ii*) inhibition of hypertrophy induced directly by AngII stimulation as well as by indirect secretion of IL-6 cytokines; (*iii*) no effect on perivascular inflammation is observed. NF-κB inhibition thus confirms the concept of targeting intracellular signaling cascades downstream of receptors activated by inflammatory peptides and cytokines including AngII and IL6 in order to block cardiac remodeling leading to heart failure.

Zusammenfassung

Auf zellulärer Ebene begegnet das Herz einer zusätzlichen Belastung durch Bluthochdruck oder einem Verlust an Zellmasse, wie bei einem Infarkt, durch die Hypertrophie der erhaltenen Kardiomyozyten. Diesem Prozess steht der Verlust an Kardiomyozyten durch den programmierten Zelltod, die Apoptose, gegenüber. Der Transkriptionsfaktor NF-κB ist im Rahmen von *In-vitro*-Versuchen mit beiden Prozessen in Verbindung gebracht worden: NF-κB reguliert einige Überlebensfaktoren, die der Apoptose entgegenwirken – andererseits ließ sich durch Inhibition von NF-κB auch die Hypertrophie von Kardiomyozyten unterdrücken. Um den Effekt einer NF-κB-Inhibition *in vivo* zu überprüfen, wurde ein transgenes Mausmodell mit herzpezifischer NF-κB-Inhibition unter Verwendung des Cre/lox-Verfahrens etabliert.

Die Mäuse zeigten keinen basalen Phänotyp. Nach Infusion von Angiotensin II als Hypertrophiestimulus zeigten Kontrollmäuse eine deutliche Verdickung der Herzwände, eine Zunahme der Zelloberfläche sowie eine Reexpression fetaler Gene. Diese Veränderungen waren in den Tieren mit NF-κB-Inhibition signifikant gedämpft. Die durch Angiotensin II induzierte, perivaskuläre Entzündungsreaktion war hingegen durch die NF-κB-Inhibition nicht beeinflusst. Diese Beobachtungen implizieren, dass die auf Kardiomyozyten beschränkte Inhibition von NF-κB die

myokardiale Hypertrophie, nicht jedoch die Apoptose oder die Entzündungsreaktion beeinflusst. Um die Signalpfade zu untersuchen, die die NF-κB-Aktivierung in Kardiomyozyten steuern, wurden isolierte Kardiomyozyten untersucht. Diese zeigten nach AngII eine leichte NF-κB-Aktivierung, hingegen zeigten IL-6-Zytokine eine starke Aktivierung. Es ist bekannt, dass Angiotensin II die Freisetzung von IL-6-Zytokinen aus Fibroblasten stimuliert. DNA-Genchip-Experimente erwiesen zudem, dass die Expression des IL-6-Rezeptors gp130 in Zellen mit NF-κB-Inhibition deutlich erniedrigt war. Zusammengefasst zeigen diese Untersuchungen, dass die NF-κB-Inhibition zu einer Blockade der Hypertrophie nach Angiotensin II ausreicht, ohne eine gesteigerte Apoptose-Rate zu induzieren. Dabei wird NF-κB direkt durch Angiotensin II, aber auch indirekt durch IL-6-Zytokine aus den umliegenden Fibroblasten stimuliert. Diese Beobachtungen unterstützen das Konzept, die myokardiale Hypertrophie als Vorstufe der Herzinsuffizienz möglichst gezielt zu unterdrücken, da die Apoptose von Kardiomyozyten erst im späteren Stadium der Herzschwäche an Bedeutung gewinnt.

1. Molecular Signal Transduction Pathways in Heart Failure Development

Heart failure has become the major reason for disability in the elderly population. Also, treatment of advanced heart failure and related clinical symptoms has become the single most costly medical syndrome in cardiology (for a reference, see *ESC Guidelines* "Heart failure"). Current treatment has been developed clinically rather than by modern molecular medicine approaches. New treatment strategies first warrant a better understanding of the cellular signal transduction pathways involved in the development of heart failure.

From a pathophysiological point of view, maladaptive myocardial hypertrophy and cardiomyocyte apoptosis precede heart dilation and functional failure both in ischemic heart disease as well as in the second most common form, heart failure related to chronic arterial hypertension. The genetic mechanisms involved in the development of heart failure include an increase in myocardial mass as well as an alteration of gene expression also involving survival gene expression. Recent investigation has centered on the identification of the molecular pathways that initiate and perpetuate cardiac hypertrophy in view of the potential for rational drug design. Specifically, targeting cardiac hypertrophy would allow to prevent the development of heart failure much earlier than current therapeutic approaches.

On a cellular level, left ventricular (LV) remodeling induced by tissue damage through ischemia/reperfusion or pressure overload involves interstitial fibrosis, cardiomyocyte apoptosis and hypertrophy (Michael et al. 1999). Activation of signaling cascades through angiotensin II (AngII) and catecholamines like isoproterenol induce cardiac hypertrophy, deterioration of LV function and heart failure (Oishi et al. 2003). The molecular pathways of cardiac hypertrophy after pathophysiological stress mediated through stimulation of G-protein receptors have been extensively characterized (Haq et al. 2001, Zhang et al. 2003b). Ca^{2+}-induced activation of calcineurin followed by nuclear translocation of the transcription factor NF-AT integrates several important upstream pathways (De Windt et al. 2001, Molkentin et al. 1998). In addition, phosphorylation of the serin/threonin kinase AKT (Condorelli et al. 2002) as well as activation of the mitogen activated protein kinases ERK and p38 (Clerk et al. 2001) were shown to be essential in this process. Recently published data further implicate glycogen synthase kinase 3 β (GSK3β), Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) (Zhang et al. 2003a) as well as class II histone deacetylases (Zhang et al. 2002) in cardiac hypertrophy.

Downstream of these signaling molecules several transcription factors were found to contribute to cardiac remodeling including NFAT (Molkentin et al. 1998), GATA 4 (Morisco et al. 2001), CREB (Fentzke et al. 1998), NF-κB (Purcell et al. 2001), STAT 3 (Tone et

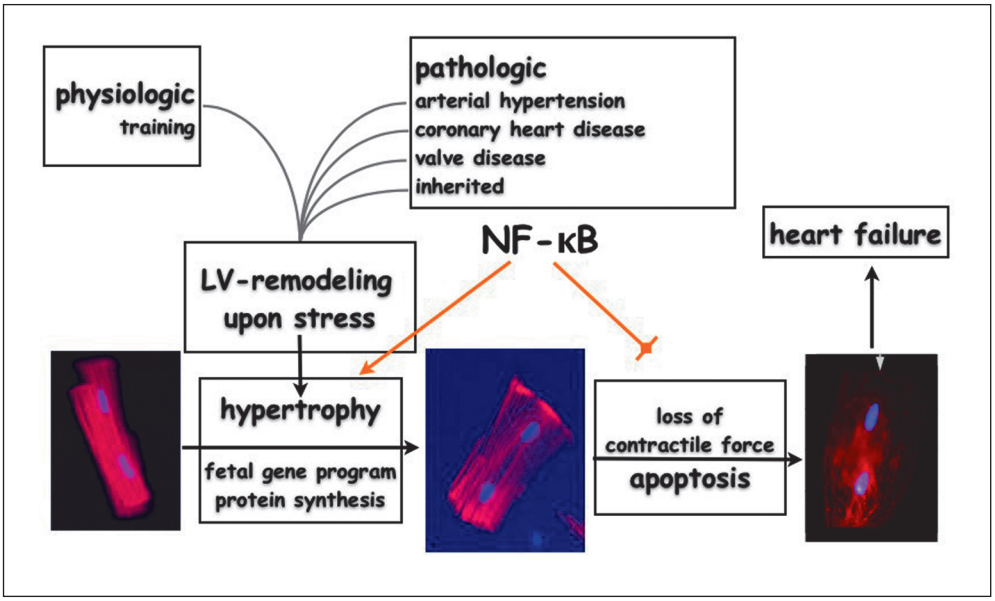

Fig. 1 Pathogenesis of heart failure: cellular level

al. 1998), β-catenin (Haq et al. 2003) and MEF2 (Passier et al. 2000). Genetic mouse models demonstrated rapid development of heart failure after heart-specific deletion of CREB (Fentzke et al. 1998) or the STAT3-activating receptor gp130 (Hirota et al. 1999) after pathophysiological stress. It is currently unclear, which of the above mentioned signaling pathways may be a suitable target for pharmaceutical intervention preventing pathophysiological cardiac remodeling resulting in heart failure, while leaving adaptive pathways intact.

The signaling pathways involved in cardiac hypertrophy resemble those of cell proliferation. As adult cardiomyocytes are irreversibly post-mitotic, cell size, protein content and numbers of nuclei increase without a change in absolute cell numbers. Cyclin-dependent kinases, histone deacetylases, members of the 14-3-3 family of proteins as well as RNA helicases have all been implicated in cardiomyocyte hypertrophy (Liu et al. 2002, McKinsey et al. 2000, Vega et al. 2003). As inhibition of cardiac hypertrophy was demonstrated to prevent the development of heart failure despite continuous stress signals, intensive efforts were recently directed towards the identification of new molecular targets to inhibit cardiomyocyte hypertrophy (Esposito et al. 2002).

2. Role of Transcription Factor NF-κB and CREB in Left Ventricular Remodeling

The group focuses on the role of specific transcription factors in cardiac hypertrophy, namely CREB and NF-κB. While NF-κB did not influence hypertrophy induced by hypoxia/reoxygenation in isolated rat neonatal cardiomyocytes, GPCR-mediated cardiomyocyte hypertrophy was abrogated *in vivo* (El Jamali et al. 2004).

2.1 Hypertrophy Induced by Hypoxia/Reoxygenation In Vitro Is Linked to CREB Activation Downstream of GSK3β

In vivo, left ventricular remodeling after myocardial infarction involves ventricular hypertrophy generally attributed to increased cardiac workload. We hypothesized hypoxia/reoxygenation to directly induce cardiomyocyte hypertrophy and studied several kinases and transcription factors in isolated cardiomyocytes possibly involved.

Hypoxia for 6 h followed by 42 h reoxygenation induced cardiomyocyte hypertrophy as assessed by 3H leucine incorporation and immunohistochemical analysis. Pharmacological inhibition of reactive oxygen species (ROS), serine/threonine kinase AKT and ERK abolished reoxygenation-induced hypertrophy. In addition, a β2-adrenergic receptor (β2-AR) antagonist as well as Gi inhibitor pertussis toxin blocked reoxygenation-induced hypertrophy. Hypoxia for 6 h increased transcription factors CREB, NF-κB, and GATA DNA-binding activities. However, only CREB DNA binding was sustained during reoxygenation. Inhibition of PI3-kinase, ERK and PKA abrogated reoxygenation-induced CREB DNA binding without affecting CREB serine-133 phosphorylation. These same pathways were found to regulate hypoxia/reoxygenation-induced GSK3β kinase activity and CREB serine-129 phosphorylation. GSK3β mutants resistant to phosphorylation blocked the stimulation of CRE-dependent transcription induced by hypoxia/reoxygenation. Transfection of cardiomyocytes with a dominant negative mutant of CREB abrogated hypoxia/reoxygenation-induced hypertrophy.

These data suggest that hypoxia/reoxygenation induces cardiomyocyte hypertrophy through activation of CREB. Inactivation of GSK3β by hypoxia/reoxygenation possibly integrating PI3-kinase and ERK pathways downstream of β2-AR and ROS, is a prerequisite for CRE-dependent transcription. Transient hypoxia may contribute to cardiac hypertrophy in ischemic heart disease independent of cardiac workload.

2.2 Requirement of NF-κB in Angiotensin II and Isoproterenol Induced Cardiac Hypertrophy

In vitro experiments have proposed a role of transcription factor NF-κB in cardiomyocyte hypertrophy as well as protection against apoptosis. The net effect on cardiac remodeling *in vivo* under common stress stimuli is currently unclear.

We have generated mice with cardiomyocyte-restricted expression of the NF-κB super-repressor IκBαΔN (ΔN^{MHC}) by use of the Cre/lox technique. ΔN^{MHC} mice displayed an attenuated hypertrophic response compared to control mice upon infusion of angiotensin II (AngII) or isoproterenol (Iso) by micro-osmotic pumps, as determined by echocardiography (left ventricular wall dimensions: control+AngII: × 1.5±0.1 fold vs. sham; DNMHC+AngII: × 1.1±0.1 vs. sham, $P<0.05$, $n\geq9$), heart weight and histological analysis. Real-time RT-PCR showed significantly reduced expression of hypertrophy markers β-myosin heavy chain (MHC) and atrial natriuretic peptide (ANP) in AngII-treated ΔN^{MHC} mice ($P<0.05$ vs. control+AngII, $n=4$). Neither cardiomyocyte apoptosis nor left ventricular dilatation was observed. In cultured adult rat cardiomyocytes, NF-κB DNA-binding activity was increased by both, AngII and IL-6 related cytokines. The latter are known to be released by cardiac fibroblasts upon AngII stimulation and could thus locally increase the NF-κB response of cardiomyocytes. Finally, results from *in vitro* and *in vivo* experiments suggest a role of NF-κB in the regulation of prohypertrophic IL-6 receptor gp130 on mRNA and protein levels.

These results indicate that targeted inhibition of NF-κB in cardiomyocytes *in vivo* is sufficient to impair AngII- and Iso-induced hypertrophy without increasing the susceptibility to apoptosis.

3. Conclusion

These data support the notion, that inhibiting the first step towards heart failure development, namely cardiac hypertrophy, is sufficient to abrogate progression to heart failure. Especially regarding the double effect of NF-κB on apoptosis and hypertrophy, the presented data emphasize the resistance of not-hypertrophied cardiomyocyte to external stress.

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Cytokines and Heart Failure

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Abstract

The involvement of cytokines and chemokines in heart failure (HF) is based on strong and different evidences both on experimental and clinical grounds. The roles played by cytokines are various and somewhat contrasting. Cytokines such as TNFα or IL-6 are elaborated and secreted soon after myocardial infarction (MI) and can influence cardiac myocyte survival/death by apoptosis or necrosis. Chronically cytokines can mediate repair and remodeling through activation of matrix metalloproteinases, collagen deposition and regulate integrins and angiogenesis and endothelial progenitor cell mobilization (i.e. VEGF, SDF-1, EPO, G-CSF, GM-CSF). Accordingly, the actions of inflammatory cytokines can be favourable (= healing) or unfavourable (= cardiac rupture, negative inotropism, chamber dilation). Cytokines may exert their negative or positive effects on cardiac myocytes either directly or indirectly, mediated by their action on vascular cells. In later stages of HF, cytokines may play a role in the development of cardiac cachexia through impairment of the metabolism of skeletal muscle. Elevated circulating concentrations of cytokines such as TNFα, IL-6, CRP and other acute phase reactants have been reported to predict unfavourable outcome in patients with HF. More recently high CRP, TNFα and IL-6 have been shown to predict the propensity to develop HF. Even if the involvement of inflammatory/immune response in acute myocardial infarction and HF pathophysiology has been studied/documented along the last 4 decades, still no single molecule specifically targeted to inhibit inflammatory cytokines has been approved for clinical use yet. The recent failure of etanercept, a protein specifically directed against TNFα, in HF highlights the complexity of trying to inhibit the pleiotropic cytokine pathways. A previous relevant failure was vesnarinone, a PDE inhibitor found to significantly decrease circulating TNFα, but ineffective in improving survival of patients with HF (VEST trial). The reporting of ancillary anti-inflammatory anti-cytokine actions of different classes of drugs beneficial in ischemic and cardiac disease and HF such as RAAS inhibitors, beta-blockers and statins does not allow to conclude that anti-inflammatory action is beneficial. On the other hand, the lack of efficacy of anti-inflammatory drugs does not imply that inflammation is not involved in the pathophysiology of HF.

Zusammenfassung

Für die Beteiligung von Zytokinen und Chemokinen am Herzversagen liegen aussagekräftige und differenzierte Beweise aus experimentellen und klinischen Quellen vor. Zytokine spielen dabei verschiedene und teils widersprüchliche Rollen. Zytokine, wie z. B. TNFα oder IL-6, werden bereits in der frühen Phase nach dem Herzinfarkt produziert und abgesondert und können das Überleben oder Absterben der Kardiomyozyten durch Apoptose bzw. Nekrose beeinflussen. In chronischen Zuständen können Zytokine Reparatur- und Remodellierungsprozesse durch Aktivierung von Matrix-Metalloproteinasen und Kollagenablagerung vermitteln bzw. Integrine und Angiogenese sowie die Mobilisierung von Endothel-Progenitorzellen (d. h. VEGF, SDF-1, EPO, G-CSF, GM-CSF) regulieren. Dementsprechend können die Funktionen der Entzündungszytokine günstig (= heilend) oder ungünstig (= Herzruptur, negative Inotropie, Kammererweiterung) sein. Zytokine können ihre negative/positive Wirkung auf Kardiomyozyten entweder direkt oder indirekt, über Beeinflussung von Gefäßzellen, ausüben. In späteren Phasen des Herzversagens spielen wahrscheinlich Zytokine durch Störung des Skelettmuskelstoffwechsels eine Rolle bei der Entstehung der Kardiokachexie. Nach vorliegenden Berichten deuten erhöhte Werte von Zytokinen, z. B. von TNFα, IL-6, CRP, und anderen Akutphasenreaktanten im Kreislauf auf einen ungünstigen Ausgang bei Patienten mit Herzversagen hin. In jüngster Zeit konnte auch nachgewiesen werden, dass hohe Werte von CRP, TNFα and IL-6 auf eine verstärkte Neigung zur Ausbildung von Herzversagen hinweisen. Obwohl der Einfluss der Entzündungs-/Immunreaktion bei akutem Myokardinfarkt auf die Pathophysiologie des Herzversagens seit 40 Jahren untersucht/dokumentiert wird, steht noch immer kein Einzelmolekül für die gezielte Inhibierung von Entzündungszytokinen zum klinischen Einsatz zur Verfügung. Die jüngsten ne-

gativen Erfahrungen mit Etanercept, einem Protein mit spezifischer Wirkung auf TNFα, bei Herzversagen unterstreichen die Komplexität der Wege zur Inhibierung pleiotroper Zytokine. Ähnlich negative Resultate wurden zuvor mit Vesnarinon erzielt, einem PDE-Inhibitor, der zu einer signifikanten Senkung des zirkulierenden TNFα führte, jedoch wirkungslos bei der Verbesserung der Überlebenschancen der Patienten mit Herzversagen blieb (VEST-Versuch). Die vorliegenden Berichte über Antientzündungs- und Antizytokin-Nebeneffekte unterschiedlicher Klassen von Medikamenten mit positiven Wirkungen bei Ischämie und Herzkrankheit sowie Herzversagen, z. B. RAAS-Inhibitoren, Beta-Blocker und Statine, gestatten nicht den Schluss, dass die entzündungshemmende Wirkung sich wirklich vorteilhaft auswirkt. Andererseits bedeutet die fehlende Wirksamkeit der entzündungshemmenden Präparate nicht, dass Entzündungen bei der Pathophysiologie des Herzversagens keine Rolle spielen.

1. Introduction

Heart failure (HF) is a complex disorder in which different systems participate. The pathophysiologic role of neurohormones in the progression of HF has been firmly established leading to new treatment modalities such as angiotensin converting enzyme (ACE) inhibitors and beta-blockers. However, despite best recommended therapies, chronic HF is a progressive disease with high morbidity and mortality, suggesting that important pathogenic mechanisms remain unmodified by the present treatment modalities.

Persistent inflammation may represent such unmodified mechanisms. Thus, since the initial observation by Levine et al. (1990), numerous studies have demonstrated that HF patients have raised plasma/serum levels of inflammatory cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6 as well as several chemokines, e.g. monocyte chemoattractant peptide (MCP)-1, IL-8 and macrophage inflammatory protein (MIP)-1α. Importantly, the rise in inflammatory mediators seems not to be accompanied by a corresponding increase in anti-inflammatory cytokines such as IL-10, resulting in an inflammatory net effect. These inflammatory mediators are not only increased in the circulation, but enhanced expression has also been found within the failing myocardium (Mann 2002).

The involvement of cytokines and chemokines in HF is based on strong and different evidences both on experimental and clinical grounds.

The roles played by cytokines are various and somewhat contrasting. Cytokines such as TNFα, IL-1 or IL-6 are elaborated and secreted soon after myocardial infarction and can influence cardiac myocyte survival/death by apoptosis or necrosis. Chronically, cytokines can mediate repair and remodeling through activation of matrix metalloproteinases, collagen deposition and regulate integrins and angiogenesis and endothelial progenitor cell mobilization (i.e. VEGF, SDF-1, EPO, G-CSF, GM-CSF). Accordingly, the actions of inflammatory cytokines can be favorable (= healing) or unfavorable (= cardiac rupture, negative inotropism, chamber dilation).

Inflammatory cytokines may exert their effects on cardiac myocytes either directly (Finkel et al. 1992) or indirectly, mediated by their action on vascular cells (Barry et al. 1994, Bozkurt et al. 1998).

In later stages of HF, cytokines may play a role in the development of cardiac cachexia through impairment of the metabolism of skeletal muscle, tissue wasting and weight loss.

2. Circulating Cytokines as Prognostic Markers in HF

Several studies have reported raised plasma concentrations of inflammatory cytokines and chemokines in direct relation to deterioration of functional class (i.e. *New York Heart Asso-*

ciation Classification) and left ventricular function (Aukrust et al. 1998, 1999, Damås et al. 2000). Elevated circulating concentrations of cytokines such as TNFα, IL-1, IL-6 have been reported to predict unfavorable outcome in patients with HF (Torre-Amione et al. 1996). For example, in a large population of HF patients (1200 patients, the cytokine database from the Vesnarinone trial) circulating levels of inflammatory cytokines (i.e. TNFα and IL-6) and cytokine receptors (i.e. soluble TNF receptors [TNFRs]) were found to be independent predictors of mortality in patients with advanced HF (Deswal et al. 2001). These clinical data further support the notion that raised levels of cytokines in HF patients are not only epiphenomena, but may reflect important pathogenic mechanisms in these patients.

3. Origins of Elevated Cytokines in HF

Several hypotheses have been suggested to describe the origin of immune activation in HF. The production of pro-inflammatory cytokines has mostly been attributed to secretion by mononuclear cells, although the myocardium seems to be another important source. Some evidence suggests that catecholamines augment this myocardial cytokine production. The concepts trying to explain increased production of pro-inflammatory mediators comprize response to myocardial injury and underperfusion of peripheral tissues. It has been proposed that increased bowel wall oedema causes translocation of bacterial endotoxin (LPS) from the gut which eventually yields pro-inflammatory cytokine (TNFα in particular) production from monocytes in the bloodstream and possibly other tissues. Attempts to prove any of the aforementioned hypotheses, however, yielded only indirect evidence. Thus, the elevation of the plasma concentrations of several pro-inflammatory mediators in acute myocarditis and acute myocardial infarction may hint towards the tissue injury hypothesis. Also, peripheral IL-6 spillover was found to be increased in patients with HF when comparing arterial and venous plasma concentrations, thus indicating a peripheral cytokine production. This, however, has not been demonstrated for TNFα. In contrast with the "infectious hypothesis" stays the lack of benefit from antimicrobial therapy in HF and in atherosclerosis.

4. Downstream of Cytokines, Acute Phase Proteins in HF

The most widely known member in cardiovascular medicine is the short pentraxin C-reactive protein, which by specifically binding to bacterial polysaccharides plays a role in innate immunity (Pepys and Berger 2001).

It has been shown that human C-reactive protein (CRP), the classical acute-phase protein that binds to ligands exposed in damaged tissue and then activates complement, increases myocardial and cerebral infarct size in rats subjected to coronary or cerebral artery ligation, respectively. Whether these effects in acute settings may be relevant also under chronic conditions such as those found in HF is not fully understood. However, CRP is elevated in patients with HF, and there is a direct relationship between elevated plasma CRP and the progression of HF. Higher plasma CRP is associated with worse hemodynamic and neurohormonal profile and a poorer quality of life. CRP is an independent but a relatively weak predictor for adverse clinical outcomes, independent of ischemic/non-ischemic etiology, and measurement of CRP

may provide additional prognostic information as an adjunct for global risk assessment in patients with HF (Anand et al. 2005).

In community studies, plasma CRP predicted the development of HF and other adverse events (Ridker et al. 2000).

A long pentraxin, PTX3, has been recently found to be elevated in chronic HF. Pentraxin-3 (PTX3) is a prototypic long pentraxin produced mainly by dendritic cells, macrophages, and endothelial cells in response to primary inflammatory stimuli. In rodents, after systemic administration of microbial products and inflammatory cytokines or ligation of the left coronary artery to model acute MI, PTX3 is expressed at very high levels in the heart. Moreover, PTX3 is present in atherosclerotic lesions and in small-vessel vasculitis in humans, and it is induced by oxidized LDL in smooth muscle cells (Mantovani et al. 2006).

PTX3 was shown to peak in plasma around 7 hours after the onset of symptoms in patients with MI and to decrease thereafter toward baseline in a few days. In the same context, plasma CRP increased, but it peaked much later, between 24 and 48 hours after symptom onset. Because the short pentraxin CRP is produced mainly by the liver in response to IL-6 and PTX3 by the heart and vasculature in response to primary inflammatory stimuli, we hypothesized that PTX3 could be an acute-phase reactant more closely related than CRP to cardiac injuries such as myocardial infarction and therefore could be a sensitive and specific prognostic indicator in this context (Latini et al. 2004).

5. Therapeutic Applications

Even if the involvement of inflammatory/immune response in acute myocardial infarction and HF pathophysiology has been studied/documented along the last 4 decades, still no one single molecule specifically targeted to inhibit inflammatory cytokines has been approved for clinical use yet.

The recent failure of etanercept, a protein specifically directed against TNFα, in HF highlights the complexity of trying to inhibit the pleiotropic cytokine pathways.

A previous relevant failure was vesnarinone, a PDE inhibitor found to significantly decrease circulating TNFα, but ineffective in improving survival of patients with HF (VEST trial).

The reporting of ancillary anti-inflammatory anti-cytokine actions of different classes of drugs beneficial in ischemic and cardiac disease and HF such as RAAS inhibitors, beta-blockers and statins does not allow to conclude that anti-inflammatory action is beneficial. On the other hand, the lack of efficacy of anti-inflammatory drugs does not imply that inflammation is not involved in the pathophysiology of HF.

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Heart Failure is a Neurohumoral Disease

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With 3 Figures

Abstract

Progression of heart failure is mediated largely via persisting activation of various neuroendocrine systems. We show a striking improvement of healing processes by selective mineralocorticoid receptor blockade after experimental myocardial infarction (MI). In rats treated with the selective aldosterone receptor antagonist eplerenone starting immediately after coronary ligation, left ventricular dilatation was significantly improved. Macrophage infiltration in the infarcted LV myocardium of eplerenone-treated rats was significantly greater at 48 hours, but significantly reduced at 5 days after MI. Eplerenone treatment increased neovascularization, and was associated with significantly increased Factor XIII in the infarct zone. In rats early after large MI we demonstrate reduced mobilization of endothelial progenitor cells (EPC) and elevated markers of oxidative stress within the bone marrow. Statin treatment and ACE inhibition increased circulating EPC levels, and were associated with improvement of bone marrow molecular alterations.

Zusammenfassung

Die Progression der Herzinsuffizienz wird vor allem durch die persistierende Aktivierung verschiedener neuroendokriner Systeme getriggert. Wir beobachteten eine deutliche Verbesserung der frühen Heilungsprozesse durch sofortige selektive Mineralokortikoidrezeptor-Blockade bei Ratten mit Myokardinfarkt (MI). Durch unmittelbare Behandlung mit dem selektiven Aldosteronantagonisten Eplerenon nach Koronarligatur wurde die linksventrikuläre Dilatation signifikant vermindert. Die Makrophagendichte im Infarktareal war 48 Stunden nach MI bei Eplerenon-behandelten Ratten signifikant erhöht, jedoch signifikant reduziert nach fünf Tagen. Eplerenon-Behandlung verstärkte die Neovaskularisation und war verbunden mit signifikant erhöhten Faktor-XIII-Spiegeln in der Infarktzone. Drei Tage nach großem MI konnten wir eine reduzierte Mobilisierung endothelialer Progenitorzellen (EPC) und erhöhte Marker oxidativen Stresses im Knochenmark zeigen. Statin-Behandlung oder ACE-Hemmung erhöhten die zirkulierenden EPC-Spiegel und verbesserten die molekularen Veränderungen im Knochenmark.

Progression of heart failure is mediated largely via persisting activation of various neuroendocrine systems (Bauersachs and Ertl 2006). Neurohumoral inhibition using beta-blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, and aldosterone antagonists accounts for the markedly improved prognosis in patients with heart failure over the last two decades (Swedberg et al. 2005, Jorde 2006). Although combined application of neurohumoral inhibitors is limited by side effects and pre-existing conditions such as hypotension and renal insufficiency especially in the elderly, the SENIORS trial indicates that selected beta-blockers may be given with good safety and efficacy even in the very elderly (Flather et al. 2005).

We have shown that aldosterone receptor blockade plus ACE inhibition confers additional protection against left ventricular remodeling in rats with chronic heart failure after large myocardial infarction (MI) (Fraccarollo et al. 2003). Now, we show a striking improve-

ment of healing processes by immediate selective mineralocorticoid receptor blockade after MI. In rats treated with the selective aldosterone receptor antagonist Eplerenone starting immediately after coronary ligation, left ventricular function as well as dilatation was significantly improved after seven days (Bauersachs et al. 2006). Infarct expansion index was significantly reduced, while scar thickness was enhanced by immediate eplerenone treatment post MI (Fig. 1). Macrophage infiltration into the infarcted LV myocardium of eplerenone-treated rats was significantly greater at 48 hours, but significantly reduced at 5 days after MI compared to placebo. Immunohistochemical staining 7 days post-MI demonstrated increased capillary density and thin-walled pericyte-poor vascular structures at the peri-infarct region after eplerenone treatment. Increased neovascularization was associated with significantly increased Factor XIII in the infarct zone post infarction in rats treated with eplerenone. Factor XIII is a potent stimulator of angiogenesis, and promotes healing processes after MI, as factor XIII knock-out mice die from myocardial rupture within the first days post MI (Nahrendorf et al. 2006). Eplerenone also reduced increased superoxide anion production in the infarct area 3 days after MI.

Antioxidant effects also appear to account at least in part for the improvement of LV remodeling and function achieved by statin treatment in CHF. We demonstrated a significant reduction of the detrimental fibrosis and hypertrophy of the surviving LV myocardium in rats after MI which was associated with reduced protein nitrotyrosines, an indicator of oxidative

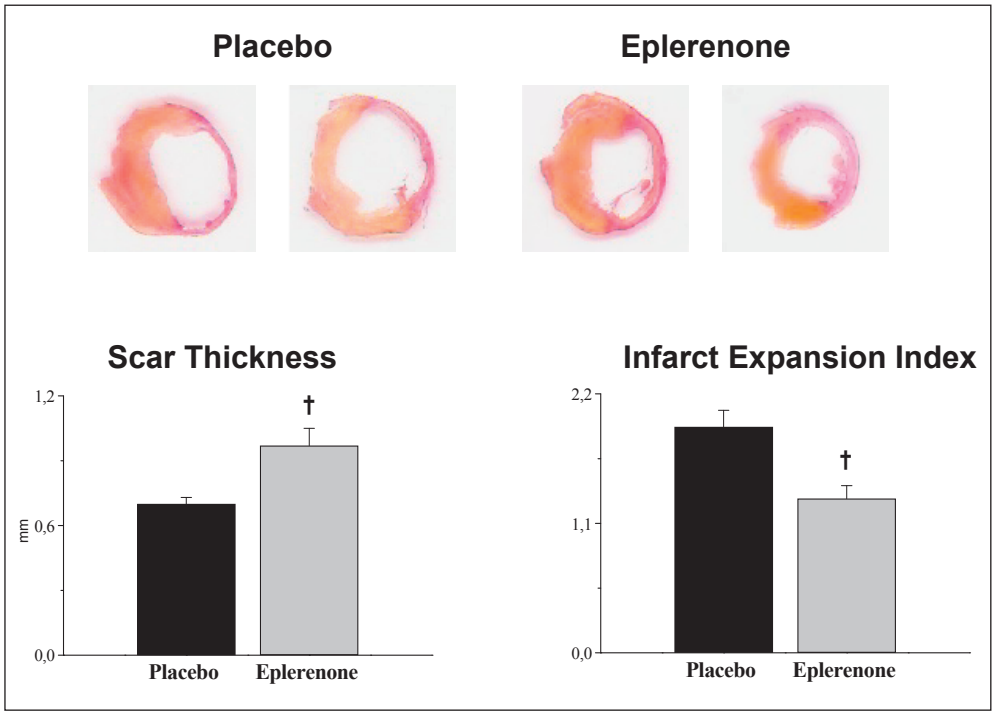

Fig. 1 Representative left ventricular sections, infarct expansion index and scar thickness 7 days after large myocardial infarction in rats treated either with placebo or eplerenone immediately after coronary ligation. *p<0.05 vs. sham, † p<0.05 vs MI placebo

stress (Bauersachs et al. 2001). Recent data from our group demonstrated reduced mobilization of endothelial progenitor cells (EPC) in rats early after large MI (Fig. 2) which was associated with elevated markers of oxidative stress within the bone marrow in these animals (Thum et al. 2006). Statin treatment markedly increased EPC mobilization and eNOS activity within bone marrow, and reduced oxidative stress (Fig. 3). In contrast, ACE inhibition increased bone marrow ERK phosphorylation and MMP-9 activity, also resulting in a significant increase in circulating EPC levels. Augmented EPC levels in the early postinfarction phase by ACE inhibition or statin treatment were associated with improved cardiac function and increased capillary density in the peri-infarct area 7 days after MI.

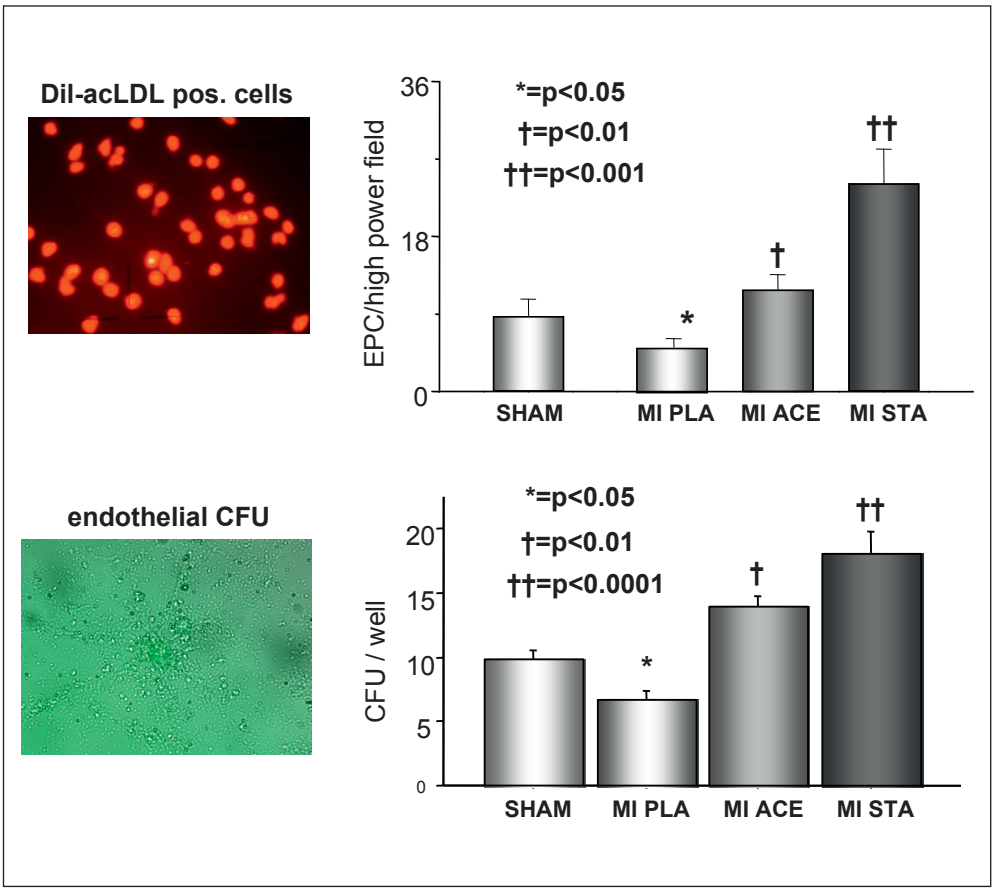

Fig. 2 Circulating endothelial progenitor cells 3 days after sham-operation or large myocardial infarction (MI) in rats treated either with placebo (MI PLA), ACE inhibition (MI ACE) or statin (MI STA). *Top*: dil-acLDL/UEA-1 positive cells; *bottom*: colony forming units (CFU). *p<0.05 vs. sham; † p<0.05, ††p<0.001, vs MI PLA.

In addition to the prevention of adverse remodeling of the surviving myocardium after MI by chronic therapies directed against neurohumoral activation, our data suggest that immediate neurohumoral inhibition after MI beneficially promotes early healing processes.

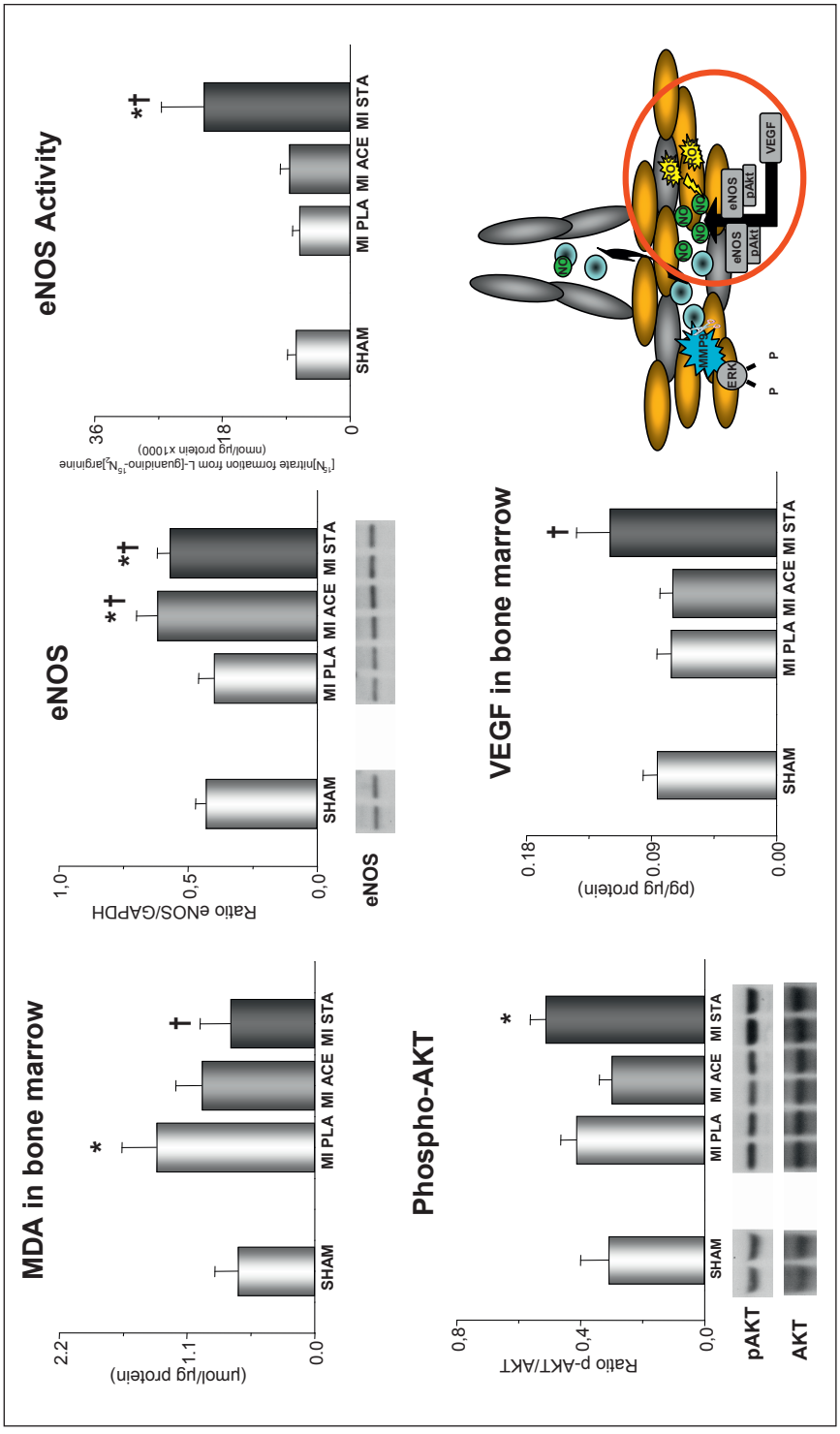

Fig. 3 MDA-TBA adducts as an index of bone marrow reactive oxygen species formation, eNOS protein expression (Western blot), calcium-dependent NOS activity (conversion of L-[guanidino-$^{15}N_2$]arginine to ^{15}N-nitrate), Akt phosphorylation (Western blot), and VEGF concentration (ELISA) in bone marrow extracts 3 days after sham-operation or large myocardial infarction (MI) in rats treated either with placebo (MI PLA), ACE inhibition (MI ACE) or statin (MI STA). *p<0.05 vs. sham; † p<0.05 vs MI PLA.

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Inflammation in Non-inflammatory Diseases

Manolis Pasparakis (Cologne)

Deregulated activation of the immune system is directly involved in the pathogenesis of a multitude of diseases. While the important role of immune deregulation in the pathogenesis of diseases such as multiple sclerosis and rheumatoid arthritis was recognized since many years, a number of studies in the last years have implicated inflammation as a crucial pathogenic component in diseases that were not previously considered as classical "inflammatory" disorders, such as type II diabetes, atherosclerosis and cancer. Accumulating evidence suggests that the interaction between the immune system and other cell types including epithelial, endothelial, stromal and parenchymal tissue cells is a critical factor in the initiation and progression of such diseases. Consequently, signaling pathways previously known to regulate immune responses are now recognized as important players in the pathogenesis of non-classical inflammatory diseases.

Amongst numerous signaling cascades, the NF-κB pathway is unique in its unusual and rapid mechanism of activation, the wide variety of stimuli leading to its induction and the large number of genes it regulates. The NF-κB signaling pathway functions in essentially all mammalian cell types and is activated in response to injury, infection, inflammation and other stressful conditions requiring rapid reprogramming of gene expression. Genes regulated by NF-κB include cytokines and chemokines, adhesion molecules, regulators of cell survival, proliferation and apoptosis, acute phase proteins and proteins important for protection from oxidative stress. Activation of NF-κB is mediated by the IκB kinase (IKK), which consists of the IKK1 and IKK2 catalytic subunits and a regulatory subunit called NEMO.

NF-κB activation has been implicated in the pathogenesis of a number of diseases, including inflammatory, metabolic and degenerative disorders and cancer. The role of NF-κB in disease pathogenesis remains controversial and may vary depending on the specific disease. NF-κB activity may promote inflammation and tissue damage by inducing the expression of proinflammatory mediators and effector molecules, but on the other hand it also protects cells from cytokine-induced death by regulating genes with critical anti-apoptotic and anti-oxidant activities. The net effect of NF-κB activation in disease pathogenesis is likely to be determined by a balance between both opposing activities and will probably play different roles in different cell types. In immune cells induction of NF-κB is important for cell activation and elicitation of immune responses that are beneficial for host defence but may be harmful during degenerative and inflammatory diseases. In non-immune cells on the other hand, activation of NF-κB may be critical for the protection of the cells from apoptosis induced by proinflammatory mediators such as TNF. Analysis of the cell-specific function of the NF-κB

pathway *in vivo* is crucial for understanding the role of this signaling cascade in physiological and pathological processes.

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Heart Failure is a Calcium Disease

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Myocardial remodeling in heart failure involves major alterations in intracellular Ca handling which contribute to contractile dysfunction. However, the role of altered Ca homeostasis in the genesis and progression of heart failure is a matter of ongoing discussion.

In human end-stage heart failure, defective excitation-contraction (EC) coupling contributes to both contractile dysfunction and triggered arrhythmias. L-type Ca current regulation is disturbed, intracellular Ca reuptake into the sarcoplasmic reticulum reduced, and the expression and activity of the sarcolemmal Na/Ca exchanger is increased. In consequence, reduced systolic and increased diastolic Ca concentrations contribute to systolic and diastolic contractile dysfunction. Of interest, defective gating of the sarcoplasmic reticulum Ca release channel (RyR2) has been recently described, most probably as a result of PKA/CaMKII-mediated hyperphosphorylation. In consequence, Ca "leaks" from the SR into the cytosol during diastole. This uncoordinated Ca release contributes to contractile dysfunction, but may also induce triggered arrhythmias by diastolic activation of the electrogenic Na/Ca exchanger. Altered intracellular Ca handling is aggravated by disturbed intracellular Na homeostasis in the failing human heart which contributes to diastolic Ca overload. Changes in intracellular Ca and Na handling also underlie the pathological force-frequency-relationship, i.e., a frequency-dependent decline in systolic and increase in diastolic tension in the failing human heart.

Recently, the concept that disturbed Ca handling may also precipitate and aggravate heart failure has been put forward. (Rare) human mutations in the phospholamban gene, resulting in a superinhibitory function of the protein, may result in familial forms of dilated cardiomyopathy. In addition, there is a distinct subcellular local control of Ca homeostasis which appears to be separate from EC coupling processes. For example, Ca is tightly controlled in the nuclear envelope and nucleus. IP3-receptor mediated Ca release from the nuclear envelope may activate nuclear CamKIIδ_B which phosphorylates nuclear histone deacetylases (HDAC), thereby inducing nuclear HDAC export and derepression of gene transcription. Similarly, Ca-mediated activation of the cytosolic phosphatase calcineurin results in transcription-factor activation and induction of maladaptive gene expression.

In consequence, altered intracellular Ca homeostasis is not only a result of pathological remodeling but is also a key signal involved in induction and maintenance of potentially pathological growth signals and remodeling.

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Heart Failure is a Viral Disease

Matthias Pauschinger (Nürnberg)

Dilated cardiomyopathy (DCM) is one major cause of progressive heart failure affecting especially young people with an incidence of 40 : 100 000. A viral immune pathogenesis is suspected in as many as (approximately ?) 60 % of DCM cases. Using sensitive molecular-biological methods such as *in situ* hybridization or the polymerase chain reaction, these methods enable the detection of viral genome of different viruses in the myocardium. Using this approach Kühl and colleagues could detect persistence of viral genome in the myocardium in 165 of 245 DCM patients (67.4 %). In addition, many murine myocarditis models could proof the concept of the viral etiopathogenesis in DCM. The clinical impact of viral persistence in this disease entity could be demonstrated by Why and colleagues in 1994. This study demonstrated for the first time the link between viral persistence and worse prognosis in DCM patients. This link between viral persistence and clinical outcome is also shown in the study by Kühl and colleagues. This study in 172 DCM patients clearly demonstrates that spontaneous clearance of viral genome is linked to a significant improvement in left ventricular function whereas this improvement was not shown in patients with viral persistence. Due to this relevant clinical data a pilot antiviral treatment trial with β-interferone was performed in 22 virus positive DCM patients showing a significant improvement in NYHA classification as well as a significant improvement of left ventricular function. Based on the favourable results of this pivotal pilot study, a randomized, double-blind, placebo-controlled European-wide multicenter study (Betaferon [interferon-β] in patients with Chronic Viral Cardiomyopathy [BICC-Study]) was initiated with very promising first data. Therefore, the virus linked etiopathogenicity in a high amount of DCM patients is very likely, and this knowledge will lead to new therapeutic options.

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Superoxide and Healing in the Cardiovascular System

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NADPH Oxidases as Pivotal Sources of ROS in Cardiac Disease

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Abstract

The main pathological effects of oxidative stress have traditionally been considered to be free radical-induced oxidation and damage, resulting in cell dysfunction, necrosis and/or apoptosis. More recently, it has become evident that reactive oxygen species (ROS) also have more subtle effects, in particular the highly specific modulation of intracellular signaling pathways and proteins (redox signaling). Among the many sources of ROS, the NADPH oxidases are especially well suited for redox signaling in that their primary function is ROS production which occurs in a highly specialized process specifically initiated by diverse stimuli. All NADPH oxidases are multimeric enzymes containing a core catalytic Nox subunit and a lower molecular weight $p22^{phox}$ subunit. Five distinct Nox isoforms, Nox1–5, are expressed in a tissue-specific pattern, with Nox1,2 and 4 abundantly expressed in cardiovascular cells. The Nox subunit catalyzes electron transfer from NADPH to molecular O_2, resulting in superoxide formation. The classical Nox2 (or $gp91^{phox}$) oxidase is activated through the association of cytosolic regulatory units, $p67^{phox}$, Rac, $p47^{phox}$ and $p40^{phox}$, with the core enzyme. Post-translational modification of $p47^{phox}$ and Rac, downstream of agonist-receptor initiated signal transduction cascades, is critical in this process. Whereas the general process for Nox1 activation is broadly similar to Nox2, Nox4 appears to be constitutively active and not regulated by these cytosolic subunits. Oxidase activation is implicated in downstream activation of protein kinases (e.g., ERK1/2, Akt), transcription factors (e.g., NF-κB) and proteases (e.g., MMPs). The involvement of Noxs in cardiac pathophysiology will be used to illustrate how Nox oxidase-dependent signaling impacts on disease phenotype.

Zusammenfassung

Als die wichtigsten pathologischen Auswirkungen von oxidativem Stress werden traditionell durch freie Radikale induzierte Oxidation bzw. Schädigung mit nachfolgender Zellfunktionsstörung, Nekrose und/oder Apoptose angesehen. Neuere Forschungsergebnisse haben gezeigt, dass reaktive Sauerstoffarten (ROS) auch eher subtile Wirkungen erzielen, insbesondere die hochspezifische Modulierung intrazellulärer Signalwege und Proteine (Redoxsignalisierung). Unter den zahlreichen Quellen von ROS sind die NADPH-Oxidasen besonders gut für eine solche Redoxsignalisierung geeignet, da ihre wichtigste Funktion in der ROS-Produktion liegt, die in einem hochspezialisierten Prozess erfolgt, der durch verschiedene Stimuli ausgelöst wird. Bei allen NADPH-Oxidasen handelt es sich um multimere Enzyme mit einer katalytischen Nox-Untereinheit als Kern und einer Untereinheit $p22^{phox}$ mit niedrigerem Molekulargewicht. Fünf verschiedene Nox-Isoformen, Nox 1–5, werden in einem gewebespezifischen Muster exprimiert, wobei die Expression von Nox1, 2 und 4 überwiegend in kardiovaskulären Zellen erfolgt. Die Nox-Untereinheit katalysiert den Elektronentransfer von NADPH auf molekularen O_2, wodurch es zur Bildung von Superoxid kommt. Die klassische Oxidase Nox2 (bzw. $gp91^{phox}$) wird durch die Assoziation des Kernenzyms mit zytosolischen Regulatoreinheiten, $p67^{phox}$, Rac, $p47^{phox}$ und $p40^{phox}$, aktiviert. Die post-translatorische Modifizierung von $p47^{phox}$ und Rac, *downstream* der durch Agonistrezeptor initiierten Signaltransduktionskaskaden, ist bei diesem Prozess die kritische Größe. Während der generelle Ablauf der Aktivierung von Nox1 im weitesten Sinn der von Nox2 ähnlich ist, scheint Nox4 dagegen konstitutiv aktiv zu sein und nicht der Regulierung durch diese zytosolischen Untereinheiten zu unterliegen. Die Oxidaseaktivierung ist in der nachgeschalteten Aktivierung von Proteinkinasen (z. B. ERK1/2, Akt), Transkriptionsfaktoren (z. B. NF-κB) und Proteasen (z. B. MMPs) impliziert. Die Wirkungsweise der Nox-Proteine in der Kardiopathophysiologie kann dazu genutzt werden, zu zeigen, wie Nox-Oxidase-abhängige Signalübermittlung den Phänotyp der Krankheit beeinflusst.

1. Introduction

Reactive oxygen species (ROS) are involved in many pathophysiological processes and as the name suggests are chemically highly reactive. ROS include free radicals such as superoxide (O_2^-) as well as more stable species such as hydrogen peroxide (H_2O_2).

Traditionally ROS were considered to be involved mainly in radical-induced oxidation and damage, resulting in cell dysfunction, necrosis and/or apoptosis. More recently, it has become evident that their biological effects depend upon the specific moiety generated, its localization and the relative balance between levels generated and the activity of antioxidant systems. ROS modulate the activity of proteins such as ion channels and enzymes as well as diverse intracellular signaling pathways (commonly termed redox signaling). Additionally, O_2^- inactivates nitric oxide (NO) thus regulating its bioavailability.

There are several potential sources of ROS, namely mitochondria, cytochrome P450-based enzymes, xanthine oxidase, NADPH oxidases and uncoupled NO synthases. Among these, NADPH oxidases appear to be the only enzymes whose primary function is ROS generation. This provides an attractive way in which the cell could tightly regulate redox signaling (Lambeth 2004). Furthermore, ROS derived from NADPH oxidase can amplify ROS production from other enzymes, e.g., by uncoupling NO synthases or by oxidation of xanthine oxidase (Landmesser et al. 2003).

2. NADPH Oxidase Structure, Isoforms and Mechanism of Action

The NADPH oxidase complex was first described in phagocytes where it plays a vital role in non-specific host defense by generating a large burst of O_2^- (the "oxidative burst") (Lambeth 2004). However, more recently, non-phagocytic cells have also been found to contain NADPH oxidase activity. In the cardiovascular system, NADPH oxidases generate ROS constitutively in endothelial cells (Bayraktutan et al. 2000), vascular smooth muscle cells (VSMC), adventitial and cardiac fibroblasts (Pagano et al. 1998), and cardiomyocytes (Bendall et al. 2002). In addition, basal ROS production can be augmented by various stimuli (Lambeth et al. 2000). NADPH oxidase-derived ROS is considered to be an important second messenger in cardiac hypertrophy and the remodeling process leading to heart failure (Murdoch et al. 2006).

The classic phagocytic oxidase comprizes a core membrane-bound catalytic Nox (for NADPH oxidase, formerly known as $gp91^{phox}$) domain that generates O_2^- by transferring an electron from NADPH to molecular O_2 and a lower molecular weight subunit called $p22^{phox}$ which associate in a heterodimer (Lambeth 2004). Activation of the oxidase requires the association of a number of cytosolic subunits, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac1 or 2, with the core heterodimer. It is now known that there is a family of NADPH oxidases each based on a different Nox isoform and encoded by separate genes. These Noxs have been classified into three groups according to their predicted structure: (*i*) Nox1-Nox4, which contain 6 transmembrane domains; (*ii*) Nox5, which has an additional N-terminal Ca^{2+}-binding domain; (*iii*) and Duox1 and Duox2, which include both the Ca^{2+}-binding domain and a further peroxidase-like domain (Cave et al. 2005). The Nox isoforms are expressed in a cell and tissue-specific manner. In the cardiovascular system, Nox1 is expressed mostly in VSMC, Nox2 in endothelial cells, cardiomyocytes and fibroblasts, and Nox4 quite widely in most cell types. Nox5 has been reported to be expressed in human endothelial cells where it may influence cell proliferation but is not

expressed in rodents (Belaiba et al. 2007). Cells often express more than one Nox isoform suggesting that there may be isoform-specific effects (Dworakowski et al. 2006).

The production of intracellular ROS by Noxs is tightly regulated. Activation of the classical Nox2 NADPH oxidase is controlled at the level of translocation of cytosolic regulatory units ($p67^{phox}$, Rac, $p47^{phox}$ and $p40^{phox}$) and their binding to the core Nox subunit (Lambeth 2004). The $p67^{phox}$ subunit is thought to be the key activator of the electron transfer process whereas $p47^{phox}$ is required for $p67^{phox}$ translocation and is considered to be the "organizer". Rac translocates independently to the membrane upon activation, where it interacts with $p67^{phox}$ and regulates electron transfer (Bokoch and Zhao 2006). Posttranslational modification of $p47^{phox}$ and Rac are critical for controling NADPH oxidase activity (Diebold et al. 2004). Recently, isoforms of $p47^{phox}$ and $p67^{phox}$ were discovered, named NoxO1 (for Nox Organizer 1) and NoxA1 (for Nox Activator 1) respectively. These are important for Nox1 activity although their precise roles in the cardiovascular system remain unclear. In contrast to Nox1 and Nox2, the Nox4-based oxidase does not require either $p47^{phox}$, $p67^{phox}$ or Rac for its activity, and may be constitutively active.

The activation mechanisms described above may be initiated by (*i*) G-protein-coupled receptor agonists e.g. angiotensin II and endothelin-1; (*ii*) cytokines, e.g. TNFα (tumor necrosis factor α); (*iii*) growth factors, e.g. VEGF (vascular endothelial growth factor); (*iv*) metabolic factors, e.g. insulin, oxidized low-density lipoprotein; (*v*) hypoxia-reperfusion; and (*vi*) mechanical stimuli e.g. oscillatory shear stress (Li and Shah 2004). Many of these activators are particularly important in cardiovascular pathophysiology.

3. Cardiovascular Redox Signaling Involving NADPH Oxidase

An increase in ROS production following oxidase activation may influence diverse downstream redox-sensitive signal transduction pathways, e.g., (*i*) protein kinases such the MAPK family, PKC, PKD, JAK, ERK1/2, Akt, (*ii*) transcription factor activity (e.g. AP-1, NF-κB) (*iii*) enzyme activity (e.g. MMP) and (*iv*) direct effects on enzymes, receptors and ion channels. Good evidence now supports a role for NADPH oxidases as second messengers in endothelial dysfunction, migration, angiogenesis, atherosclerosis, vascular smooth muscle hypertrophy, cardiac hypertrophy and remodeling (Griendling 2004). ROS may also induce specific changes in the function of proteins involved in myocardial excitation-contraction coupling, e.g., the sarcoplasmic reticulum Ca^{2+} ATPase pump (SERCA2a), ryanodine receptor and contractile proteins (Yano et al. 2004).

4. NADPH Oxidases in Cardiac Disease

4.1 NADPH Oxidases in Left Ventricular Hypertrophy

ROS generated by NADPH oxidases have been demonstrated as important in the pathogenesis and pathophysiology of left ventricular hypertrophy (LVH). Many stimuli which induce hypertrophy of isolated cardiomyocytes have been shown to work at least in part through either NADPH oxidase-derived ROS generation or to have a requirement for Rac1 (which is needed for Nox2 activation) (Higuchi et al. 2003). For example, angiotensin II-induced myo-

cyte hypertrophy requires Nox2 and Rac1 (Nakagami et al. 2003, Wenzel et al. 2001). The role of NADPH oxidases *in vivo* was investigated using mice deficient in the Nox2 NADPH oxidase ($Nox2^{-/-}$ mice). In a model of cardiac hypertrophy induced by short-term (7–14 day) suppressor infusion of angiotensin II, Bendall et al. (2002) found that increases in heart/body weight ratio, myocyte area and mRNA expression of ANF and βMHC were all markedly inhibited in $Nox2^{-/-}$ mice compared to wild-type controls. This was associated with an absence of angiotensin II-induced increases in myocardial NADPH oxidase activity in $Nox2^{-/-}$ mice. These data suggest that Nox2 oxidase is required for short-term angiotensin II-induced cardiac hypertrophy. In contrast to this, $Nox2^{-/-}$ mice were not protected against hypertrophy induced by a pressor dose of angiotensin II (Johar et al. 2006). Similarly, in LVH induced by aortic constriction, $Nox2^{-/-}$ mice displayed the same level of hypertrophy as their wild-type controls (Byrne et al. 2003, Maytin et al. 2004). This was accompanied by similar increases in hypertrophic molecular markers at the RNA level as well as increases in ROS production in both knockout and wild-type mice. Taken together, these results indicate that although Nox2 appears to be important in cellular studies and in models of suppressor angiotensin II-induced hypertrophy *in vivo*, it seems not to be essential for pressure overload-induced hypertrophy. Nevertheless, Nox2 does have a significant role in other aspects of pressure overload LVH. Nox2-deficient mice subjected to artic banding were found to be protected against LV systolic and diastolic dysfunction observed in wild-type animals, as assessed by detailed pressure-volume analyzes of LV contractile function. The protection against contractile dysfunction was also evident at the level of isolated single cardiomyocytes (Grieve et al. 2006). Thus, it appears that Nox2 may affect distinct components of the phenotype of the hypertrophying heart independent of the increase in muscle mass *per se*. The cell-specific contribution of Nox2 to these effects remains to be defined since it is expressed in several cell types including cardiomyocytes, endothelial cells and infiltrating inflammatory cells.

Another important question is which signaling pathways are involved in the effects of Nox2 NADPH oxidase on cardiac hypertrophy. In many cell types, the small GTPase RAS is a redox-sensitive signaling switch, which in isolated ventricular myocytes undergoes a thioredoxin-1 sensitive postranslational oxidative modification upon α-adrenergic receptor hypertrophic stimulation (Kuster et al. 2005). There is also evidence for the involvement of redox-sensitive activation of ERK1/2 in the context of α-adrenergic agonist-induced cardiomyocyte hypertrophy (Xiao et al. 2002). Rac1 located specifically in the cardiomyocyte is involved in angiotensin II induced activation of apoptosis signal-related kinase 1 (ASK1) and NF-κB, another potential pro-hypertrophic pathway (Satoh et al. 2006). Interestingly, ASK1 knockout mice have a similar phenotype to Nox2 deficient mice upon exposure to short term suppressor angiotensin II infusion (Bendall et al. 2002).

4.2 NADPH Oxidase in Cardiac Remodeling after Myocardial Infarction

The importance of oxidative stress in adverse remodeling is well recognized. Post-MI myocardium obtained from experimental models and human patients shows an increase in mRNA levels of $p22^{phox}$ and Nox2 mRNA (Fukui et al. 2001). This corresponds with an increase in markers of oxidative stress in experimental MI models (Hill and Singal 1996), as well as attenuation of adverse LV remodeling by various antioxidant treatment (e.g., probucol, dimethylthiourea or genetic manipulation (Kinugawa et al. 2000, Sia et al. 2002). Antioxidant treatment appears to reduce LV remodeling by attenuating increases in collagen volume frac-

tion, MMP activity and myocyte size, which also improves cardiac function. A direct involvement of Nox2 has been suggested by recent work from our laboratory which found reduced post-MI LV remodeling in Nox2 deficient mice compared to wild-type (Looi et al. 2008). On the other hand, infarct size after permanent *in vivo* coronary ligation remains the same between NADPH oxidase deficient mice and wild-type controls (Hoffmeyer et al. 2000). In contrast, Nox2 may also have a beneficial role in the setting of acute myocardial ischemia. Recently, Nox2 was found to contribute to ischemic myocardial preconditioning of isolated hearts in response to transient global ischemia (Bell et al. 2005).

4.3 NADPH Oxidase in Cardiac Fibrosis

Interstitial cardiac fibrosis commonly occurs as part of post-MI remodeling as well as in the setting of pressure overload (e.g., hypertension). The pro-fibrotic effects of ROS are well recognized. Expression of profibrotic genes and the balance between MMPs and their inhibitors TIMPs influences collagen levels and thus fibrosis. In the vasculature, ROS derived from NADPH oxidase in response to mechanical stretch or angiotensin II can activate MMPs (Grote et al. 2003, Luchtefeld et al. 2005). There is strong evidence indicating that activation of Nox2 drives cardiac fibrosis. In models of LVH induced by pressure overload and pressor or suppressor angiotensin II infusion, Nox2-deficient mice were found to have a reduction in connective tissue growth factor CTGF, pro-fibrotic genes such as procollagen-1 and 111, in MMP-2 activity, and in overall interstitial cardiac fibrosis (Bendall et al. 2002, Grieve et al. 2006, Johar et al. 2006). Furthermore, aldosterone-driven interstitial cardiac fibrosis in rats was associated with increased oxidative stress together with increased ventricular Nox2 expression. Chronic treatment with apocynin, an NADPH oxidase inhibitor, blunted cardiac fibrosis (Park et al. 2004, Sun et al. 2002). These results provide strong evidence for Nox2 driving cardiac fibrosis; however, the precise mechanisms involved remain to be established.

5. Conclusions

Recent studies implicate NADPH oxidases as important sources of ROS that regulate redox-sensitive pathophysiological processes contributing to LVH and cardiac remodeling. NADPH oxidases are activated by several stimuli known to be important in LVH, fibrosis and remodeling. Furthermore, there are many redox-sensitive pathways that may be activated or inhibited by ROS derived from NADPH oxidase and affect distinct components of the overall response in a tightly regulated manner depending upon the stimulus. We have focused in this brief article on the role of NADPH oxidase specifically in the heart; however, it also has important roles in the rest of the cardiovascular system. A better understanding of the regulation of NADPH oxidases and the mechanisms through which they modulate redox signaling could potentially suggest new therapeutic strategies.

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Cytochrome P450 2C is a Functionally Significant Source of Reactive Oxygen Species

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With 2 Figures

Abstract

In the porcine coronary artery a cytochrome P450 (CYP) enzyme, homologous to CYP 2C8/9, has been linked to the nitric oxide (NO) and prostacyclin-independent relaxations generally attributed to the endothelium-derived hyperpolarizing factor. Under normal conditions this enzyme generates vasodilator epoxyeicosatrienoic acids but the enzyme can also generate reactive oxygen species which scavenge endothelium-derived NO thus decreasing its bioavailability and attenuating vasodilator responses. This is a pathophysiologically relevant phenomenon as the endothelial dysfunction (decrease in the acetylcholine-induced, NO-mediated relaxation) manifest in patients with coronary artery disease can be reversed using the CYP 2C9 inhibitor sulfaphenazole.

Zusammenfassung

In Koronararterien des Schweins wurde ein Cytochrom P450 (CYP)-Enzym identifiziert, das homolog zu den menschlichen CYP2C8/2C9 ist, und mit der NO- und Prostazyklin-unabhängigen Vasodilatation, die man dem endothelialen hyperpolarisierenden Faktor zuschreibt, in Verbindung gebracht wird. Unter Normalbedingungen synthetisiert dieses Enzym vasodilatorisch wirkende Epoxyeicosatriensäuren. Das Enzym kann jedoch auch reaktive Sauerstoffspezies synthetisieren, welche das endothelial gebildete NO abfangen und dadurch dessen Bioverfügbarkeit und in Folge auch seine vasodilatatorischen Eigenschaften reduzieren. Dies ist ein pathophysiologisch relevantes Phänomen, da die endotheliale Dysfunktion (Verringerung der Acetylcholin-induzierten, NO-vermittelten Relaxation), wie sie bei Patienten mit Koronarer Herzkrankheit zu beobachten ist, durch Gabe des CYP2C9 Inhibitors Sulfaphenazol rückgängiggemacht werden kann.

1. Introduction

Local vascular tone is generally determined by a variety of factors such as neurotransmitters released from autonomic nerves, circulating vasoactive compounds, tissue metabolites and endothelium-derived autacoids. The best characterized autacoids are the potent vasodilators nitric oxide (NO) and prostacyclin (PGI_2) and the vasoconstrictor peptide endothelin. Several studies have, however, convincingly demonstrated the existence of an NO/PGI_2-independent component of endothelium-dependent relaxation in various arterial beds, most notably in mesenteric, carotid, coronary and renal arteries. Since the NO/PGI_2-independent vasodilatation originally described was co-incident with vascular smooth muscle hyperpolarization, and was abolished by depolarizing concentrations of potassium, it was proposed to be mediated by an endothelium-derived hyperpolarizing factor or "EDHF" (Quilley et al. 1997).

2. Cytochrome P450 and the Endothelium-derived Hyperpolarizing Factor

The hypothesis that the EDHF produced in some vascular beds may be a cytochrome P450 (CYP)-derived metabolite of arachidonic acid was initially developed based on experiments showing that the application of exogenous arachidonic acid to isolated vascular segments induced relaxation. In addition, in many arteries EDHF-mediated responses were inhibited by compounds which inhibit phospholipase A_2, the enzyme responsible for the liberation of arachidonic acid from membrane phospholipids. As EDHF and PGI_2 were known to be distinct, an arachidonic acid metabolizing enzyme other than cyclooxygenase was proposed to be responsible for the generation of EDHF. Therefore, either a lipoxygenase or CYP product became a likely candidate (Pinto et al. 1987, Rubanyi and Vanhoutte 1987). Recently, data relying on techniques other than the pharmacological inhibition of CYP have considerably strengthened the hypothesis that CYP activity is an integral component of the EDHF response. RT-PCR, Western blotting and immunofluorescence techniques, for example, have been used to demonstrate that coronary endothelial cells express CYP epoxygenases (epoxyeicosatrienoic acid or EET-producing isozymes) including, CYP 2C8, CYP 2C9 and CYP 2J2 (Fisslthaler et al. 1999, 2000, Node et al. 1999). The functional relevance of CYP activity has been addressed by enhancing the expression of CYP 2C epoxygenases using β-naphthoflavone. The β-naphthoflavone-induced increase in CYP protein not only increased the synthesis of EETs by endothelial cells, but also enhanced the bradykinin-induced, EDHF-mediated hyperpolarization and relaxation of intact coronary artery segments (Popp et al. 1996, Fisslthaler et al. 1999, 2000). Perhaps the most convincing evidence obtained to date in support of the hypothesis that the EDHF synthase in porcine coronary arteries is a CYP enzyme, has been provided by the use of antisense oligonucleotides against the coding region of CYP 2C8/9. Incubation of porcine coronary arteries with antisense, but not sense or scrambled, oligonucleotides markedly reduced CYP 2C mRNA and protein and attenuated bradykinin-induced, EDHF-mediated hyperpolarization and relaxation without compromising responsiveness to endogenously produced NO or an NO donor (Fisslthaler et al. 1999). This inhibitory effect of antisense oligonucleotides provided the first non-pharmacological evidence that the EDHF produced by porcine coronary arteries is a CYP-dependent metabolite of arachidonic acid, or rather, that a CYP metabolite is an essential permissive factor for EDHF-mediated vascular responses. A similar approach has also been used to show that EDHF-mediated responses in isolated resistance arteries from hamster gracilis muscle can also be attributed to the activation of a CYP 2C epoxygenase (Bolz et al. 2000).

3. Cytochrome P450 and the Production of Reactive Oxygen Species

Superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals can be generated during the CYP reaction cycle when the electrons for the reduction of the central heme iron are transferred on the activated bound oxygen molecule (Fig. 1). Indeed, the continuous production of reactive oxygen species (ROS) appears to be one of the most important outcomes of NADPH consumption by microsomal mono-oxygenases implying that these enzymes contribute significantly to the cellular production of oxygen-derived free radicals (Davydov 2001). Given that oxidative stress is now appreciated to play a significant role in the early stages of vascular disease and inflammation, we hypothesized that CYP epoxygenases ex-

pressed in endothelial cells may contribute to the generation of oxygen-derived free radicals within the vascular wall.

Antisense oligonucleotides, derived from the cDNA sequences of human CYP 2C8 and 2C9, were used to down-regulate CYP 2C expression in isolated segments of porcine coronary artery (Fisslthaler et al. 1999). However, while the antisense oligonucleotide treatment attenuated the EDHF-mediated relaxation of porcine coronary arteries, the NO-mediated relaxation was improved. A similar improvement in NO-mediated relaxation was observed in the presence of the selective CYP 2C9 inhibitor sulfaphenazole. To determine whether or not the putative coronary EDHF synthase generates ROS, supersomes isolated from cells overexpressing either CYP 2C8 or 2C9 were incubated with the oxidative sensitive fluorogenic reagent oxyBURST. ROS generation was observed using both CYP 2C8- and 2C9-containing

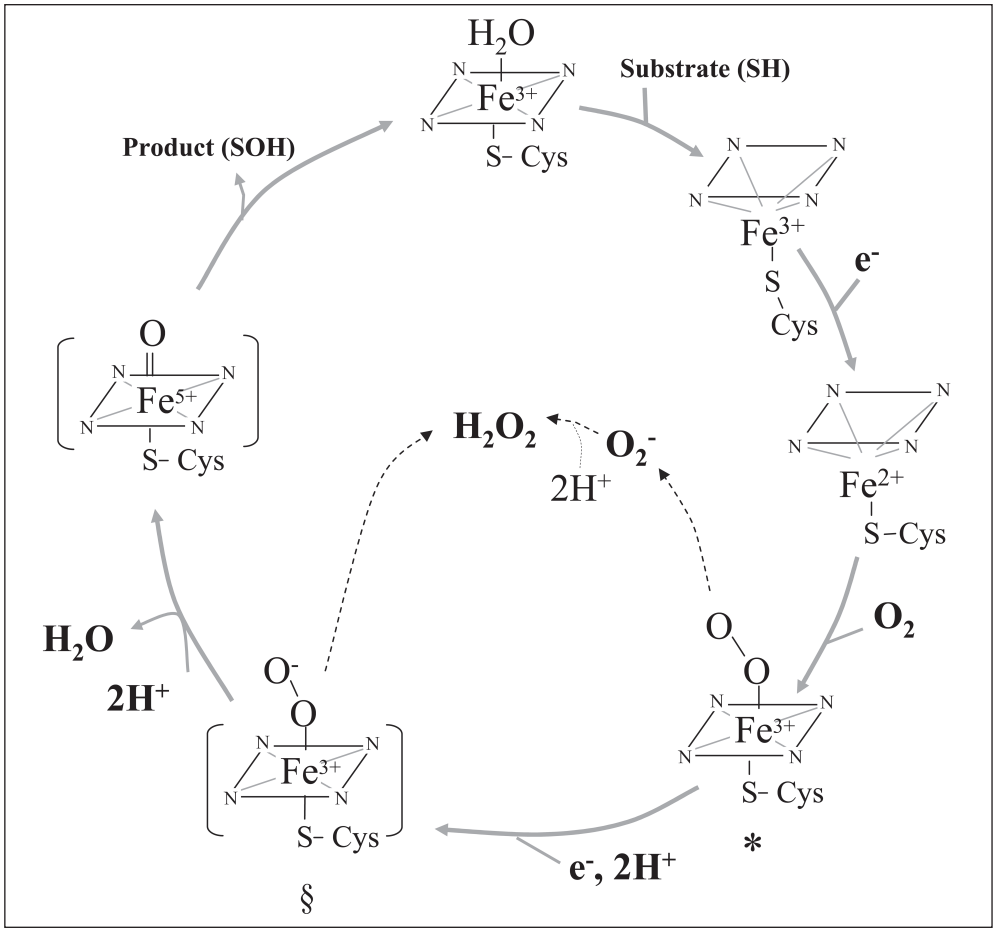

Fig. 1 Scheme of the catalytic cycle of CYP mono-oxygenase. The pathways marked as dotted lines produce oxygen-derived free radicals. In one branch a superoxide anion (O_2^-) is released as a result of the decay of the one-electron-reduced ternary complex (*). The second reactive oxygen species-producing pathway is the protonation of the peroxycytochrome P450 (§). For further details see Davydov (2001).

supersomes. The generation of ROS by both CYP enzymes was abolished by DPI while sulfaphenazole selectively inhibited ROS generation by CYP 2C9. To ensure that sulfaphenazole did not affect ROS generation by the NADPH oxidase or xanthine oxidase, the generation of reactive oxygen species by human leukocytes and a purified preparation of xanthine oxidase were assessed using compound 5-enhanced chemiluminescence and compared with that of CYP 2C9-containing supersomes. Sulfaphenazole failed to affect ROS generation by xanthine oxidase or the NADPH oxidase under either basal conditions or following stimulation with phorbol 12-myristate 13-acetate, but inhibited CYP 2C9-induced compound 5-chemiluminescence.

Since CYP 2C is expressed in native porcine coronary artery endothelial cells, sections of porcine coronary artery were stained with dihydroethidine. A fluorescent signal was detected in both endothelial and smooth muscle cells. However, after treatment of arterial segments with CYP 2C antisense oligonucleotides, CYP 2C protein levels were decreased and the fluorescent ethidine signal in the endothelium was markedly attenuated (Fig. 2). CYP 2C antisense oligonucleotides did not affect the fluorescent signal in the vascular smooth muscle. Incubation of arterial rings with sulfaphenazole also selectively attenuated the ethidine fluorescent signal detected in endothelial cells without affecting the signal in either the media or adventitia. As 11,12-EET, a major CYP 2C9 product detectable in porcine coronary artery endothelial cells (Fisslthaler et al. 1999), exerts anti-inflammatory effects on TNF-α-stimulated endothelial cells by preventing the activation of NF-κB (Node et al. 1999), we determined the effects of CYP induction and CYP 2C9 over-expression on the activity of NF-κB. Enhancing CYP expression by incubating endothelial cells with the Ca^{2+} antagonist nifedipine (Fisslthaler et al. 2000), increased endothelial generation of 11,12-EET by 453 ± 40 % (n = 4) and ROS production by 283 ± 22 % (n = 3) compared to non-transfected endothelial cells. Nifedipine treatment also increased both the basal and TNF-α-induced increase in NF-κB DNA-binding. The expression of vascular cell adhesion molecule-1 (VCAM-1) is controlled by NF-κB (Spiecker et al. 1998) and is reported to be increased by ROS (Soares et al. 1998) but decreased by 11,12-EET (Node et al. 1999). To determine which of these influences predominates in CYP expressing cells we assessed the expression of VCAM-1 in human endothelial cells transfected with CYP 2C9. VCAM-1 was not detected in unstimulated endothelial cells, but was clearly evident 10 hours after transfection of endothelial cells with CYP 2C9, an effect not observed in transfected cells treated with sulfaphenazole. In native porcine coronary endothelial cells, which express CYP 2C9 and stain ethidine-positive, VCAM-1 was also detected. Treatment with CYP 2C9 antisense oligonucleotides markedly attenuated the basal expression of VCAM-1.

The most frequently studied sources of ROS in endothelial cells are the NADPH oxidase, xanthine oxidase, cyclooxygenase and the endothelial NO synthase (Kojda and Harrison 1999). It is only relatively recently that CYP enzymes, some of which may generate ROS (White and Coon 1980, Bondy and Naderi 1994, Puntarulo and Cederbaum 1998) have been identified in the vasculature, both in endothelial and in vascular smooth muscle cells (for review see Fleming 2001). In the porcine coronary artery, a CYP 2C epoxygenase may be the dominant source of endothelial O_2^- *in vivo*, as it can be continuously activated by the rhythmic vessel distension that occurs during the cardiac cycle (Popp et al. 1998, Fisslthaler et al. 2001). Ischemia-reperfusion injury in a rat model has also been linked to an increase in the generation of O_2^- by a CYP epoxygenase (Granville et al. 2004). CYP-derived ROS formation is clinically relevant and decreases the bioavailability of NO. In hu-

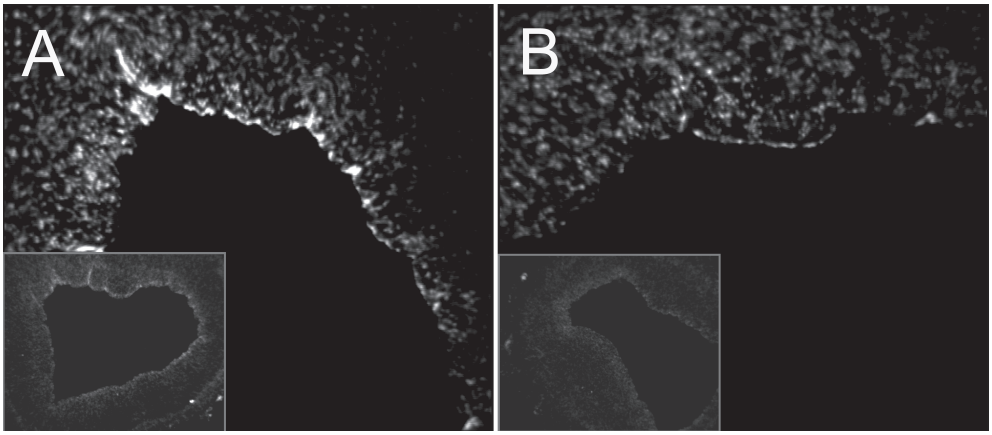

Fig. 2 *In situ* detection of ROS production in the porcine coronary artery. (*A*) and (*B*), Fluorescent photomicrographs obtained at identical settings of sections of the same porcine coronary artery labeled with the oxidative dye hydroethidine 18 hours after treatment with (*A*), control and (*B*), antisense CYP 2C oligonucleotides. The inserts show an overview of the signal obtained in a separate experiment and the results presented are representative of data obtained in 6 separate experiments. From Fleming et al. 2001

mans with manifest coronary artery disease, sulfaphenazole was able to restore acetylcholine-induced, NO-mediated vasodilatation (Fichtlscherer et al. 2004).

Different CYP isoforms seem to generate varying amounts of oxygen-derived free radicals (Puntarulo and Cederbaum 1998). For example, the pro-apoptotic effects of CYP 2E1 in glutathione-depleted cells has been linked to enhanced CYP-associated oxidative stress (Wu and Cederbaum 2001), whereas bovine aortic endothelial cells transfected with CYP 2J2 are protected against the oxidative stress induced by hypoxia and re-oxygenation (Yang et al. 2001). Apparent differences in ROS generation by different CYP enzymes may also reflect the extent of electron leakage from the protein complex i.e., the tightness of enzyme coupling to the cytochrome P450 reductase or cytochrome b_5 (Gruenke et al. 1995, Davydov 2001).

4. Summary

The CYP 2C epoxygenase, expressed in coronary endothelial cells, is not only crucial for the generation of the potent vasorelaxant 11, 12-EET, but is a potential major source of ROS within the coronary wall. Thus, while the anti-inflammatory CYP product, EDHF/EET, may be the dominant endothelium-derived vasoactive autacoid in states associated with a manifest "endothelial dysfunction", the enhanced activation of the CYP-like EDHF synthase may eventually be detrimental to vascular homeostasis, as a consequence of the simultaneous generation of EETs and ROS. However, it is currently unclear what determines the switch between a CYP-derived vasodilator such as an EET and the CYP-derived generation of O_2^-. Moreover, since endothelial CYP activity and expression can be stimulated by hormonal as well as haemodynamic stimuli, such as cyclic stretch (Popp et al. 1998, Fissthaler et al.

2001), the activation of CYP 2C in endothelial cells may participate to the stretch-induced generation of O_2^-, which has until now been attributed to the activation of the NADPH oxidase (Hishikawa et al. 1997, Hishikawa and Luscher 1997).

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Xanthine Oxidase as Pivotal Source of Reactive Oxygen Species

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Immunohistochemical studies have documented the presence of xanthine oxidase in vascular endothelial and smooth muscle cells of human vessels. We have observed increased endothelium-bound xanthine oxidase activity in patients with coronary disease and chronic heart failure (CHF) by using electron spin resonance spectroscopy that was related to endothelial dysfunction in these patients. Myocardial xanthine oxidase levels were found to be increased in patients with CHF and in experimental heart failure providing a relevant source of reactive oxygen species (ROS). Recent studies further support the concept that xanthine oxidase activation contributes to endothelial dysfunction and left ventricular remodeling/dysfunction in coronary disease and heart failure, thereby affecting prognosis. The enzyme is synthesized as xanthine dehydrogenase that uses NAD^+ as an electron acceptor and has to be converted to xanthine oxidase that uses molecular oxygen as the preferred electron acceptor to become a source of O_2^- and hydrogen peroxide. Several mechanisms causing the conversion of xanthine dehydrogenase to xanthine oxidase in endothelial cells have been identified. Interestingly, increased ROS production from other oxidant enzymes, such as the NAD(P)H oxidase, triggers conversion of the enzyme to xanthine oxidase to augment vascular and myocardial oxidant stress, most likely by oxidizing cysteines in the enzyme.

Xanthine oxidase inhibition may represent a novel avenue for the treatment of cardiovascular disease. In this respect, several recent experimental studies have evaluated the effect of xanthine oxidase inhibition by allopurinol or its metabolite oxypurinol in experimental models of heart failure and in small studies of patients with CHF. Both in patients with CHF and in experimental heart failure, xanthine oxidase inhibition improved myocardial efficiency. In experimental heart failure xanthine oxidase inhibition reduced left ventricular (LV) remodeling and improved LV function. It should be noted, however, that some of the current treatment strategies in patients with coronary disease or heart failure, in particular ACE inhibition or AT1-receptor blockade, have an impact on xanthine oxidase activation, that may importantly contribute to their therapeutic efficacy, which in turn, limits the specific inhibition of xanthine oxidase by drugs.

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General Aspects of Healing and Inflammation

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Role of CD73-derived Adenosine in Acute and Chronic Inflammation

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We have recently reported that mice with targeted deletion of ecto-5'-nucleotidase/CD73 are characterized by reduced coronary flow, enhanced platelet activation and increased adherence of monocytes to the endothelium. In the present study we have investigated the molecular mechanisms by which this proinflammatory response is mediated and explored whether CD73-derived adenosine modulates neointima formation in an acute injury model, and inhibits its development of arteriosclerosis in ApoE/CD73$^{-/-}$ double mutants.

CD73$^{-/-}$ mice exibit increased luminal staining for vascular adhesion molecule (VCAM)-1 in carotid arteries and increased expression of VCAM-1 transcripts and protein in whole carotid lysates. Endothelial cells cultured from CD73$^{-/-}$ show an up-regulation of mRNA and protein expression of VCAM-1, which was associated with in creased nuclear factor (NF)-κB activity. Measurement of expression of the A_1, A_{2a}, A_{2b} and A_3 receptor by RTPCR in the aorta and freshly harvested aortic endothelial cells revealed substantial downregulation of the A_1 receptor (20% of control) while all other adenosine receptors remained unchanged. *Ex-vivo* perfusion of carotid arteries shows that the increased monocytes arrest in carotid arteries of CD73$^{-/-}$ is mediated by α4β1 integrin. After wire-induced injury of the carotid artery, CD73 expression was upregulated in WT mice, while neointima formation and macrophage content was increased in CD73$^{-/-}$ mice, concomitant with elevated NF-κB activation, luminal VCAM-1 expression and soluble VCAM-1 concentration. Treatment of mice with the specific A_{2a} receptor agonist ATL-146e reversed the increased VCAM-1 transcript and protein expression in CD73$^{-/-}$-derived endothelial cells. Most importantly, ATL-145e fully prevented wire-induced neointima formation in CD73$^{-/-}$ mice.

To explore whether CD73-derived adenosine also modulates chronic inflammation we have generated ApoE/CD73$^{-/-}$ double mutants and found in 6 month old animals kept on normal diet that the development of arteriosclerotic lesions in the aorta was 2.5 fold higher compared with ApoE controls. Measurement of various cytokines in plasma substantiates the increased inflammatory state in the double knockout.

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Our data demonstrate that CD73-derived adenosine through activation of A_{2a} receptors protects against vascular inflammation, monocyte recruitment and neointima formation. Endogenously formed adenosine is also important in limiting the progression of arteriosclerosis.

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Gender Aspects in Cardiovascular Healing and Heart Failure

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Clinical and animal studies suggest that estrogen receptors (ER) are involved in the development of myocardial hypertrophy and heart failure and that ER are able to modulate the outcome in experimental myocardial infarction (MI). Clinical findings suggest that fibrosis in the cardiovascular system plays a greater role in women in comparison with men. Fibrosis may control healing processees after MI.

Sex differences in the regulation of the extracellular matrix have already been shown in animal models. Fibrosis is differently regulated in the injured male and female heart and kidney. Mice with diabetes, with hypertension and with heart failure exhibit sex differences in organ fibrosis. In the left ventricle of ovarrectomised rats an increase in collagen content and a decrease in Col III and MMP2 content were observed. Substitution with estrogen reversed these changes and led to a decrease of Col I/Col III ratio and to an increase in MMP2-expression. Inhibition of biosynthesis of Col I by estrogen was also shown in isolated mouse-mesangial cells and cardiac fibroblasts. Estrogen modulated collagen synthesis also in human vascular cells and in mouse retinal cells. The effect of estrogen may be partially due to the interaction of activated estrogen receptors with AP1 or NF-κB binding sites in the MMP2 promotor. We presented experimental findings showing a regulation of extracellular matrix in cardiac fibroblasts by estrogen.

ERα mRNA and protein is increased in end stage human heart failure. ERα in the human heart is localized to the cytoplasm, sarcolemma, intercalated discs and nuclei of cardiomyocytes, but also to the nuclei of fibroblasts. Immunofluorescence studies demonstrate colocalization of ERα with β-catenin at the intercalated disc in control hearts and immunoprecipitation studies confirm complex formation of both proteins. Interestingly, the ERα/β-catenin colocalization was lost at the intercalated disc in DCM hearts. Thus, the ERα/β-catenin colocalization in the intercalated disc may be of functional relevance and a loss of this association may play a role in the progression of heart failure in DCM.

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Innate Immunity, Hypoxia Inducible Factor-1α and Wound Healing: Implications for Therapeutic Angiogenesis

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Restoring blood flow to the site of injury is a necessary prerequisite for successful repair of wounded tissue. It is now recognized that there are multiple links between pathogen recognition, increased angiogenesis, and tissue repair, which include activation of an initial innate immune response, one of the first lines of defense following tissue injury. Although best known for its role in the cellular response to hypoxic stress, including activation of multiple complementary pro-angiogenic pathways, Hypoxia Inducible Factor-1α (HIF-1α) has recently been shown to play also a pivotal role in accelerating and sustaining the innate immune response to infection and wound repair. Indeed, HIF-1α can be activated by specific inflammation-related signaling pathways even in the absence of hypoxic stress. To evaluate the possibility that a constitutively active formulation of HIF-1α delivered in an adenoviral vector, termed "Ad2/HIF-1α/VP16", might have therapeutic applications, animal experiments were performed that documented improved blood flow and tissue repair following injury with this pro-angiogenic construct. Phase I clinical trials of Ad2/HIF-1α/VP16 have now been completed in patients with advanced coronary artery disease, and in patients with critical limb ischemia, including patients with refractory leg ulceration. Preliminary bioactivity data in humans suggest that ischemic wound healing may be accelerated by this constitutively active HIF-1α construct.

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Molecular Hypoxia Signaling in Ischemia

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Tissue deprivation of oxygen (hypoxia) is common in cardiovascular diseases. We have generated mice deficient for the HIF prolyl-hydroxylases PHD1, PHD2 and PHD3, three recently identified oxygen sensors of mammalian tissues. PHD2$^{-/-}$ embryos succumbed at mid-gestation (E12.5) showing fatal placentation defects, but PHD1$^{-/-}$ and PHD3$^{-/-}$ mice were healthy and fertile. In order to determine the relative contribution of each PHD to hypoxic tissue responses, PHD-deficient mice were exposed to various pathophysiological conditions leading to tissue hypoxia. We found that loss of PHD1, but not PHD3 (PHD3$^{-/-}$) or PHD2 (PHD2$^{+/-}$), provides specific protection against ischemic skeletal muscle necrosis in a murine model of hind-limb ischemia. This myo-protective effect was not attributable to increased baseline vascularization or enhanced residual perfusion after vascular ligation. Oxidative energy metabolism was strikingly preserved in the musculature of PHD1$^{-/-}$ mice after prolonged ischemia, allowing muscle fibers to maintain sufficient levels of energy rich phosphates. We found that ischemia-induced oxidative stress was substantial in WT, but negligible in PHD1$^{-/-}$ muscle. Remarkably, the protective effect of PHD1 was abrogated in PHD1$^{-/-}$ HIF-2$^{+/-}$ mice. We conclude that PHD1 is an important regulator of the response to oxidative stress in the skeletal muscle, and that HIF-2α acts as a major effector of PHD1 in the response to oxidative damage. Selective PHD-inhibitors may therefore offer novel therapeutic opportunities for diseases characterized by oxidative stress.

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NO and NOS in Heart Disease

Regulation of Myocardial Contractility by Akt in Normal and Failing Heart

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(La Jolla, CA, and Milan)

Our group first demonstrated that Akt positively regulates cardiac inotropism. In particular, we showed that Akt is deeply involved in regulating Ca^{2+} metabolism in cardiac myocytes. We proved that overexpression of the E40K Akt mutant increases myocyte contractility by enhancing L-type Ca^{2+} channel current. Moreover, sarcoplasmic reticulum Ca^{2+} uptake is improved by Akt activation. In fact, we found that Akt increases SERCA2a protein levels by regulating its translation in wild type animals.

In an unpublished set of experiments, we found that Akt improves cardiac function in different types of mouse models of heart failure. This effect seems to be due to an increased mRNA translation of critical molecules involved in sarcomere contraction. We found that protein synthesis is depressed in advanced heart failure; Akt overexpression, through the activation of mTOR pathway, enhances protein synthesis, preventing heart failure.

The role of Akt in the control of Ca^{2+} current was also determined by generating models of inducible cardiac-specific knock-out mice. We found that ablation of PDK1, a kinase upstream of Akt, induces acute heart failure, dramatically affecting Ca^{2+} transients and plasmamembrane Ca^{2+} transport through L-Type Ca^{2+} channels.

Our experiments demonstrate that Akt is a critical molecule for Ca^{2+} homeostasis in the cardiac myocyte and that it regulates cardiac myocyte function through mechanisms which include but are not limited to Ca^{2+} metabolism control in the normal and failing heart.

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Neuronal NO Synthase (nNOS) and its Regulators in the Heart

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Nitric oxide (NO) has recently been shown to be a major regulator of cardiac signaling. nNOS (neuronal NO synthase) is an isoform of NOS subserving functions that are clearly distinct from eNOS and iNOS, notably regulation of β-adrenergic signal transduction as well as of mitochondrial oxygen consumption. nNOS is calcium-calmodulin dependent, and we have therefore sought to determine whether the sarcolemmal calcium pump is a regulator of nNOS.

The plasma membrane calcium ATPase (sarcolemmal calcium pump, PMCA) is regarded as a calcium transporter that is not directly involved in E-C coupling. However, we have previously shown *in vitro* that in non-cardiac cells PMCA4 is involved in signaling *via* its interaction with neuronal nitric oxide synthase (nNOS). By generating and studying a PMCA4 null mutant mouse line (*Pmca4*$^{-/-}$) we have now shown that PMCA4 modulates cardiac function *in vivo* through its regulator interaction with nNOS, but that it does not have a role in E-C coupling. Calcium was extruded normally from *Pmca4*$^{-/-}$ cardiomyocytes, but *in vivo*, both contractility and speed of relaxation were significantly elevated. Overexpression of PMCA4 reduced the β-adrenergic contractile response *in vivo*, however, when we overexpressed PMCA mutant ct120, which does not interact with nNOS, these changes did not occur. We have also demonstrated that PMCA4 ablation abolishes the nNOS-dependent diastolic dysfunction accompanying β-adrenergic induced cardiac hypertrophy.

In conclusion, we have demonstrated that PMCA4 carries signals in the myocardium which are relevant to both physiological heart function and the progression of diastolic dysfunction in cardiac hypertrophy. These signals are independent of E-C coupling and are mediated by nNOS. In addition to providing novel insights into the regulation of NOS, our results provide the first clear evidence that there are distinct transporters and hence mechanisms for the two functions of calcium in the heart, contraction and signaling.

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Cardiac Endothelial-type Nitric Oxide Synthase (eNOS) in Myocardial Biology

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The role of nitric oxide as a regulator of cardiac function was suggested in the early nineties, but a consensual view of its main functions in cardiac physiology is only emerging on the basis of experiments using systemic or cardiomyocyte-specific genetic deletion or overexpression of the three nitric oxide synthases (NOS). Unlike exogenous, pharmacologic NO donors, signaling by endogenous NO is restricted to intracellular effectors co-localized with each NOS in specific subcellular compartments, e.g., in caveolae where eNOS co-localizes with caveolin-3 in cardiomyocytes. These both ensures coordinate signaling by the three NOS isoforms on distinct (but sometimes overlapping) aspects of cardiomyocyte function and helps to reconcile previous apparently contradictory observations based on the use of non-isoform-specific NOS inhibitors. In particular, eNOS sustains normal EC coupling and contributes to the early and late phases of the Frank-Starling mechanism of the heart. It also attenuates the β1-/β2-adrenergic increase in inotropy and chronotropy, and reinforces the post-synaptic vagal control of cardiac contraction. By doing so, eNOS protects the heart against excessive stimulation by catecholamines, just as an "endogenous beta-blocker". In the ischemic and failing myocardium, induced iNOS further reinforces this effect, as does eNOS coupled to overexpressed β3-adrenoceptors. In addition to their direct regulation of contractility, the cardiac NOS exert pleiotropic effects on the cardiac cell biology, such as modulation of oxygen consumption, substrate utilization, sensitivity to apoptosis, regenerative potential and hypertrophic remodeling. The latter was recently put into question on the basis of apparently discordant effects of eNOS to protect from, or aggravate left ventricular hypertrophy in response to pressure overload. Consideration of the post-translational regulation of eNOS activity, e.g., its propensity to produce superoxide anions instead of NO upon enzyme uncoupling, may help to resolve this apparent contradiction. Accordingly, pharmacologic modulation of cardiac eNOS activity (and protection from uncoupling) in stress situations should provide new, attractive therapeutic approaches of myocardial diseases beyond the effects currently achieved with exogenous NO donors. These may be particularly powerful to enhance cardiac regeneration from resident cardiac stem cells.

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Healing, Inflammation, and Vascular Diseases

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Cell-Cell Interactions in the Vascular Wall

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Abstract

The interactions of circulating leukocytes and platelets with cells of the vascular wall during their inflammatory or atherogenic recruitment are governed by the sequential involvement of multiple adhesion molecules and chemokines. In particular, this extended abstract will cover the mechanistic role of platelet-derived chemokines and their receptors, e.g. RANTES and CCR5, and the role of platelet and vascular adhesion molecules, e.g. P-selectin and junctional adhesion molecule-A, in supporting arterial recruitment of mononuclear cells during atherosclerotic and neointimal lesion formation. In case of the chemokines, the intricate interplay in the functional actions of these molecules may be defined by an "interactome".

Zusammenfassung

Die Interaktionen zirkulierender Leukozyten und Thrombozyten mit Zellen der Gefäßwand während der entzündlichen oder atherogenen Rekrutierung wird durch die sequentielle Beteiligung multipler Adhäsionsmoleküle und Chemokine kontrolliert. Insbesondere wird diese Zusammenfassung die mechanistische Rolle thrombozytärer Chemokine und ihrer Rezeptoren, z. B. von RANTES und CCR5, sowie thrombozytärer und vaskulärer Adhäsionsmoleküle, z. B. von P-Selektin und junktionalem Adhäsionsmolekül JAM-A, bei der Rekrutierung mononukleärer Zellen in die arterielle Gefäßwand und der Ausbildung neointimaler und atherosklerotischer Läsionen behandeln. Im Fall der Chemokine kann das komplexe Zusammenspiel der funktionell relevanten Interaktionen in einem „Interaktom" definiert werden.

1. Introduction

An impressive body of work has established the current paradigm of atherosclerosis as an inflammatory process that promotes lesion development and progression (Zernecke and Weber 2005). Early atheroma formation is characterized by leukocyte recruitment and expression of inflammatory mediators which is confounded in the context of hyperlipidemia. The emigration of mononuclear cells is also critical for vascular remodeling and neointima formation following vascular injury and is governed by sequential interactions of adhesion and signal molecules, e.g. chemokines. Beyond a balance of pro- and anti-inflammatory cytokines, understanding the role of effector chemokines, which attract leukocyte subsets and are expressed with apparent redundancy in vascular lesions, provide more specific mechanistic clues defining different stages and qualities of atherosclerotic lesions (Weber et al. 2004). The function of chemokines in distinct steps during mononuclear cell recruitment to vascular lesions has been studied in transgenic mice and other suitable models, displaying a high degree of specialization, both synergistic and antagonistic interactions, which can be defined by the chemokine "interactome" (Weber and Koenen 2006). The importance of platelet

chemokines deposited on endothelium to trigger inflammatory monocyte arrest, differences in the involvement of chemokines between native and neointimal lesion formation, and the contribution of (junctional) immunoglobulin adhesion molecules in coordinating atherogenic cell interactions are particularly noteworthy (Weber 2003, 2004). A novel role of chemokines and platelets in the recruitment of vascular progenitors during neointimal hyperplasia and in the recovery of endothelial denudation underscores their relevance for atherosclerotic disease. The molecular complexity of vascular cell recruitment may allow a more selective therapeutic targeting of different atherosclerotic conditions.

2. Platelets, Chemokines and their Receptors in Vascular Lesion Formation

The family of chemokines regulates the activation of leukocytes and coordinates their trafficking to sites of inflammation and during immune surveillance. Besides the widely appreciated and reviewed role of vascular chemokines, which can exert specialized functions (Weber 2004), platelet-derived chemokines have recently been involved in the atherogenic cell recruitment, neointimal and atherosclerotic lesion formation (Weber 2005). The deposition of the chemokine RANTES by platelets has been shown to trigger shear-resistant monocyte arrest on inflamed or atherosclerotic endothelium (von Hundelshausen et al. 2001). The delivery of RANTES by platelets may epitomize a novel principle relevant to inflammatory or atherogenic monocyte recruitment from the circulation. Our data further suggest that RANTES deposition and subsequent monocyte arrest are promoted by platelet P-selectin and involved in wire-induced intimal hyperplasia, and that blocking RANTES receptors attenuates neointima formation and macrophage infiltration in apolipoprotein E (ApoE)-deficient mice (Schober et al. 2002). This mechanism represents an important component explaining the protection against neointimal growth in P-selectin-deficient mice and may entail a novel approach to the treatment of restenosis or atherosclerosis by the administration of chemokine receptor antagonists. Results from another study further insinuate that circulating activated platelets and platelet-leukocyte/monocyte aggregates promote formation of atherosclerotic lesions in $ApoE^{-/-}$ mice. This role of activated platelets in atherosclerosis is attributed to platelet P-selectin-mediated delivery of platelet-derived proinflammatory factors to monocytes/leukocytes and the vessel wall (Huo et al. 2003). Circulating platelet microparticles (PMP) may serve as a finely tuned transcellular delivery system for RANTES, triggering monocyte arrest to inflamed and atherosclerotic endothelium, introducing a novel mechanism for platelet-dependent monocyte recruitment in inflammation and atherosclerosis (Mause et al. 2005). The blockade or deficiency of PMP-expressed adhesion receptors demonstrated differential requirement of P-selectin, glycoprotein Ib (GPIb), GPIIb/IIIa, and junctional adhesion molecule-A for PMP interactions with endothelium, PMP-dependent RANTES deposition, and subsequent monocyte arrest.

3. Functional and Heterophilic Oligomerization of Chemokines

The heptahelical-receptor-binding and function of chemokines is thought to be governed by their interaction with cell surface proteoglycans and oligomer formation. For instance, the formation of higher order aggregates of RANTES bound to the endothelial surface is critical

for triggering the arrest of monocytes and activated T cells on activated endothelium (Baltus et al. 2003). Recent studies revealed that heterophilic interactions between chemokines can significantly modify their biological activities and through these we can gain initial insights into the structural basis underlying this novel regulatory mechanism. Our results suggest that heterophilic interactions with platelet factor 4 require structural motifs important in RANTES oligomerization and amplify RANTES-triggered effects on monocyte adhesion (von Hundelshausen et al. 2005). This may have implications for the modulation of inflammatory recruitment by platelet-derived chemokines. We have proposed the concept of a functional "interactome", constituted by a variety of heterophilic chemokine-chemokine interactions in particular microenvironments. This model could establish how signals either synergistic or antagonistic conferred by various chemokines are integrated for the combinatorial control of leukocyte responses (Weber and Koenen 2006). This will also be validated in suitable *in vivo* models.

4. The Special Role of Junctional Adhesion Molecule (JAM)-A in Atherogenic Recruitment

The exact molecular mechanisms of transendothelial diapedesis of leukocytes are still only incompletely understood (Weber 2003). We have identified the immunoglobulin superfamily member JAM-A as a counter-receptor for LFA-1 that is ideally situated to guide and control the apical access for transendothelial migration during inflammatory or atherogenic leukocyte recruitment (Ostermann et al. 2002). Additional studies have provided the first evidence that the I-domain of LFA-1 contains a functional and cation-dependent binding site for JAM-A (Fraemohs et al. 2004). Soluble forms of JAM-A can be effectively applied to inhibit distinct steps of mononuclear cell recruitment on inflamed or atherosclerotic endothelium. In conjunction with its expression and re-distribution on the apical surface of atherosclerotic endothelium, this suggests a functional contribution of JAM-A to atherogenesis (Ostermann et al. 2005). The recombination of JAM-A-deficient mice into an atherogenic background (ApoE-deficient mice) indeed revealed a crucial role of JAM-A in accelerated lesion formation and monocyte infiltration in atherosclerosis-prone mice (Zernecke et al. 2005). One mechanism underlying reduced recruitment was implied by findings that the luminal expression of the arrest chemokine RANTES in injured arteries and its endothelial deposition by activated platelets *in vitro* were diminished by JAM-A deficiency.

5. The Axis Stromal Cell-derived Factor (SDF)-1/CXCR4 in Neointimal Lesion Formation

Circulating smooth muscle progenitor cells have been identified as a source for neointimal smooth muscle cells after various types of injuries to the vessel wall contributing to neointimal hyperplasia implying a fundamental role of these progenitors in the vascular response to injury. Recent studies have provided insights into the molecular mechanisms involved in mobilization and local recruitment of smooth muscle progenitor cells. The CXC-chemokine SDF-1α and its receptor CXCR4 have been identified as the central signaling axis regulating the homing of smooth muscle progenitor cells to the injured vessel wall. The origin of

neointimal smooth muscle cells (SMCs) has previously been attributed to the local medial vessel wall layer. The finding that SMCs can be derived from circulating or adventitial SMC precursors after mechanical injury, heart transplantation and in genuine atherosclerosis has extended the concept of neointimal formation considerably (Schober et al. 2006). Interestingly, the upregulation of SDF-1 expression was observed in the vessel wall and increased plasma SDF-1 have been observed early after wire-induced endothelial denudation and have been associated with the degree of injury and subsequent SMC apoptosis. Blocking SDF-1 in ApoE-deficient mice after wire-injury resulted in a significant reduction in neointimal area by inhibiting the accumulation of bone marrow-derived SMC progenitors in the neointima (Schober et al. 2003, Zernecke et al. 2005). The effect of SDF-1 on SMC progenitor recruitment is dependent on the expression of CXCR4 on bone marrow cells, as neointimal hyperplasia as well as the neointimal SMC content is diminished in $ApoE^{-/-}$ mice after bone marrow reconstitution with fetal hematopoietic stem cells obtained from $CXCR4^{-/-}$ mice (Zernecke et al. 2005). In contrast, monocyte and monocyte-derived foam cell content is not affected by blocking SDF-1. Moreover, lentiviral antagonist transfer revealed that the local SDF-1 expression is critical for neointimal growth in the injured artery. In early SMC progenitor cell recruitment after injury, SDF-1 is presented by surface-adherent platelets and can significantly increase the P-selectin-dependent arrest of progenitor cells to the injured vessel. While most $lin^-/sca\text{-}1^+$ SMC progenitor cells attracted by SDF-1 express the PDGF-β receptor, which has been found on fetal SMCs, the SDF-1-dependent mobilization and recruitment was observed for both $c\text{-}kit^+$ and $c\text{-}kit^-$ SMC progenitors, however $c\text{-}kit^-/PDGF\text{-}\beta^+/lin^-/sca\text{-}1^+$ progenitors preferentially gave rise to neointimal SMCs. These findings epitomize the importance of SDF-1 for tissue repair and identify a prime target to limit neointimal lesion development. A potentially stabilizing role in native atherosclerosis remains to be defined (Schober et al. 2006) and subject to future investigation.

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Vascular Inflammation in Diabetes mellitus

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Abstract

Extensive and premature atherosclerosis occurs in diabetes (macro-vascular complications), and this is accompanied by small vessel disease (micro-vasculopathy), causing damages in the retina, the nervous system, the kidney and other organs. An understanding of the mechanisms leading to macro- and micro-vascular disease in diabetes requires the search of specific triggers. Investigations in vascular and molecular biology in the last ten years have identified at least three diabetic triggers, namely glucose, the advanced glycation end-products, and insulin. Glucose itself, when present in concentrations compatible with diabetic hyperglycemia, leads to acute endothelial changes characterized by the acquisition of a pro-thrombotic, pro-atherogenic phenotype. When hyperglycemia is prolonged, it also leads to the non-enzymatic transformation of otherwise innocuous proteins, such as serum albumin, leading to the formation of advanced glycation end-products. These act at the level of extracellular matrix and changing intracellular signal transduction pathways, as well as extracellular signaling molecules, interacting with at least one characterized receptor termed Receptor for AGEs (RAGE). By this receptor-mediated stimulation, endothelial cells express adhesion molecules and tissue factor. Since RAGE is characteristically upregulated by the presence of its ligands, AGE stimulation leads to a vicious circle of local vascular inflammation, recruiting several types of leukocytes, including monocytes, lymphocytes and neutrophils into the vessel wall and the surrounding tissues, potentially explaining some features of end-organ damage accompanying diabetes. Finally, insulin, at low micromolar concentrations, is itself able to selectively increase the expression of vascular cell adhesion molecule-1, promoting macrovascular disease. This occurs through a MAP-kinase mediated pathway, prevailing – in situations of insulin resistance and type-2 diabetes – over another intracellular signaling pathway, the PI3 kinase pathway leading to increased availability of nitric oxide and by itself being anti-atherogenic. Knowledge of these mechanisms may help in devising new therapeutic strategies in diabetes and interpreting the somewhat puzzling results of trials of intensive glycemic control.

Zusammenfassung

Ausgedehnte und frühzeitige Atherosklerose tritt bei Diabetes (makrovaskuläre Komplikationen) auf und wird von Erkrankungen der Mikrogefäße (Mikrovaskulopathie) begleitet, die zu Schäden an der Retina, dem Nervensystem, den Nieren und an anderen Organen führen. Die Aufklärung der Mechanismen, welche zu Erkrankungen der Makro- bzw. Mikrogefäße bei Diabetes führen, erfordert die Suche nach spezifischen Auslösern. Untersuchungen im Bereich der Gefäß- und Molekularbiologie der letzten zehn Jahre haben zu Erkenntnissen über mindestens drei Diabetesauslöser geführt, und zwar Glucose, die fortgeschrittenen Glykationsendprodukte sowie Insulin. Glucose selbst, wenn sie in Konzentrationen vorhanden ist, die diabetischer Hyperglykämie entsprechen, führt zu akuten Veränderungen des Endothels, die durch den Erwerb eines prothrombotischen, proatherogenen Phänotyps charakterisiert sind. Bei andauernder Hyperglykämie führt sie auch zu einer nichtenzymatischen Transformation von ansonsten harmlosen Proteinen, z. B. Serumalbumin, was zur Bildung fortgeschrittener Glykationsendprodukte führt. Diese wirken auf der Ebene der extrazellulären Matrix, indem sie die intrazellulären Wege der Signalweiterleitung verändern, sowie als extrazelluläre Signalübertragungsmoleküle, die mit mindestens einem beschriebenen Rezeptor, dem sogenannten Rezeptor für AGE (RAGE), in Wechselwirkung treten. Durch diese Rezeptor-vermittelte Stimulation kommt es bei den Endothelzellen zur Expression von Adhäsionsmolekülen und Gewebefaktor. Da RAGE typischerweise in Gegenwart seiner Liganden hochreguliert wird, führt die AGE-Stimulation zu einem Teufelskreis von lokaler Gefäßentzündung, die mehrere Leukozytenarten auf den Plan ruft, einschließlich Monozyten, Lymphozyten und Neutrophilen in der Gefäßwand und den umgebenden Geweben. Daraus erklären sich wahrscheinlich einige Erscheinungen der Endorganschädigung als Begleiterscheinung des Diabetes. Schließlich ist Insulin bei niedermolekularen Konzentra-

tionen selbst in der Lage, selektiv die Expression des Gefäßzellenadhäsionsmoleküls 1 zu verstärken und damit die Makrogefäßkrankheit zu fördern. Dies erfolgt über einen MAP-Kinase-vermittelten Weg, der sich bei bestehender Insulinresistenz und Diabetes Typ 2 gegenüber einem anderen intrazellulären Signalübertragungsweg, dem Kinase PI3-Weg, durchsetzt, der zu erhöhter Verfügbarkeit von Stickstoffmonoxid führt und so selbst antiatherogen ist. Die Kenntnis dieser Mechanismen kann bei der Festlegung neuer Therapiestrategien bei Diabetes und der Deutung der etwas rätselhaften Ergebnisse von Versuchen zu intensiver Glykämiekontrolle helfen.

Macro- and micro-vascular complications are both main causes of morbidity and mortality in type-2 diabetes (Beckman et al. 2002), but macrovascular disease, essentially an accelerated form of atherosclerosis, precedes, even by as many as ten years, the development of diabetic hyperglycemia (Turner and Holman 1996), and broadly characterizes the insulin resistance syndrome, where high insulin levels are needed to provide glycemic control (Stout 1996). High glucose (Cosentino et al. 2003) and glucose-induced protein and lipid modifications – the advanced glycation endproducts (Basta et al. 2004) – are considered main triggers of mechanisms leading to vascular complications once diabetic hyperglycemia has ensued. However, what causes atherosclerosis in insulin resistance, at least partially independent of often associated risk factors such as hypertriglyceridemia, low levels of high-density lipoproteins (HDL) and hypertension (Laakso 1996, Despres et al. 1996), remains a matter of controversy.

Syndromes of insulin resistance are characterized by hyperinsulinemia, and this has long been speculated to be causally linked to vascular disease (Despres et al. 1996, Pyorala 1979, Stout 1990). On endothelial cells, insulin induces the expression and release of plasminogen activator inhibitor(PAI)-1(10) and of various growth factors and cytokines, mostly through a pathway linked to the activation of mitogen-activated protein kinases (MAPK) (Cusi et al. 2000, Soop et al. 2002). However insulin, through an insulin receptor substrate(IRS)-1/phosphatidyl inositol(PI)-3 kinase-calcium/calmodulin-dependent pathway (Zeng et al. 2000), also promotes a rapid increase in nitric oxide (NO) bioavailability (Zeng and Quon 1996), able to suppress the expression of endothelial leukocyte adhesion molecules (De Caterina et al. 1995). This has led to the concept of insulin as an anti-atherogenic hormone (Dandona et al. 2002). We have recently shown, however, that insulin, at pathophysiologically relevant concentrations, leads *per se* to increased expression of vascular cell adhesion molecule-1-(VCAM-1) (Madonna et al. 2004), probably the adhesion molecule most relevant to the development of atherosclerosis (Cybulsky et al. 2001), in a system where insulin may still increase NO bioavailability. We have also shown that this effect can be potentiated by the PI-3-kinase inhibitor wortmannin (Madonna et al. 2004), leading to postulate a balance in endothelial insulin signaling, linking insulin with endothelial VCAM-1 expression, likely modulated in conditions of insulin resistance (Jiang et al. 1999). Because insulin's ability to induce VCAM-1 expression is a plausible explanation for macrovascular disease accompanying hyperinsulinemic conditions, we have then examined potential molecular mechanisms involved in this specific pattern of endothelial activation.

Human umbilical vein endothelial cells were incubated with insulin (0–24 h) ± inhibitors of signaling pathways potentially involved. At pathophysiological concentrations (10^{-9}–10^{-7} mol/l), insulin selectively induced VCAM-1 expression (enzyme immunoassays, flow-cytometry, immunocytochemistry and immunoblotting). Incubation of endothelial cells with inhibitors of ERK1/2 failed to affect insulin-induced VCAM-1 expression. Conversely, the p38 mitogen activated protein (MAP) kinase inhibitors SB203580 and SB202190, the protein

kinase C(PKC)-β inhibitor LY379196 and, partially, the c-Jun NH_2-terminal kinase (JNK) inhibitor SP600127, all tested at concentrations around their IC_{50} for inhibition of substrate phosphorylation, decreased insulin effect on VCAM-1. Gene silencing by small interfering RNA significantly reduced the expression of p38MAPK, and this was accompanied by suppression of insulin-stimulated VCAM-1 expression. Treatment with insulin also led to activation of NF-κB.

In conclusion: Pathophysiological insulin concentrations increase VCAM-1 expression and activate NF-κB. This mostly occurs through stimulation of p38MAPK, with additional effects of PKC-β. Inflammation is thought to play a key role in the pathophysiological mechanisms of atherosclerosis occurring in insulin-resistance and obesity (Festa et al. 2000). Our current findings are likely relevant to an understanding of the central, unresolved question of whether compensatory hyperinsulinemia has any detrimental action *per se* in insulin resistance, or is simply a marker of this disease state (Baron 1996, Schmidt and Stern 2000). Our results point to a pathogenetic role of pathophysiological and pharmacological insulin concentrations in vascular disease. On the other hand, our data support further research on the use of specific inhibitors of the MAPK and PKC pathways (or of their downstream effectors, such as NF-κB), as novel pharmacological agents for treatment of diabetic vascular disease.

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Drug-eluting Stents – Promises and Pitfalls

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With 1 Figure

Abstract

Drug-eluting stents (DES) have become the most widely used stents to prevent restenosis and to reduce the need for repeated revascularization. There is a time-dependent effect of drug-eluting stents on endothelial function in the persistent region which is associated with endothelial dysfunction.

Zusammenfassung

Medikament-abgebende Stents (DES) sind heute die am weitesten verwendete Stentart zur Reduzierung der Restenose und Verhinderung einer Reintervention. Sie haben eine negative Wirkung auf die Endothelfunktion im Persistenzbereich.

Drug-eluting stents (DES) have become the most widely used stents to prevent restenosis and to reduce the need for repeated revascularization. In our country up to 90 % of these stents (2006–2007) are implanted in patients with coronary artery disease and have dramatically reduced the need for re-interventions but have increased the costs of coronary interventions. Despite this success, late and very late stent thrombosis have become a problem because patients stop dual antiplatelet therapy or have to stop it due to minor surgical interventions which may lead to the risk of sudden stent thrombosis. Previous examinations of autopsy samples have shown that up to 70 % of these drug-eluting stents are not or incompletely re-endothelialized and, thus, represent the basis for late and very late stent thrombosis. Early or subacute stent thrombosis has a mortality risk of 3–8 %, whereas late and very late stent thrombosis bares a risk which is ten times higher, namely 40–50 % of mortality rate. Currently, patients are advised to adhere to a 6 or better 12 months dual antiplatelet therapy and in cases with high risk to take this medication life-long.

Not only the risk of late or very late stent thrombosis is increased in these patients but there is endothelial dysfunction in the peristent region which has been observed in patients with Sirolimus- but also with Paclitaxel-eluting stents (Fig. 1).

During supine bicycle exercise patients with drug-eluting stents demonstrated active vasoconstriction in the proximal and distal peristent (10 mm) region during exercise which was reversible after sublingual nitroglycerin. These data indicate that endothelial dysfunction is responsible for this paradoxical vasoconstriction adjacent to drug-eluting stents because in a group of patients with control (bare-metal) stents no abnormal vasoconstrictory response was observed. When these data are plotted against the time interval after stent implantation, an inverse linear relationship was observed indicating that endothelial dysfunction may dis-

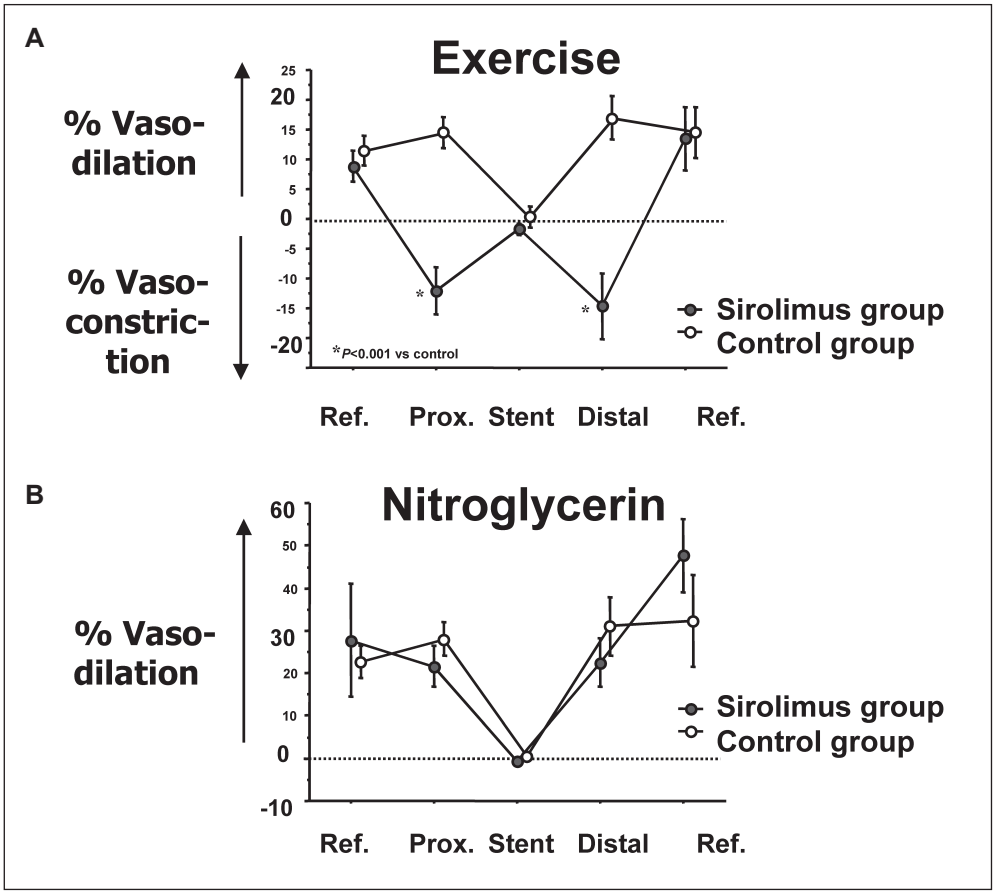

Fig. 1 Line chart with percent changes of the mean cross-sectional lumen area in the sirolimus and control groups during exercise (*A*) and after nitroglycerin application (*B*). Mean values ± SEM are shown for the proximal reference segment (Prox.Ref.), the proximal segment (Prox.), the stent segment (Stent), the distal segment (Distal), and the distal reference segment (Distal Ref.). The stent segment does not elicit any vasomotion. The sirolismus group shows exercise-induced vasoconstriction of the proximal (–12 ± 4 %) and distal (–15 ± 6 %) segment to the stent, whereas the control group demonstrates exercise-induced vasodilation of the respective segments (15 ± 3 % and 17 ± 4 %) ($p < 0.001$). The proximal and distal reference segments dilate during exercise in both groups. After nitroglycerin application, all the segments (except for the stent) show maximal vasodilation. (Togni et al. 2005)

appear after one to two years of implantation (vaseular healing). This abnormal vasomotor response may, however, represent a new target for re-occurrence of vascular damage and atherosclerosis.

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Chemokines in Atherosclerosis

Israel Charo (San Francisco, CA)

Our laboratory focuses on the early events in the development of atherosclerotic heart disease. Atherosclerosis (commonly known as "hardening of the arteries") actually begins very early in life. Autopsies of combat casualties during the Korean War revealed extensive fatty streaks (the earliest recognizable sign of atherosclerosis) in young men in their early twenties. Fatty streaks are composed of scavenger-type cells, known as macrophages, that have ingested large quantities of lipids. Our high-fat Western diet is the source of these lipids, and circulating white blood cells, known as monocytes, are the precursors of these lipid-laden macrophages.

Research in our laboratory attempts to clarify why monocytes leave the bloodstream and enter the artery wall to ingest lipids, and thereby to contribute to forming fatty-streaks. A chemokine protein, known as monocyte chemoattractant protein 1 (MCP-1), was found to be made by cells in the artery wall in response to a high-fat diet. This specialized protein is responsible for recruiting monocyte/macrophages out of the blood stream. MCP-1 is thus a key molecular link between high-fat diets and the beginnings of atherosclerosis.

To understand how MCP-1 contributes to heart disease, we started by identifying its specific receptor and cloning its gene. This receptor, known as CCR2 (chemokine receptor 2), is expressed on monocytes and directs their migration to sites where MCP-1 is present. To demonstrate the importance of MCP-1 and CCR2 in atherosclerosis, we turned to mouse models of this disease. Using a genetic engineering technique called homologous recombination, we created mice that are genetically deficient in CCR2 (CCR2 knockout mice) and found that they are resistant to diet-induced atherosclerosis. These studies established the importance of CCR2 in the development of atherosclerosis, and provide the rationale for the current effort in many of the large pharmaceutical companies to develop drugs that block CCR2 activation by MCP-1.

We have also found that the chemokine known as fractalkine drives part of the atherosclerotic response to high-fat diet. Studies in fractalkine receptor (CX3CR1) knockout mice revealed that they were resistant to atherosclerotic lesion formation. Studies are now under way to understand that relative contributions of CCR2 and CX3CR1 to atherogenesis.

Recent work in the laboratory has focused on the role of chemokines in monocyte homeostasis. We have found that CCR2 null mice have a significant deficit in the number of circu-

lating monocytes, and that this is due to a need for CCR2 in monocyte mobilization from the bone marrow.

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Healing, Inflammation, and Stem Cell Therapy in Different Organs

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Neurotrophic Factor Signaling in Neural Stem Cell Differentiation and Survival: The Role of Bag1 as a Scaffold for B-RAF/Akt Function on the Surface of Mitochondria

Stefan Wiese [1], Bettina Holtmann [1], Rudolf Götz[2], and Michael Sendtner[1]

With 2 Figures

Abstract

Differentiation and survival of developing neurons depends on signaling molecules from innervated target and glial cells. These neurotrophic factors belong to several gene families, and the survival effect of these proteins is mediated through specific receptors. These signaling pathways modulate cell division, growth and survival in many cell types, and RAF kinases play an essential role in coordinating these processes.

Zusammenfassung

Differenzierung und Überleben sich entwickelnder Neuronen werden von Signalmolekülen aus innervierten Target- und Gliazellen bestimmt. Diese neurotrophen Faktoren gehören zu verschiedenen Genfamilien. Der Überlebenseffekt dieser Proteine wird durch spezifische Rezeptoren vermittelt. Diese Signalübertragungswege modulieren Zellteilung, Wachstum und Überleben vieler Zellarten. RAF-Kinasen spielen bei der Koordinierung dieser Prozesse eine wichtige Rolle. In diesem Beitrag werden die Signalübertragungswege in isolierten neutralen Stammzellen und Nervenzellen an Hand mehrerer Mausmutanten beschrieben.

Differentiation and survival of developing neurons depends on signaling molecules from innervated target and glial cells. These neurotrophic factors belong to several gene families including the neurotrophin family of nerve growth factor (NGF) related molecules, the ciliary neurotropic factor (CNTF) family of pluripotent cytokines, and growth factors, such as insulin-like growth factor I (IGF-I), with multiple actions in many tissues. The survival effect of these proteins is mediated through specific receptors that activate downstream signaling pathways, in particular the MAPK and PI-3K/Akt pathways.

These signaling pathways modulate cell division, growth and survival in many cell types including neural stem cells, neurons, cardiomyocytes and corresponding stem cells in the heart. RAF kinases play an essential role in coordinating these signaling pathways. In mammals, the RAF-kinase family includes 3 members, A-RAF, B-RAF and C-RAF-1. Gene knockout mice for B-RAF or C-RAF die during embryonic development between day 12 and 15, showing multiple organ defects, in particular in the cardiovascular system (Wojnowski et al. 1997, 1998, 2000). Therefore, we have isolated motoneurons from E12 C-RAF- and B-RAF-deficient mice and investigated their capacity to respond to neurotrophic factors for survival. Neurons isolated from mice lacking C-RAF can survive in the presence of neurotrophic factors. Survival in response to these factors is indistinguishable from wild-type

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cells. In contrast, B-RAF-deficient sensory or motor neurons have lost their responsiveness to neurotrophic factors (Wiese et al. 2001). This appears interesting, as the B-RAF-kinase, in contrast to C-RAF, is activated by extracellular signals which lead to elevated cAMP. Thus, B-RAF could be an important integrator of neurotrophic factors and neural activity through neurotransmitter receptors.

In neural stem cells and neurons, B-RAF is localized mainly at mitochondria, and co-localizes with Bag1, a co-chaperone for Hsp70. Bag1 has also been found to interact with C-RAF, Bcl-2 and other proteins. In order to determine how Bag1, B-RAF and other proteins work together in regulating neural differentiation and survival, we have generated Bag1 gene knockout mice. The first 2 coding exons of Bag1 were deleted in order to disrupt the expression of all isoforms of the corresponding Bag1 protein (Goetz et al. 2005). Homozygous Bag1$^{-/-}$ mice die around embryonic day 13. At that time, the embryos showed normal body size. Histological analysis revealed massive apoptosis in the fetal liver and the developing nervous system. The fetal liver of Bag1$^{-/-}$ mice was smaller than in wild-type mice, and TUNEL as well as propidium iodide staining revealed massive apoptosis, suggestive of a severe defect in hematopoiesis. The Bag1$^{-/-}$ embryos were also anemic as a result of defective erythropoiesis in the fetal liver. Similar defects in neuroepithelial stem cells survival were also observed in the forebrain vesicles (Fig. 1).

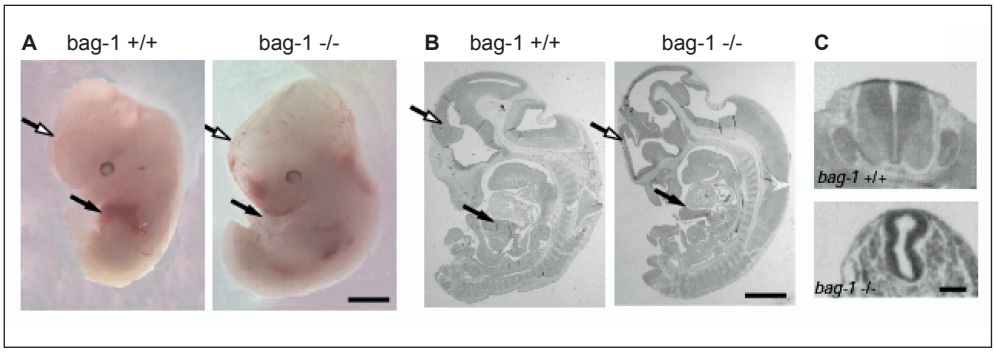

Fig. 1 12.5 day old Bag1$^{-/-}$ embryos show severe defects in size of forebrain vesicles and neural tube formation. (*A*) Size of Bag1$^{-/-}$ embryos is normal at E12.5, but erythropoiesis in the fetal liver (black arrow) is reduced, and forebrain vesicles (open arrows) are macroscopically not detectable. (*B*) Open arrows indicate reduced size of forebrain vesicles. (*C*) The neural tube is smaller, and the lateral part dos not properly form in E12.5 Bag1$^{-/-}$ embryos (from Goetz et al. 2005)

Therefore, we isolated neural stem cells from E11.5 forebrain of Bag1$^{-/-}$ and control embryos. Growth and survival of the stem cells in neurospheres did not differ between Bag1$^{-/-}$ and control stem cells. However, when the cells were dissociated and transferred to laminin coated dishes in order to induce differentiation, differences became apparent. The number of apoptotic cells after 24 h on laminin was massively enhanced in Bag1$^{-/-}$ stem cell cultures (Fig. 2*C*). Differential analysis of the Pax6 positive neural stem cells, the early differentiating neurons that are positive for the marker Doublecortin (Dc) or differentiated neurons that can be identified with neurofilament-M antibodies revealed that particularly the Pax6 positive stem cells showed enhanced vulnerability in the absence of Bag1.

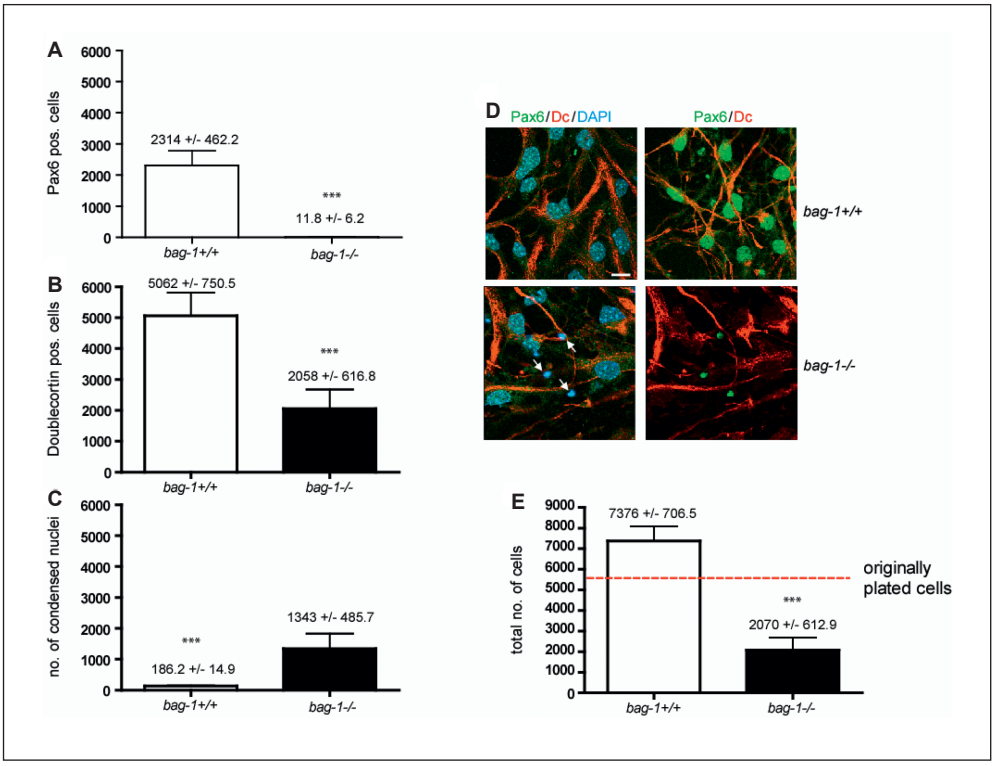

Fig. 2 Enhanced apoptosis of differentiating neurons from Bag1$^{-/-}$ Pax6-positive neural stem cells. (*A*) Quantitative analysis of Pax6-positive cells in cultures of differentiating Bag1$^{+/+}$ and Bag1$^{-/-}$ neural stem cells after 2 days on Poly-L-Lysine. (*B*) Quantitative analysis of Doublecortin (Dc)-positive cells in cultures of differentiating Bag1$^{+/+}$ and Bag1$^{-/-}$ neural stem cells after 2 days on Poly-L-Lysine. (*C*) Quantification of cells with condensed nuclei in Bag1$^{-/-}$ and control cultures of neural stem cells. (*D*) Images of Bag1$^{+/+}$ and Bag1$^{-/-}$ cultures of neural stem cells after 2 days under differentiating conditions on Poly-L-Lysine. Arrows point to condensed nuclei with Pax6 immunoreactivity. (*E*) The number of originally plated neural stem cells in Bag1$^{-/-}$ cultures is significantly reduced after 48 h under differentiating conditions.

It appeared that in particular the dividing Pax6 positive stem cells died at high numbers, and that the depletion of these stem cells was responsible for reduced numbers of neurons in the forebrain and neural tube of Bag1 deficient mouse embryos. These data reveal that neural stem cells, at a time when they divide asymmetrically to generate a neuron from one daughter cell and renew a stem cell from the other daughter cell, are particularly vulnerable in the absence of Bag1, suggesting that Bag1 is required in a signaling cascade that prevents apoptosis of these stem cells during this critical developmental period. The phenotype in the liver of Bag1$^{-/-}$ mice indicates that such signaling is not only required for neural stem cells but also for other stem cell populations such as hematopoietic stem cells.

In order to study the signaling pathway that is responsible for the enhanced apoptosis of Bag1$^{-/-}$ stem cells, the MAPK, RAF and PI-3/K-Akt pathways were analyzed. Lack of Bag1 does not disturb the primary function of Akt or RAF as the phosphorylation of their target proteins forkhead homologous transcription factor and Erk1/2 in the liver and brain of Bag1$^{-/-}$ embryos was normal.

However, the formation of a complex that is formed by Akt, B-RAF and Bag1 on the surface of mitochondria was disrupted. The Akt and B-RAF kinases did not co-localize anymore on the surface of mitochondria. As a consequence Bad phosphorylation at Ser136, a target for the complex of Akt and RAF on the surface of mitochondria, was absent. This defect corresponds to the limited capacity of self-renewal of stem cells during early development.

Future studies have to show whether this signaling pathway also regulates the capacity for self-renewal, mitosis and differentiation of stem cells in the postnatal nervous system and other organs in which renewal of cells from a stem cell population is critical for proper organ function. During the course of these studies, not only mouse mutants in which the genes for Bag1 and/or B-RAF can be deleted in a cell- or tissue-specific manner, but also upstream regulators for these pathways will be studied. Parallels between the mechanisms that guide differentiation and survival of early neurons, in particular motoneurons and cardiomyocytes, could guide the way in this context. Remarkably, the transcription factor islet-1, which plays a key role in orchestrating differentiation of motoneurons in the spinal cord (Ericson et al. 1992, Pfaff et al. 1996), is also expressed in a subset of cardiomyogenic progenitor cells (Yuan and Schoenwolf 2000) and expression of this transcription factor is downregulated both in motoneurons and cardiomyocytes when these cells differentiate (Yang et al. 2006). After differentiation, both the early motoneurons and cardiomyocytes respond to specific growth factors. The neurotrophic factors that support survival of embryonic motoneurons have been characterized in detail and include members of the neurotrophin, the ciliary neurotrophic factor (CNTF) family of pluripotent cytokines and pluripotent growth factors such as insulin-like growth factor-I. Thus, not surprisingly, cardiotrophin-1 (CT-1), a neurotrophic factor of the CNTF gene family, has first been discovered as a growth and survival factor for cultured cardiomyocytes (Pennica et al. 1995) before its physiological function as a survival factor for developing motoneurons has been revealed by the generation of CT-1 gene knockout mice (Oppenheim et al. 2001). When deletion of CT-1 is combined with gene defects in CNTF and LIF, these mice show more severe defects including enhanced loss of muscle strength and enhanced fatiguing in the running wheel (Holtmann et al. 2005). These mouse models could also be a useful tool to study signaling requirements for survival, self renewal and differentiation of cardiomyogenic stem cells as well as the requirements of differentiated cardiomyocytes for their maintenance.

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Nova Acta Leopoldina NF *95*, Nr. 351, 109–110 (2008)

Stem Cells and Hematologic Disorders

Hermann Einsele (Wuerzburg)

Abstract

Transplantation of hematopoetic stem cells is an important treatment modality in hematology/oncology. The use of immunotherapy for cancer treatment is a promising strategy. Allogeneic stem cell transplantation (allo-SCT) is the most widely used form of immunotherapy. The present experience shows clearly that the allo-SCT strategy should be further developed and refined because of the potential benefit that can be expected in some patients' sub-groups.

Zusammenfassung

Die Transplantation von hämatopoetischen Stammzellen ist ein wichtiger Bestandteil der Therapie von hämatologischen Neoplasien. Die Anwendung der Immuntherapie bei der Krebsbehandlung ist eine aussichtsreiche Strategie. Allogene Stammzellentransplantation (allo-SCT) ist die verbreitetste Form der Immuntherapie. Vorliegende Erfahrungen weisen eindeutig aus, dass die Allo-SCT-Strategie wegen des bei einigen Teilgruppen von Patienten zu erwartenden Nutzens weiterentwickelt und verfeinert werden sollte.

Cancer represents a major problem of public health. New therapeutic approaches are needed to complete the present strategies.

Several clinical studies in the early 1980s showed that the dose escalation of chemotherapy and/or radiation therapy made increased tumor reduction possible, in some cases even overcame resistance to certain mitotic toxins. However, when the chemotherapeutic and/or radiation dose was increased, aplasia took considerable time so that patients with this treatment were confronted with serious infectious complications. To make intensive chemotherapy more patient compliant and, most of all, to reduce the complications in connection with the protracted neutropoenia and thrombocytopenia, the application of bone marrow stem cells established itself as supporting action in the sense of an autologous stem cell transplantation in haematology and oncology.

For several haematological forms of neoplasia, notably multiple myelom and Non-Hodgkin lymphoma, high-dose therapy with autologous stem cell transplantation is a critical therapeutic approach for first-line treatment or in case of a relapse after primary treatment. In the meantime mobilization chemotherapy and mobilizing cytokine treatment successfully release sufficient numbers of stem cells from the bone marrow in the peripheral blood so that stem cells can be obtained from peripheral blood by leukapharesis and the removal of bone marrow under full general anaesthesia in the operation theatre is no longer necessary.

The first transplantation of allogenic stem cells dates much earlier, to the beginning of the 1970s. In contrast with autologous stem cell transplantation, the stem cells are obtained from another donor who in most cases has the same HLA, i.e., tissue characteristics, as the

patient. Here again, transfusion of haematopoetic stem cells of another donor is needed to avoid protracted aplasia due to chemotherapy and radiation, which is also applied as part of allogenic transplantation. In addition to this, allogenic stem cell transplantation involves the transfer of a new immune system which can act very efficiently against remaining tumor cells. The immune effector cells transferred from the donor detect the tumor cells as being foreign and attack them. This response is referred to as transplant against tumor or transplant against leukaemia response.

During the immunological reactions against the tumor cells, immunological reactions against other recipient cells also occur. Mainly in the acute phase, the so called transplant against host reaction, these responses are directed against the skin, the liver and the intestine. If such a reaction occurs, the patient must be treated intensively with immunosuppressive drugs. Meanwhile, allogenic stem cell transplantation has become an established method of treatment, most of all of acute leukaemia, but also of certain immunodeficiency syndromes. New methods with reduced chemotherapy and radiation now make it possible to treat elderly patients with allogenic stem cell transplantation. In addition to this, the reduced-dose conditioning therapy with allogenic stem cell transplantation is now increasingly also being evaluated in the therapy of recurrent lymphoma and myeloma and certain solid tumors. The immune effector mechanisms essential to tumor control (α- and β-TCR cells, λ-TCR cells, natural killer cells, etc.) are the topic of intensive research.

The use of immunotherapy for cancer treatment is a promising strategy. Allogeneic stem cell transplantation (allo-SCT) is the most widely used form of immunotherapy. The allogeneic immune effectors infused with the graft can recognize and eradicate the patients' malignant cells. The curative potential of allo-SCT is classically based on the cytoreduction of tumor cells induced during the high dose chemo- and/or radiotherapy-based conditioning regimen and the immune control mediated by allogeneic immune effectors (graft versus tumor effect, GVT). Allo-SCT is currently under important modifications because of better understanding of the GVT mechanisms and the development of new treatment techniques: (*i*) allo-SCT of peripheral blood stem cells after mobilization with G-CSF; (*ii*) donor lymphocyte infusion (DLI); and (*iii*) development of reduced intensity conditioning regimens aiming to reduce the toxicity while favoring the immune component of the anti-tumor effect. In addition to its established efficacy in hematological malignancies, several arguments are in favor of the use of allo-SCT also in neoplastic solid tumors. Promising results from advanced cancer patients were already obtained. The present experience shows clearly that the allo-SCT strategy should be developed and refined because of the potential benefit that can be expected in some patients' sub-groups.

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Hematopoietic Progenitor/Stem Cell Activities are Increased by Transient HDAC-Inhibitor Treatment

Albrecht M. Müller and Duttu Vallabhapurapu (Wuerzburg)

DNA and chromatin modifications are important for the establishment and maintenance of cell type-specific gene expression patterns that constitute cellular identities. One type of modification, the acetylation of histones occurs reversibly on lysine ε-NH_3^+ groups of core histones. Hyperacetylated histones are associated with active chromatin domains, whereas hypoacetylated histones are enriched in non-transcribed loci. The level of histone acetylation depends on the opposing activities of histone acetyltransferases and deacetylases (HDACs). Previously we reported that altering the epigenotype of neurosphere cells followed by transplantation enables the generation of neurosphere-derived haematopoietic cells. To analyze whether progenitor/stem cell activities in bone marrow can be increased by HDAC-inhibitor treatment, murine bone marrow cells were transiently treated with HDAC-inhibitors (Trichostatin A, hydroxamic acid analogues of trapoxin [SW68, SW70] and valproic acid) followed by *in vitro* and *in vivo* analysis of hematopoietic activities.

We observe that bone marrow cells are sensitive to HDAC-inhibitor treatment as assessed by PI and CFSE labeling studies in terms of cell death and proliferation respectively. In combined lineage-marker/PI staining we find a selective survival of immature cells following HDAC-inhibitor treatment due to the preferential death of mature cells. Bone marrow cultures treated with different HDAC inhibitors yielded greater numbers of $lin^-/ckit^+$ cells as compared to untreated cultures along with higher colony forming cell (CFC) numbers. Importantly, the increase in CFC numbers is both detected if CFC calculations are based on cell numbers prior or post HDAC inhibitor treatment indicating an actual increase in CFCs. The HDAC inhibitor-mediated increase in CFCs is reduced in BM cell cultures from $HoxB4^{-/-}$ mice suggesting an involvement of HoxB4. In competitive repopulation studies we observe increased hematopoietic engraftment potential in treated compared to untreated bone marrow cultures. We conclude that transient treatment of bone marrow cells with HDAC-inhibitors results in an increase of hematopoietic progenitor/stem cell numbers. These results substantiate the potentiality of chromatin modifications for the regulation of hematopoietic progenitor/stem cells.

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Stem Cells and Cardiac Diseases

Piero Anversa (Valhalla, NY)

The identification of cardiac progenitor cells in small and large mammals raises the possibility that the human heart contains a population of stem cells capable of generating cardiomyocytes and coronary vessels. The characterization of human cardiac stem cells (hCSCs) would have unprecedented clinical implications for the management of the failing heart. We established the conditions for the isolation and expansion of c-kit-positive hCSCs from small fragments of myocardium discarded at surgery. Additionally, we tested whether these cells have the ability to form functionally competent human myocardium after infarction in immunodeficient and immunosuppressed animals independently of cell fusion. Here we reported the identification *in vitro* of a class of human c-kit-positive cardiac cells which possess the fundamental properties of stem cells: they are self-renewing, clonogenic and multipotent. hCSCs differentiate predominantly into cardiomyocytes and to a lesser extent into smooth muscle cells and endothelial cells. When locally injected in the infarcted myocardium of immunodeficient mice and immunosuppressed rats, hCSCs generate a chimeric heart, which contains human myocardium composed of myocytes, coronary resistance arterioles and capillary profiles. Importantly, the differentiated human cardiac cells possess only one set of human sex chromosomes excluding cell fusion. Although the human myocardium shows an immature phenotype, it contracts regionally and contributes to the improvement in the hemodynamic performance of the infarcted left ventricle. In conclusion, hCSCs can be isolated and expanded *in vitro* from samples of myocardium, which is a prerequisite for autologous stem cell therapy in humans.

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Pro and Contra: Stem Cell Therapy in the Heart

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Adult Stem Cell Therapy in the Heart – All Hype and no Hope?

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Abstract

Stem cells derived from the bone marrow have been predicted to induce a favorable impact on the performance of the infarcted heart. Whereas early animal studies suggested a transdifferentiation of adult stem cells into cardiomyocytes, more recent studies have severely challenged this notion and indicated that only embryonic stem cells (ESC) might demonstrate the ability to replace the damaged myocard. Most of the experiments performed thus far have focused on the dramatic changes in the destiny, i.e. differentiation program of adult stem cells. There is an absolute paucity of data on the cellular and molecular processes involved in the complex cascade of (trans-) differentiation. Even the very first steps have not yet been elucidated, which are migration of the stem cells towards their niche and the interaction with the surrounding cells in the microenvironment. Before these are known, it is premature to translate the observations in animal models into clinical trials. Despite the lack of pathophysiologic evidence, many clinical studies have, in the meantime, been conducted, with contradicting results. Most of the trials examining the use of stem cells for cardiovascular diseases have severe limitations that render interpretation impossible. These include lack of controls, proper randomization, blinding, and lack of systematic assessment of infarct size or left ventricular function prior to administration of therapy. The follow-up period was mostly of short duration and most studies were performed with a small number of patients. A randomized study that included an adequate number of patients and with an adequate follow-up period (REVIVAL-2) showed no significant difference to controls. Moreover, a few studies have indicated an increase in restenosis in the intervention (stem cell) group. Hence it is premature to consider the clinical application of adult stem cells for heart diseases. More experimental evidence from the laboratory is required to address the plethora of questions before well-designed and definitive clinical trials can be performed.

Zusammenfassung

Von aus dem Knochenmark gewonnenen Stammzellen wird angenommen, dass sie eine positive Wirkung auf die Leistung des Herzens nach Infarkt haben. Auf der Basis früher Tierstudien war eine Transdifferentiation von adulten Stammzellen zu Kardiomyozyten angenommen worden. Neuere Untersuchungen widersprachen dieser Vorstellung und ergaben, dass nur embryonale Stammzellen (ESC) geschädigtes Myokard ersetzen können. Die Mehrzahl der bisher durchgeführten Untersuchungen konzentrierte sich auf die dramatischen Veränderungen im Differenzierungsprogramm der adulten Stammzellen. Dagegen besteht ein Mangel an Daten zu den Prozessen auf zellulärer bzw. molekularer Ebene, die Teil der komplexen Abläufe bei der (Trans-)Differenzierung sind. Selbst die allerersten Schritte sind bisher noch ungeklärt, so etwa die Migration der Stammzellen in ihre Nische und die Interaktion mit den umgebenden Zellen in der Mikroumwelt. Solange diese Vorgänge nicht verstanden sind, wäre es verfrüht, Beobachtungen an Tiermodellen in klinische Versuche zu übertragen. Ungeachtet der Defizite im Verständnis der Pathophysiologie wurden in der Zwischenzeit zahlreiche klinische Studien durchgeführt, durchaus mit widersprüchlichen Ergebnissen. Die Mehrzahl der Studien zum Einsatz von Stammzellen bei kardiovaskulären Erkrankungen weisen so weitgehende Einschränkungen auf, dass eine Interpretation unmöglich ist. Dazu zählen u. a. fehlende Kontrollen, Mängel in der Randomisierung bzw. dem Versuchsdesign sowie das Fehlen einer systematischen Bewertung der Infarktgröße bzw. der linken Herzkammerfunktion vor Therapiebeginn. Der Nachsorgezeitraum war meist sehr kurz, und die Mehrzahl der Studien erfolgte mit einer kleinen Patientenzahl. Eine Studie mit einer ausreichend großen Patientenpopulation und einer Nachsorgeperiode von angemessener Dauer (REVIVAL-2) ergab keine signifikanten Unterschiede zu der Kontrollgruppe. Ferner zeigte sich bei einigen Studien eine Zunahme der Restenose bei der Interventions-(Stammzellen-)gruppe. Daher sind Erwägungen zum klinischen Einsatz adulter Stammzellen für die Behandlung von Herzkrankheiten verfrüht. Es bedarf weiterer experimenteller Erkenntnisse im Labormaßstab zu einer Vielzahl von Fragen, ehe gründlich angelegte und definitive klinische Untersuchungen durchgeführt werden können.

1. Introduction

Hematopoietic stem cells (HSC) are multipotent stem cells that give rise to all the different lineages of the blood-forming cells. Recently it has been proposed that bone marrow might serve as a reservoir for cardomyogenic precursors. Several studies in animal models and a multitude of clinical trials suggested that stem cells from the marrow could be used to repair cardiac injury and that the hematopoietic stem cells could contribute to myocardial repair and regeneration. This concept of stem cell plasticity has been highly controversial. Albeit a few early clinical trials have suggested encouraging results, several randomized clinical trials on the cardomyogenic potential of hematpoietic stem cells have not been conclusive.

Based on the pathophysiologic evidence and on the results of the clinical trials we have little evidence for the direct cardiac regeneration by HSC.

For modern medical decision making, such as the use of hematopoietic stem cells for myocardial ischaemic diseases, in the age of evidence-based medicine, the following paradigm should apply: (*i*) a clear understanding of the pathophysiologic mechanism; (*ii*) a proper use of information derived from clinical trials that are systematic, reproducible and unbiased; (*iii*) an understanding of certain rules of evidence for a proper assessment of the medical literature.

2. What is the Pathophysiologic Evidence?

Most of the evidence provided for transdifferentiation of adult stem cells has been inadequate. The concept that bone marrow stem cells have plasticity to differentiate into different cell types depending on their local environment challenged a long-held dogma of developmental biology. A series of experiments by Wagers et al. critically examined the hypothesis that HSC can give rise to non-hematopoietic cells *in vivo*. Their experiments found little contribution of HSC to non-hematopoietic tissue. In the analysis of cardiac tissue not a single donor HSC-derived cardiomyocyte was detected. In 2002 the concept of fusion emerged as an alternative explanation for the so called transdifferentiation events. Both Terara et al. and Ying et al. found that in co-culture experiments with bone marrow cells and embryonic stem cells, or with neural cells and embryonic stem cells, tetraploid hybrids could be detected that adopt embryonic stem cell characteristics.

The most critical issue is that none of the reports claiming transdifferentiation of bone marrow stem cells to cardiomyocytes has included any evidence that the crucial and specific cardiac marker proteins are present in any appreciable quantities. The heart muscle is a multicellular muscle system coupled by cardiac-specific intercellular junctions ("intercalated discs"), anchoring the contractile myofibrils. The skeletal muscle is syncytial, i.e. a multinucleate system without any intercellular junctions. The most specific molecular markers of the cardiomyocyte include the elements of the intercalated disc (desmoplakin, plakoglobin, plakophilin-2, desmoglein-2). A point mutation in plakophilin-2, for example, would cause a rhythmogenic right ventricular cardiomyopathy. It is surprising that none of the animal studies have examined the so-called transdifferentiated cardiomyocytes in this light. Most of the results from more recent animal studies have raised questions about the reproducibility and validity of the original study by Orlic et al. (2001). Combined, they demonstrate that local implantation of HSC-enriched bone marrow into ischaemic myocardium did not result in myocardial regeneration.

3. Clinical Studies

Until recently, most of the trials performed have been in small case series of phase I or pilot studies, examining the safety and feasibility of bone marrow cell treatment for ischemic heart disease. Few of these studies were controlled, let alone randomized.

Several randomized trials have now been reported. Among them, only the trials of the Frankfurt Group (Top Care) have shown benefits for patients receiving HSC treatment. The following major issues are very critical among the early phase studies and even in the large scale randomized trials:

(*i*) They use very different types of cells;
(*ii*) the delivery of the cells was heterogeneous, i.e. via catheter, by injection into infracted areas and
(*iii*) the time point of delivery of HSC varied also greatly – immediately after a myocardial infarction, within 14 days after myocardial infarction, from two weeks to several months after the initial event, etc.

In all the studies there is a lack of systematic assessment of infarct size or left ventricular function prior to intervention and the duration of follow-up is usually short.

The ASTAMI (autologous stem cell transplantation in acute myocardial infarction) trial showed no benefit for the stem cell group. In fact control examination at 6 months showed that the left ventricular ejection fraction was increased in the control group. In the BOOST (Bone Marrow Transfer to Enhance ST-elevation Infarct Regeneration) trial, the benefits of bone marrow cells could be sustained at 18 months. However, within this period there was also improvement of global left ventricular function in the control group. The comparison to the stem cell group was then no longer significant. The REVIVAL-2 examined whether stem cell mobilization by granulocyte-colony stimulating factor (G-CSF) was beneficial in patients with acute myocardial infarction. This well-designed, randomized, controlled trial showed no benefit for the group receiving G-CSF.

4. Conclusion

Despite all the above mentioned controversies, cell transplantation for myocardial repair has been translated from the bench to the bed side. There is now an absolute need for fundamentally sound animal experiments to assess which type of cells might be beneficial and the basic biologic mechanisms of improvement induced by HSC must be explored. At the same time there is an absolute need for larger, randomized, double-blind clinical trials to assess whether cell transplantation is a useful therapy. Very little is known about the long-term fate of transplanted cells and the mechanisms by which they may exert a positive effect. This area is replete with unanswered questions.

Reference

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Regeneration Therapy for Acute Myocardial Infarction

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Experimental studies suggest that the intravascular or intracardiac administration of either bone marrow or blood-derived progenitor cells may improve contractile function after experimentally induced acute myocardial infarction. Initial clinical pilot trials as well as small randomized but unblinded studies in patients with acute myocardial infarction also indicated that the intracoronary infusion of bone marrow-derived progenitor cells into the infarct artery may improve left ventricular function within four to six months.

The recently completed Repair-AMI Trial was the first double-blind, placebo-controlled multicenter trial investigating the effects of intracoronary infusion of bone marrow-derived progenitor cells versus placebo medium into the infarct artery in patients with successfully reperfused acute myocardial infarction. The results at four months follow-up indicated a significantly greater improvement in global left ventricular ejection fraction combined with a complete abrogation of left ventricular endsystolic volume expansion in the bone marrow-derived cell treated group compared to the placebo group. These findings were substantiated by MRI-analyzes in a subset of patients. There was a significant interaction between the treatment effect of bone marrow-derived cell infusion and baseline ejection fraction showing that those patients with the largest infarcts derived the most benefit from intracoronary cell therapy. Mechanistically, intracoronary Doppler flow velocity measurements revealed a complete normalization of coronary flow reserve in the infarct artery treated with intracoronary infusion of bone marrow-derived cells, whereas there was only a modest improvement in those infarct vessels infused with placebo medium. Finally, although the study was not powered to detect any significant differences in clinical endpoints, the group of patients receiving intracoronary infusion of bone marrow-derived cells had fewer cardiovascular events during the four months follow-up period compared to the patients receiving placebo medium infusion.

Taken together, evidence is emerging that the intracoronary administration of bone marrow-derived progenitor cells is associated with improved recovery of left ventricular contractile function in patients with acute myocardial infarction. Given the preferential effect in patients with large acute myocardial infarctions, studies are warranted to examine the potential effects of progenitor cell administration on morbidity and mortality in patients with extensive infarction and depressed left ventricular contractile function despite successful reperfusion

therapy. Moreover, specific attention should be paid in the future to the process of cell isolation in order to maximize functionality of the transplanted progenitor cells.

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