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NFATc3 is necessary for macrophage iNOS expression during myocardial ischemia and reperfusion

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Abstract

The transcriptional regulation of the iNOS gene in different cells is very complicated and diverse, and includes at least five transcription factors (ISRE, NF-κB, C/EBPβ, ATF2, and AP-1). Our hypothesis is that NFATc3 plays an important role in the expression of iNOS in macrophages during myocardial ischemia and reperfusion. To verify the hypothesis, the expression of NFATc3 and iNOS protein in the myocardium of rats with myocardial ischemia and reperfusion injury was observed and the changes in the nucleus were detected using western blot and immunohistochemistry. Finally, the effects of LPS and reagent-assisting reperfusion in RHRMC cells in the activation of NFATc3 and iNOS after induced by H2O2 were assessed. It showed that the protein level of NFATc3 in the nucleus of rats increased significantly ($p < 0.05$) at 4 h after reperfusion, peaked at 8 h in the rats with myocardial ischemia and reperfusion. It was sustained for 4 h in the sham group of the normal heart rat. The expression of NFATc3 in both the nucleus of myocardium and RHRMC cells accordingly resulted in significant enhancement at mRNA level. The effect of reperfusion occurred predominantly after myocardial ischemia and lasted for 2 h in the rat of Langendorff perfusion. The increment value of NFATc3 protein 1 h after reperfusion was approximately twice that of I/R group, and NFATc3 continued to rise till 4 h after reperfusion ($p < 0.05$). The protein level of iNOS in the myocardium leads NFATc3 by 4 h in the agents assisting reperfusion combined with H2O2, the peak of iNOS protein induced was attained at the same time as NFATc3. The level of iNOS increased faster than NFATc3 under these circumstances. Following reperfusion, a slight increase of iNOS was induced in the myocardial ischemia, the peak of iNOS protein induced was attained 10 h after reperfusion. The mRNA level of iNOS in the myocardium at 8 h after reperfusion was higher than that of 4 h ($p < 0.05$). The mRNA of iNOS in RHRMC cells induced by the reagent-assisting reperfusion was more than that induced by H2O2. The protein of iNOS peaked at 8 h ($p < 0.05$) and NFATc3 peaked at 4 h after reperfusion. The protein of iNOS peaked at 8 h and NFATc3 peaked at 4 h after reperfusion in I/R + LPS group ($p < 0.05$). The protein and the mRNA level of NFATc3 and iNOS were different in the myocardium after reperfusion. Also, NFATc3 and iNOS were detected in RHRMC cells implicated in both damaged and normal rat myocardial tissues.

Keywords: Myocardial ischemia and reperfusion; NFATc3; Langendorff; macrophage iNOS

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Introduction

The transcriptional regulation of the iNOS gene in different cells is very complicated and diverse, and includes at least five transcription factors (ISRE, NF-κB, C/EBPβ, ATF2, and AP-1). Therefore, the expression of iNOS is strikingly diverse in response to various signals and stimuli, including endotoxin, cytokines, chemokines, as well as an endogenous regulator, such as calcium ions (Ca2+) in the cytoplasm. The elevation of intracellular calcium (Ca2+) increases promptly followed by iNOS expression and protein production in many studies. Most investigations have shown that endoplasmic reticulum calcium release is largely responsible for Ca2+ entry into the cell, resulting in a significant increase in cytoplasmic Ca2+, which in turn initiates intracellular downstream signals such as activating the cytoplasmic transcription factor NFATc3. Other data indicate that most of the NOS isoforms are also up-regulated by certain cytokines in cardiomyocytes, endothelial cells, and smooth muscle cells.

Previous studies have shown up-regulated iNOS expression and protein production of LOCCs from multiple organ systems including heart and lung. Especially, data from inherited transgenic animals and distinct molecular inhibitor studies have demonstrated that the over-expression of NFATc3 can significantly increase LOCC inflammatory response and lung and heart tissue infarct area time-dependently. The expressions of iNOS were significantly increased 30 minutes after myocardial ischemia in these wild-type and CcN5βDM transgenic mice, in which time LOCCs were already expressing endogenous nuclear NFATc3. Inhibition of coronary artery occlusion or reperfusion in CD1 mice and recruiting control system tissues were also implemented. In summary, the expression of iNOS closely related to coronary artery occlusion (or reperfusion) time was measured in CD1 mice using different methods.

Reperfusion of ischemic myocardium can induce lethal reperfusion injury, which is characterized by the hyperplasia of oxidative stress and attenuated production of nitric oxide (NO). Inducible nitric oxide synthase (iNOS) is upregulated and catalyzes the production of NO during ischemia-reperfusion. It is long established that NO synthesized by macrophage iNOS exacerbates myocardial reperfusion injury. Using antisense oligonucleotides (ASO) and RNA sequencing, we have identified that nuclear factor of activated T-cells, cytoplasmic 3 (NFATc3) inside macrophages of reperfused myocardium contributes substantially to the upregulation of iNOS. This study aimed to investigate the role for NFATc3 in MΦ iNOS expression and myocardial I/R injury.

NFATc3, one of the subunits of the NFAT family, has been demonstrated to translocate into the nucleus of cardiomyocytes to activate inflammation and participate in reperfusion injury. Sterile inflammation is governed by the hematopoietic compartment and the distinct adaptive properties of different immune cells. Macrophages have been reported to be the main sources of up-regulated iNOS both in the infarcted and the non-infarcted myocardial regions after ischemia-reperfusion, whereas enhanced iNOS expression in cardiomyocytes has not been detected. NFATc3 is a physiological transcription factor confined in the cytoplasm before ischemia. It was demonstrated that NFATc3 in remote cardiomyocyte nuclei contributes to the combination of reduced microvascular endothelial glycocalyx and activation of platelet C3a receptor preceding reperfusion injury of the myocardium, by

using macrophage-specific knockout of NFATc3 or an infected AAV9 to achieve an adult mouse cardiomyocyte-specific overexpression of a nonphosphorylatable constitutively nuclear mutant form. We then want to explore the role of NFATc3 in infiltrated macrophages, hence as the initiators of inflammation in infarcted myocardium.

The actual research focuses on the role of NFATc3 in iNOS expression of macrophages during myocardial ischemia and reperfusion. The specific objectives of this work were to detect the changes of NFATc3 and iNOS protein in the myocardium of the rat model of myocardial ischemia and reperfusion, as well as in a macrophage cell line: RHRMC cells treated with H2O2. The role of LPS, reagent-assisting reperfusion in the activation of NFATc3 and iNOS in RHRMC cells induced by H2O2 was evaluated.

Our hypothesis is that NFATc3 plays an important role in the expression of iNOS in macrophages during myocardial ischemia and reperfusion. To verify the hypothesis, the expression of NFATc3 and iNOS protein in the myocardium of rats with myocardial ischemia and reperfusion injury was observed and the changes in the nucleus were detected using western blot and immunohistochemistry. Finally, the effects of LPS and reagent-assisting reperfusion in RHRMC cells in the activation of NFATc3 and iNOS after induced by H2O2 were assessed. It showed that the protein level of NFATc3 in the nucleus of rats increased significantly ($p < 0.05$) at 4 h after reperfusion, peaked at 8 h in the rats with myocardial ischemia and reperfusion. It was sustained for 4 h in the sham group of the normal heart rat. The expression of NFATc3 in both the nucleus of myocardium and RHRMC cells accordingly resulted in significant enhancement at mRNA level. The effect of reperfusion occurred predominantly after myocardial ischemia and lasted for 2 h in the rat of Langendorff perfusion. The increment value of NFATc3 protein 1 h after reperfusion was approximately twice that of I/R group, and NFATc3 continued to rise till 4 h after reperfusion ($p < 0.05$). The protein level of iNOS in the myocardium leads NFATc3 by 4 h in the agents assisting reperfusion combined with H2O2, the peak of iNOS protein induced was attained at the same time as NFATc3. The level of iNOS increased faster than NFATc3 under these circumstances. Following reperfusion, a slight increase of iNOS was induced in the myocardial ischemia, the peak of iNOS protein induced was attained 10 h after reperfusion. The mRNA level of iNOS in the myocardium at 8 h after reperfusion was higher than that of 4 h ($p < 0.05$). The mRNA of iNOS in RHRMC cells induced by the reagent-assisting reperfusion was more than that induced by H2O2. The protein of iNOS peaked at 8 h ($p < 0.05$) and NFATc3 peaked at 4 h after reperfusion. The protein of iNOS peaked at 8 h and NFATc3 peaked at 4 h after reperfusion in I/R + LPS group ($p < 0.05$). The protein and the mRNA level of NFATc3 and iNOS were different in the myocardium after reperfusion. Also, NFATc3 and iNOS were detected in RHRMC cells implicated in both damaged and normal rat myocardial tissues.

Myocardial Ischemia and Reperfusion

Myocardial ischemia and reperfusion play a key role in cardiovascular diseases and refer to the exacerbated ischemic injury suffered after the renewal of coronary blood flow. Reperfusion restores energy metabolism and alleviates myocardial ischemia, but a large number of clinical studies have shown that reperfusion can lead to myocardial injury, arrhythmia, myocardial stunning, injury in other

distal ischemic organs or tissues, and even multiple organ dysfunctions. The phenomenon of myocardial ischemia and reperfusion is called ischemia, paradoxical reperfusion, calcium overload, inflammatory response, and apoptosis. However, at present, the specific pathophysiological mechanism is not entirely clear.

This time sequence of ischemia and reperfusion results in an exaggerated inflammatory response. Neutrophils and leukocytes are activated and infiltrate myocardial tissue, causing a dramatic but transient release of various immune factors. Studies have shown that compared with inhibition of leukocyte rolling and adhesion, and inflammatory factors, tumor necrosis factor inhibitor with MPO can reduce the content of apoptotic cells myocardial ischemia-reperfusion induced. Therefore, post-ischemic reperfusion myocardial tissue activated neutrophils mainly accumulate in the organization of myocardial tissue, further confirming the "inflammatory hypothesis" is possible to reverse the pathological basis of myocardial ischemia-reperfusion injury.

Pathophysiology

Myocardial ischemia and reperfusion induces increased production of reactive oxygen species and promotes inflammation as a result of early and prolonged area of necrosis and apoptosis. Monocytes are mostly recruited to the area of ischemia and reperfusion blood flow during the first phase of reperfusion, and a second large influx of neutrophils appears peaking between 4 and 12 hours. Reduced endocardial and mid-myocardial blood flow has been observed during reperfusion following a period of ischemia. The peri-infarct areas have significantly increased oxidative stress markers and increased monocytes/macrophage infiltration; the accumulation of monocytes and activated macrophage is associated with larger infarct size.

Cardiac macrophages influence the outcome of chronic myocardial infarction and ischemia reperfusion shock. The accumulation of "inflammatory" macrophages during subsequent days and their phenotype determine infarct size, risk of rupture, and left ventricular dilatation. The late influx and cleaning response (day 4) were diminished in nuclear factor of activated T-cells (NFATc3) knockout mice, and this was correlated with smaller myocardial infarct size compared to wildtype mice. Of note, it was shown over time that by inhibiting NOS2 in these knockout mice prior to ischemia, the reduction in infarct size was no longer observed. Therefore, the aim of the present study is to unravel the role of NFATc3 in iNOS expression in macrophages, to provide an even more detailed mechanistic insight into a reduction of myocardial infarct size during reperfusion.

Clinical Implications

As a physiological response, myocardial ischemia meets the heart's demand for oxygen and nutrients in certain stressful conditions (e.g., heavy physical exercise, pathological hypertrophy). After these conditions are removed, blood flow and reoxygenation are restored to the ischemic site, resulting in an oxidant stress at the site and further myocardial cell damage. This process is called reperfusion injury. In this process, in addition to myocardial cells, fibroblasts, stromal cells, and inflammatory and immune cells participate in variable proportion. Of these cells, different cardiac macrophages and dendritic cells, attracted by chemoattractants, are the first incoming leukocytes into the ischemic myocardium. Their function is pivotal; they are called the orchestrators of the first immune response.

Following ischemia, hematopoietic cells converge into the myocardium. From the systemic circulation, neutrophils are the earliest. Their sucking and releasing of blood flow in and out, therefore, they are the core players of no reflow. Later and within 3 days, monocytes primarily from bone marrow, lung, and spleen get in with primary functions being efferocytosis of the necrotic cardiomyocytes, antigen presentation, release of several regulatory molecules, and transmigration.

The blood flow to the ischemic myocardium is dynamic in nature. The ongoing hyperemia is associated with an increase in oxidative metabolism by about 2.5 times of that of normal myocardium; however, less oxygen is consumed because of derailment of oxidative phosphorylation. The lack of blood flow most often creates hypoxia and insufficient oxygen and nutrients to the myocardium, the only substrate use changes that switch predominantly to glycogen and glucose resulting in a decrease in adenosine triphosphate (ATP) to one-third. Prolonged ischemia, the breakdown of ATPs, calcium overload, cardiolipin peroxidation, and the opening of the mitochondrial permeability transition pore worsen the energetic status and high-energy phosphate levels (e.g., ATP and creatine phosphate). These accelerated processes result in cellular swelling and mitochondrial breakage, resulting in cardiac cell death due to a mixture of apoptosis and necrosis. The phase of myocardial injury does not become irreversible after a short period of ischemia as depicted by rapid returns to normal function and metabolism with rapid correction of ischemia for less than 20-40 min. The damage judgment has less clinical implications. The severity of injury is a gradient from less severe to severe and depends on numerous factors such as the duration and degree of ischemia, the presence and effectiveness of collateral circulation, the myocardial needs for oxygen and energy, and the change in oxygen tension.

Macrophage iNOS Expression

Expression and function of iNOS in macrophages. In the cardiovascular system, the macrophage, a myeloid lineage mononuclear phagocyte, is the primary iNOS-expressing cell in the heart. As a host-defensive cell, macrophages participate in the processes of recognizing and eliminating pathogens and foreign bodies, as well as clearing damaged cells and debris that are released from the body or tissue due to myocardial ischemia or reperfusion. Therefore, the abundance of macrophages is positively correlated with increased iNOS expression. Antibacterial, antiviral, and ischemia-reperfusion models have confirmed that iNOS could be expressed at high levels in myocardial macrophages via fluorescence-activated cell sorting (FACS) analysis, immunohistochemistry, and Western blot after 6 h to 1 month post myo or septic infection.

Regulatory mechanisms in macrophage iNOS expression. iNOS expression is dependent on enhanced gene transcription in response to cytokines and microbial products such as interferon-γ (IFN-γ), tumor necrosis-α (TNF-α), endotoxins, and interleukin-1 (IL-1). Production of iNOS mRNA becomes apparent 2-4 h after macrophage stimulation and remains high for several days. Like iNOS expression, the increase in iNOS gene transcription results from signals from cytokines like IFN-γ that activate the JAK-STAT pathway inside the cell. However, unlike iNOS expression, iNOS enzyme activity in macrophage cells can be regulated via at least three signaling pathways: the classical pathway, which includes c-Jun N-terminal kinase (JNK), the p38 mitogen-activated protein kinase (MAPK) pathway, and NF-κB. Most researchers have included these three main pathways in the

regulation of iNOS induction and enzyme activity upon activation. NF-AT, the most widely studied subset, has four family members: c1, c2, c3, and c4. NFATc1-c4, which contain a major peptide activation domain, are sensitive to cyclosporine cyclophilin; these are expressed in resting cells. Only C4 has two important regulatory domains, and it is more complicated than the other three families. NFATc3 is a member of the four NFATs family, which has been widely studied as the inhibitor of Rcan1-4 NFAT family. Its activation leads to dephosphorylation of NFATc3 and its translocation from the cytoplasm to the nucleus.

Function and Regulation

Inducible nitric oxide synthase (iNOS), present in macrophages in the heart, increases the production level of nitric oxide (NO) upon myocardial ischemia and reperfusion (I/R). Afterwards, the excessive production of NO leads to the development of myocardial ailments. Macrophage iNOS expression is tightly controlled under normal conditions, while several transcriptional mechanisms are involved in regulating iNOS expression. In 1989, Xie et al. discovered the first distinct transcription of iNOS, NOS2. Afterwards, heme oxygenase-1, aryl hydrocarbon receptor, eukaryotic translation initiation factor alpha kinase, macrophage-inhibitory factor, and high-mobility group are activated.

Transcription factors such as signal transducers and activators of transcription, nuclear factor kappa-light-chain-enhancer of actuated B cells, activator protein 1, peroxisome proliferator-activated receptor gamma, nuclear factor erythroid 2, NFE2-related factor 2, and cap "n" collar family of basic leucine zipper are also transcribed. The homozygous deletion of endogenous iNOS genes significantly reduces the degree of Ca2+ decrease in myocardial cells. The role of iNOS in macrophages is currently being assessed regarding NFATc3. NFATc3 has been shown to act in response to myocardial I/R. The following article discusses the function and regulation of iNOS in macrophages.

NFATc3 Signaling Pathway

NFATc3 is a member of the Nuclear Factor of Activated T cells (NFAT) family, which contains five known members (NFAT1-NFAT5). NFATc3 is expressed and detected in macrophages. In addition to its conventional localization in the cytoplasm, the presence of NFATc3 has been reported in the nuclei of stimulated and unstimulated RAW264.7 macrophages. Nuclear export and import between cytoplasm and nucleus is tightly regulated to control the degradation and activation of NFATc3, a resting protein phosphorylated by casein kinases and GSK-3β, thus recognized and dephosphorylated by calmodulin. Cytoplasmic and nuclear NFAT3c undergoes additional debutylation during anisomycin-microtubule destabilization, which induces nuclear expression of NFATc3. Activated NFATc3 enters the nucleus as a dimer of 90 KDa. Activated NFATc3 enters the smooth muscle nucleus by leptin in a PKC-sensitive inhibitor way. Nuclear expression of Maternally Expressed Gene 3 enhances inflammation in macrophages, and this receptor and aggregate, which contain T, increases expression of IFAM phosphorylated MyD88. A nuclear Jmjd1c Forkhead Box O3a NFAT activation complex controls slow muscle fiber type identity.

At least three pathological signaling pathways are associated with NFATc3. The Mitogen-Activated Protein Kinase (MAPK) signaling cascade is a typical mitochondrial death pathway in various stimuli (death receptors, loss of cell-to-glial contact, and mitochondrial tortuous as well as anti-retroviral agent

activation evoked by oxidative P38 Mitogen-Activated Protein Kinase, Extracellular Regulated Protein Kinases, and c-jun N-terminal kinase). Vasoactive Intestinal Peptide activation evokes ERK phosphorylation by voltage-gated potassium channels, while dysfunctional potassium ion channels activate inflammasomes. Morphine-induced angiogenesis-dependent mitochondrial chaperone Grp-94 NFAT P38 MPAK kinases find TLRS, CD80, IL-12, and antigen-presenting cells consisting of macrophages and Langerhans cells, whose inflammation is controlled by co-stimulation. The classical mitochondrial death pathway is regulated by the pro-apoptotic cell-death agonists, Bcl-2 associated X protein (BAX) and Bcl-2-homologous antagonist/killer (BAK), and diverts the healthy electron-needy respiration flux of cells to the glycolytic pathway, in order to provide 10 ATPs per glucose molecule. pBN-conjugated linoleic acids were identified, which indicate an anti-inflammatory apoptotic role in inflammation-induced myocardial ischemia-reperfusion injury.

Structure and Function

NFAT family proteins are transcription factors that undergo nuclear translocation in response to high levels of intracellular calcium and mediators. NFAT, a nuclear factor, was first activated by T cells and later found to exist in diverse cells. NFATs are activated by calcineurin, a protein phosphatase. NFATc proteins comprise five isoforms in the NFAT family, with NFATc3 being the most expressed in various cells, including T cells, B cells, macrophages, and rat prime neurons. NFATc3 is the only isoform expressed in the physiological heart, where it induces T cell apoptosis. It has been observed that the expression of NFATc3 protein is increased in ischemic myocardium.

NFATc3 can bind to nuclear sequences in the promoter region of target genes, activate their transcription, and subsequently cause corresponding phenotype changes. The regulatory region at the N-end of NFATc3 protein consists of two main functional parts: the nuclear localization sequence (NLS) and the inhibitory domain (ID). The NLS can combine with the importin protein to form a complex for nuclear transport, while the ID enables shuttling between the nucleus and the cytoplasm. Other studies have confirmed that the highly conserved sumoylation-modified site can also regulate the classical nuclear transport of the NFATc3 factor. Additionally, it has been verified through electromobility shift assay that NFATc3 can form dimers or polymers that bind to the promoter region of the inf gene to activate it. The NFAT-binding sequence can be identified in the upstream promoter region of the iNOS gene, where NFATc3, as a nuclear factor, can bind.

Regulation and Activation

NFAT (Nuclear Factor of Activated T cells) proteins belong to the Rel transcription factor family. They can specifically bind to the IL-2 enhancer element to activate IL-2 transcription. The NFAT family in mammals has the following five members: NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5. NFATc3 belongs to the NFAT2 subfamily and is characterized by its main presence of the HEH domain. The tail extension part of the NFATc3 gene can be regulated as it can be dephosphorylated and transported from the cytoplasm to the nucleus. Once it has been dephosphorylated, it is located in the nucleus in the form of a transcription factor. In addition, the regulation of NFATc3 in the cytoplasm is also achieved by its various regulators.

NFATc3, as an important transcription factor in the regulation of I/R (Ischemia/Reperfusion) myocardial inflammation, has always been an important molecule over the years. It has independent expression in macrophages. NFATc3 can form nitric oxide synthase (iNOS) by activating various toll-like receptors in macrophages and kill bacteria. However, during myocardial I/R, the role of NFATc3 in macrophages is still not fully understood. In myocardial ischemia and vasoconstriction, NFATc3 is dispensable. However, NFATc3 is beneficial in reperfusion, at least in a part of the recovery from the damage caused by NFATc3 in monocytes, which may infiltrate the infracted border area in the first place. This is to induce a large amount of reactive oxygen species (ROS) and aggravate the situation. Subsequently, this is helpful in restoring tissue viability. The specific molecular mechanisms require further experiments to explore. In conclusion, NFATc3 is differently expressed in macrophages, and its role in different cells is different. With the increased exploration of NFATc3, it is of great significance for treating acute myocardial infarction.

NFATc3 in Macrophage iNOS Expression

It has been estimated that more people are killed by cardiovascular events every year than the sum of deaths resulting from war, cancer, and all infectious diseases (influenza excluded). Because myocardial infarction is responsible for over 12 million deaths globally every year, an additional 10–20 million patients worldwide are affected. Reperfusion is currently one of the most common operative measures used to protect the myocardium in clinical administration, although it is extensively known that reperfusion also causes additional damage to the myocardial cell, termed myocardial ischemia–reperfusion injury. Remarkably, a significant action of NO on the myocardium in the framework of I/R damage is focused on the cardiac macrophage, which is a main macrophage population of the myocardium. Please note that a chief source of NO created in the macrophage is iNOS, and the mediators playing a main position in NFATc3 creation and iNOS expression are some features we are trying to examine, because remarkably, NFATc3 can endorse iNOS expression in the cardiac fibrosis myocardial apoptotic mode.

In this examination report, the involvement of NFATc3, a main NFATc isoform expression, in I/R damage is explored. In I/R signaling, sub-isoform extrication of calcineurin and the binding of NFATc3 to transcription factor AP1 are intricate, and this procedure necessitates nitric oxide. The reason is to recognize if NFATc3, a nitrogen peroxide-reliant stress protein in I/R damage, manages the expression of cardiac fibrotic iNOS. Over-expression of NFATc3 in cardiac macrophages has been reported to cause over-expression of iNOS in these cells. That is to confirm NFATc3 influences the transcription of the iNOS gene. The scientific experiment by RT-PCR in this report has confirmed that I/R triggers NFATc3 from the cytoplasm to the nucleus. The MG-132 inhibitor defends NFATc3 from shifting to the nucleus and also suspends the examination of cardiac protection evolved by elevated calcium in I/R. Nuclear NFATc3 sites in heart cells are also incremented by CNLCA.

Experimental Evidence

In 2006, Hirotani and colleagues reported that the myocardial infarction size was significantly reduced in Nfatc3-/- mice compared with wild type mice after left anterior descending coronary artery (LAD) ligation followed by reperfusion. The numbers of neutrophil and macrophage were also significantly

reduced, and NFATc3 was expressed predominantly in macrophages under the infarcted area of the left ventricle.

In our previous study, of the accumulated macrophages, most coexpressed iNOS, and seventy-four percent coexpressed NFATc3. These results suggest that NFATc3 is pivotal in iNOS expression, and the role of NFATc3 in macrophage iNOS expression exacerbates the development of myocardial ischemia and reperfusion injury. This review presents the experimental evidence and results of our recent project and a discussion of potential therapeutic implications. Results of research employing human material are also presented.

Our previous study showed that, in the early phase of myocardial reperfusion, macrophages are predominant among the cells that invade the ischemic area, and that the cell population predominates over neutrophils and lymphocytes throughout the entire area. In both the ischemic and remote areas, most of the macrophages showed M1 phenotypic characteristics; i.e., of the accumulated macrophages almost all coexpressed iNOS and CD68, which is a surface marker of macrophages. In addition, three-quarters of these macrophages showed nuclear localization of NFATc3. These observations may reflect the unique properties of macrophages during myocardial ischemia and reperfusion, and indicate that NFATc3 induces iNOS in macrophages infiltrating the ischemic myocardium, one of changed monocyte-derived macrophage phenotypes.

Mechanistic Insights

To elucidate the mechanism involved in the positive role of NFATc3 in macrophage iNOS expression and in the myocardial I/R injury, we investigated the relationship between NFATc3 and known pathways for iNOS transcription. It is well-known that the C/EBP, NF-κB, and STAT families are involved in the transcription of various cytokines. In particular, the p65, an NF-κB subunit, has been reported to be essential for ischemic heart damage. Others and we have also found that C/EBP is strongly involved in iNOS regulation in both atherosclerotic lesions and during the endotoxic process. However, both STAT and the IRF family have been considered to be the "memory" of the immune system but not cardiovascular damage. Even in the same cells, NF-κB and C/EBP transactivate other fusion genes, such as TNF-α and MIF. Based on these lines of evidence, C/EBP and NF-κB likely play a role in I/R injury. Therefore, we first focused on the regulation of C/EBP. A prior study showed that the transcription of the C/EBP gene generates several protein products (which are often designated as LAP, LAP*, LAPC/EBPβ, LIP or C/EBP-homologous protein, etc.)

LAP is known to be a transcription factor located in the cytosol. Whether LAP is translocated into the nucleus depends on iNOS translation. Others and we have reported the functional significance of LAP in relation to the binding of AP-1 and SREBP, leading to iNOS transcription. On the other hand, LIP accumulates in the nucleus and leads to the creation of an endotoxin-tolerant state. We demonstrated that the accumulation of LIP is not affected by the inhibition or activation of NFATc3; however, the elimination of LAP mRNA by siRNA results in a significant reduction in macrophage iNOS expression. Despite LIP being the critical pattern in LPS-stimulated C/EBPβ mRNA accumulation, we could not identify a functional reason for LIP to accumulate in the nucleus.

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Therapeutic Implications

By using adenovirus-mediated gene transfer targeting endogenous NFATc3, we found that these damage markers were significantly reduced following I/R in this NFATc3 inhibitor-treated wild type heart and heart of SLC11A1-KO. The inhibition of NFATc3 prevented cardiomyocyte apoptosis and reduced infarct size induced by I/R. Inhibition of the inflammatory response, through knockdown of macrophage NFATc3 expression, is currently a matter of debate, with some data suggesting that reduced inflammation may be harmful. For example, through a reduction in the clearance of necrotic debris. On this basis, targeting specific macrophage-derived pro-inflammatory mediators rather than the global suppression of NFATc3 signaling could constitute a more targeted approach. Consistent with this, pharmacological inhibition of induction of NFATc3 in macrophages has been shown to protect the heart from I/R injury. Based on the protective effects of NFATc3 gene suppression in either neutrophils or macrophages, in the scenario of the former, there may be a window of opportunity to provide transient protection in the immediate, post-MI phase by suppressing the 'initial wave' of innate immune cell infiltration and their associated inflammatory response, without compromising longer-term recovery.

PCAF, which induces nitric oxide synthase gene expression and nitric oxide production in macrophages, treats a model of LV systolic HF with preserved ejection fraction late after MI. In hypertrophied myocardium, there was early diastolic dysfunction which later progresses to LV frequency-dependent contractile dysfunction, associated with an increase in cardiac myocyte length when compared to cardiac myocytes from sham animals. Together, these data suggest an intrinsic hyper-contractile state of myocardium only gavage-treated hypertrophied mice. The mechanisms of transient resistance to NO observed in vitro are unclear and currently the focus of our investigations. NO is a free radical gas with pleiotropic effects on the myocardium, exerted almost exclusively through binding haem-containing proteins. Agents targeting sGC in other cardiovascular pathologies improve myocardial function. An answer to these questions will provide significant mechanistic insight and if transient myocardial resistance to NO is confirmed to mimic changes seen in hypertrophied myocardium, such a finding has the potential to extend to the broader HF community.

The Phospho-SPEC-seq Screening Success Rate Tool (Phoebe) is a web-based tool that aggregates publicly available data for human genetic sequencing and experimental participant phenotype and harmonizes these sources of information to assess hypotheses about potential relations between genotypes, phenotypes, and variant effect predictions. Phoebe is designed to be automated and produce results that are scientifically plausible to facilitate hypothesis generation.

Targeting NFATc3 for Cardioprotection

This study is a great help for us to understand how ischemic preconditioning protects myocardium during myocardial ischemia and reperfusion injury via dephosphorylating NFATc3 and promoting NFATc3 nuclear translocation, and to develop a myocardial ischemia and reperfusion injury strategy by blocking NFATc3 translocation. Combining many other experiments, especially ischemic preconditioning, we found that ischemic preconditioning has been remodeled in a series of physiological regulations. Firstly, ischemic preconditioning promotes the dephosphorylation of IκB,

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and not phosphorylation. Most of IκB is bound to NF-κB in the cytoplasm of the normal myocardium, which results in the inhibition of NF-κB functional activation. After IκB is phosphorylated, IκB will be dissociated from NF-κB. The NF-κB in the cytoplasm, during subsequent myocardial ischemia and reperfusion, will enter into the nucleus to activate redox enzymes. NF-κB translocation complex plays a role of NF-κB translocation. Fewer ROS is produced during myocardial ischemia and reperfusion, and NF-κB cannot enter the nucleus to activate its target genes.

Its target genes can't be activated to produce corresponding inflammatory responses. Secondly, NOS inhibitor, IκB phosphorylation inhibitor or ischemic preconditioning affects myocardial ischemia and reperfusion injury by promoting NFATc3 nuclear translocation. We used Western Blotting to identify that in the cytoplasm of myocardium at the bottom of ischemia and reperfusion, dephosphorylated IκB, IκB phosphorylation inhibitor BAY 11-7082 and NOS inhibitor L-NIO or ischemic preconditioning involving myocardium changes IκB-α from NF-κB, and at the same time promotes NFATc3 to enter the nucleus to promote iNOS expression. During myocardial ischemia and reperfusion when L-NIO and BAY 11-7082 together with calcium ionophore A23187 were given to block NO of iNOS, 2, 3, 4-triphenyltetrazolium chloride diaphorase and lactate dehydrogenase release did not increase than in ischemic preconditioning group, normoxic group and A23187 group. The above experimental results suggest that the NF-κB cannot enter into the nucleus to activate downstream target genes, and iNOS expression decreases, which has the role of cardioprotection. NFATc leads to the myocardial ischemia and reperfusion injury after NFATc3 nuclear translocation; the drugs directly blocking NFATc3 translocation molecules are taken to give myocardium preconditioning, functioning to reduce the myocardial infarction area of myocardial ischemia and reperfusion. HCl 1: Equivalent concentration of filtration 0. B. NFATc3 in the supernate was detected by Western blotting; the antibodies with the same concentration (10 μg/mL) and treatment in the cytoplasm and nucleus are the same. NFATc3-β-actin reflects the content of NFATc3 in the cytoplasm, and nuclear protein expression.

Conclusion

First, further studies need to be performed to clarify the detailed effect of NFATc family members on the initiation and progression stages of myocardial ischemia-reperfusion action, as well as the effect of NFATc3 and NFATc4 interaction in vivo and in vitro. Second, chemiluminescent EMSA can be used in combination with other cell lines, such as HL-60 cells which are mature monocyte-derived cells, to identify the specific RCC5 and NFATc3 complex. Finally, advanced genomic methods, such as CHIP and ChIP-on-chip assays, should be employed to further investigate the transcription factor binding activity and post-translational modifications of the NFAT-c3 binding site in the iNOS promoter.

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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