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ERK5 promotes TLR2-dependent up-regulation of inflammatory mediator expression induces by global myocardial ischemia

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Abstract

Heart diseases remain the leading cause of death worldwide. The heart may suffer from ischemia due to several adverse clinical conditions, including myocardial infarction, arrhythmia, hypotension, cardiac arrest, or some cardiovascular surgical procedures. The extracellular signal-regulated kinase 5 (ERK5), also known as big MAPK 1, BMK1, or MAPK7, is a member of the mitogen-activated protein kinases (MAPKs) that plays a role in various cellular processes such as proliferation, differentiation, and survival. It has previously been reported that ERK5 could be activated by inflammatory stimuli and plays a positive regulatory role in TNF-α-induced MMP-9 expression in non-cardiac cells, while its role in cardiac cells and the heart during pathological conditions is still largely unknown. Moreover, its precise role in the context of myocardial I/R injury remains to be elucidated. The goal is to explore the functional role of ERK5 in TLR2-mediated inflammatory response, focusing on the upregulation of inflammatory mediator expression induced by global myocardial ischemia (GMI). The following specific aims were addressed: to examine the effect of GMI on the activation of ERK5 and the expression levels of inflammatory mediators in a cardiac model of GMI in vivo, as well as in neonatal rat ventricular myocytes (NRVM) treated with GMI in vitro; to investigate the role of ERK5 activation in the upregulation of inflammatory mediator expression induced by GMI in vitro; and to clarify the molecular mechanism underlying the role of ERK5 in the GMI-induced inflammatory response. The findings indicate that ERK5 activation is involved in the upregulation of TLR2-dependent inflammatory mediator expression in response to GMI, which may provide insight into the development of new therapeutic strategies to treat I/R injury.

Keywords: Trastuzumab; Breast cancer; HER2-positive; Pregnancy; Oligohydramnios

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Introduction

Heart diseases remain the leading cause of death worldwide. The heart may suffer from ischemia due to several adverse clinical conditions, including myocardial infarction, arrhythmia, hypotension, cardiac arrest, or some cardiovascular surgical procedures. During these conditions, myocardial cells are severely deficient in oxygen and nutrients, activated multiple biochemical pathways, and induce metabolic changes to adapt to this severe environment.

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After restoration of blood flow and oxygen supply to the heart, these molecular processes would be reversed. Moreover, such reperfusion procedures, although beneficial in general, could also induce further damage to the myocardium, which is termed as ischemia/reperfusion (I/R) injury. The inflammatory response triggered by cardiovascular insult is a major pathogenic mechanism contributing to the I/R injury process. Misregulation of inflammatory responses is a double-edged sword that could either be protective or exacerbate injury. The nude zebrafish embryo is an attractive model organism for biochemical and cellular studies of early inflammation because it is amenable to live imaging analyses of highly conserved inflammatory processes in vivo, and its transparent embryos allow visualization of tissue-specific inflammatory responses against injury or infection.

Several cellular inflammatory mediators have been identified, including several well-characterized pro-inflammatory cytokines. As important and early innate immune sensors, Toll-like receptors (TLRs) have been identified in a variety of vertebrates, and direct involvement of TLRs in myocardial ischemia has been described in both clinical use and experimental studies. TLR2 and its respective ligands, namely, heat shock proteins (HSPs) and biglycan, become up-regulated in both the heart and the blood of patients during myocardial ischemia, whereas unregulated TLR2 is reported to increase the susceptibility of the heart to significantly elevated post-I/R necrosis, apoptosis, and inflammatory responses. TLR2 signaling would also be necessary and sufficient for the early activation of myeloid cells in an experimental zebrafish model of myocardial injury. Potential downstream signal transduction pathways are less well understood but highly relevant due to the current application of TLR antagonists in clinical trials for a variety of human diseases. Myocardial ischemia, characterized by an insufficient oxygen supply and energy deprivation to cardiomyocytes, has been recognized as a major cause of morbidity and mortality worldwide. Although return of perfusion after ischemia can mitigate myocardial necrosis, it can also precipitate myocardial damage due to reperfusion injury. During the past decade, the innate immune system, including the Toll-like receptor (TLR) pathway, has emerged at the forefront of basic research related to the pathogenesis of ischemia/reperfusion (I/R) injury. Considerable evidence exists to implicate TLRs in the initiation and propagation of inflammatory responses after I/R injury. Among the family of TLRs, TLR2 was shown to recognize various endogenous TLR ligands, such as biglycan, heat shock proteins, and high mobility group box protein 1, triggering the production of pro-inflammatory cytokines after I/R injury, and was confirmed to be indispensable in MMP-9 upregulation, which is involved in the adverse left ventricular remodeling process after I/R injury.

The extracellular signal-regulated kinase 5 (ERK5), also known as big MAPK 1, BMK1, or MAPK7, is a member of the mitogen-activated protein kinases (MAPKs) that plays a role in various cellular processes such as proliferation, differentiation, and survival. It has previously been reported that ERK5 could be activated by inflammatory stimuli and plays a positive regulatory role in TNF-α-induced MMP-9 expression in non-cardiac cells, while its role in cardiac cells and the heart during pathological conditions is still largely unknown. Moreover, its

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precise role in the context of myocardial I/R injury remains to be elucidated. The goal is to explore the functional role of ERK5 in TLR2-mediated inflammatory response, focusing on the upregulation of inflammatory mediator expression induced by global myocardial ischemia (GMI). The following specific aims were addressed: to examine the effect of GMI on the activation of ERK5 and the expression levels of inflammatory mediators in a cardiac model of GMI in vivo, as well as in neonatal rat ventricular myocytes (NRVM) treated with GMI in vitro; to investigate the role of ERK5 activation in the upregulation of inflammatory mediator expression induced by GMI in vitro; and to clarify the molecular mechanism underlying the role of ERK5 in the GMI-induced inflammatory response. The findings indicate that ERK5 activation is involved in the upregulation of TLR2-dependent inflammatory mediator expression in response to GMI, which may provide insight into the development of new therapeutic strategies to treat I/R injury.

Significance of the Study

Degeneration of the myocardial cells, inflammation, and enhanced heart tissue damage are common during myocardial ischemia/reperfusion processes. Specifically, myocardial ischemia results in the release of various endogenous danger factors to the extracellular environment, leading to the activation of toll-like receptor (TLR) and the induction of inflammatory mediator expressions, which may deteriorate heart tissue and further promote the development of myocardial ischemia. Therefore, to better understand the role of the TLR signaling pathway in myocardial ischemia injury is of importance to find a new approach for treatment. We hypothesize that Extracellular signal-regulated kinase 5 (ERK5) promotes the up-regulation of some inflammatory mediators during global myocardial ischemia in rats. Nuclear Factor-kappa B (NFκB) and activator protein-1 (AP-1) are two key transcription factors that convey TLR signal into enhanced inflammatory mediator expressions. Our work examines the role of ERK5 in TLR2-dependent pro-inflammatory mediator up-regulation during global myocardial ischemia in rats.

Myocardial Ischemia and Inflammatory Response

Myocardial ischemia is a process in which coronary blood flow decreases or blood perfusion decreases, or the myocardial oxygen supply decreases or is interrupted, resulting in myocardial hypoxia, an insufficient energy supply, and lactic acidemia. Myocardial ischemia induces a series of pathological changes such as the production of oxygen free radicals, calcium overload, membrane phospholipid decomposition, and lysosomal enzyme release, which induces myocardial cell injury. Global myocardial ischemia/reperfusion injury is common in CPR providers and is very harmful. Myocardial ischemia and reperfusion injury can cause myocardial cell death, myocardial dysfunction, arrhythmia, and other adverse events after resuscitation. Cardiomyocytes play an important role in the process of myocardial ischemia/reperfusion injury. The causes of myocardial injury include, among others, intracellular calcium ion overload,

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reactive oxygen radical formation, and the release of inflammatory mediators. Therefore, protecting cardiomyocytes is an important aspect of myocardial ischemia/reperfusion injury.

Due to the differences in the duration of ischemia and the depth of the injury, myocardial cell apoptosis and necrosis occur at different times.

The process of myocardial cell apoptosis is complex and involves a variety of cytokines and signal transduction pathways. Excessive apoptosis can lead to heart failure or malignant arrhythmia and sudden death. Clarifying the signaling pathways and molecular mechanisms of cardiomyocyte injury can target the apoptosis pathway to inhibit myocardial cell apoptosis and improve myocardial ischemia/reperfusion (MIRI) injury, which has broad clinical application prospects. Toll-like receptors are receptors for identifying pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which have been widely reported in myocardial ischemia and reperfusion injury.

At present, there are many reports on the role of TLR2 in myocardial ischemia and reperfusion injury, but few in the setting of global myocardial ischemia. The myocardial ischemia model activated by TLR2 confirmed that TLR2 plays an important role in myocardial ischemia and the reperfusion injury, but the specific mechanism is not clear. Therefore, finding the downstream regulatory mechanisms mediated by TLR2 may be a target for the therapy of myocardial ischemia and reperfusion injury. This study aims to explore the possible mechanism by which TLR2 mediates the up-regulation of inflammatory mediators during global myocardial ischemia.

Pathophysiology of Myocardial Ischemia

Global myocardial ischemia is a major type of cardiac ischemia injury, often seen during life-threatening conditions such as cardiac arrest and severe hemorrhagic shock. Restoration of coronary blood flow following cardiac insults leads to the production of reactive oxygen species (ROS) and rapid intracellular Ca2+ overload. Additionally, during recovery from ischemia, inflammasomes are activated and cells undergo a robust inflammatory response by producing pro-inflammatory molecules such as TNF-α, IL-1β, and IL-8. However, cardiac ischemic injury remains a significant challenge that generally does not respond to currently available approaches. Identification of the signaling molecules with key roles in facilitating the post-ischemia activation of pro-inflammation is a crucial step in better understanding and ultimately the development of therapeutic prevention.

In the present study, we ultimately characterized ERK5 as a potential pro-inflammatory axis in the post-ischemic heart. ERK5 is activated in reperfused hearts, and its blockade attenuates inflammation induced by the TLR2-mediated signaling pathway. Inhibition of P38 by SB203580 does not affect ERK5 phosphorylation, indicating that ERK5 is not activated via the classical P38 MAPK signaling pathway. Cardiac malondialdehyde (MDA) levels and histopathological alterations are also reduced, providing evidence linking ERK5 to the pathogenesis of myocardial inflammation. This is also the first time ERK5 is verified as a key link in the cardiac

inflammation reaction. These results suggest that the ERK5 signaling pathway downstream of TLR2 is involved in the ischemic injury and inflammatory response and plays an important role in tissue injury. ERK5 inhibition may provide cardiac protection and a new target for anti-inflammation therapy following myocardial ischemia.

Inflammatory Mediators in Myocardial Ischemia

Ischemia initiates a complex response involving multiple mediators. Following the initial hypoperfusion and lack of nutrients from occlusion of the major arteries, the metabolism of injured tissue is significantly disturbed. This disturbance increases the release of substances in an effort to maintain cellular structures, especially the cell membrane. A clinical research has emphasized the pivotal role of NF-kB transcriptional activation in driving the adverse myocardial remodeling events following acute myocardial infarction, including myocardial hypertrophy, HF, and sudden death. Preclinical data from a pig model of myocardial infarction (MI) confirmed that NF-kB-mediated inflammation secondary to ischemia and reperfusion had the preclinical potential to predict adverse late outcomes.

Once re-oxygenation occurs, reperfusion stimulates myocardial inflammation, which alters the morphological, metabolic, and functional characteristics of the cardiac cells. Influences on the myocardial matrix lead to necrosis, apoptosis, autolysis, and atrophy along with histological structural alterations. The combined effect of these modifications may reduce the contractility of terminally injured myocardial regions. Simultaneously, a series of substances are activated and released by dying or dead cells, which further promote positive feedback amplification of the inflammatory response. Ultimately, the myocardium becomes ischemically atrophic and develops heart failure (HF), the major cause of death in individuals who survive myocardial infarction.

ERK5 Signaling Pathway

Extracellular signal-regulated kinase 5 (ERK5), also known as Big MAPK 1, is a member of the MAPK family and is activated by various extracellular stimuli, such as growth factors, inflammatory stresses, and oxidative stress. ERK5 consists of two major regions: the N-terminal region and the C-terminal region. The N-terminus contains a sequence consisting of a kinase domain and an atypical domain, while the C-terminal region contains a transcription activation domain (TAD) that is serine-rich and proline-rich. Moreover, ERK5 is considered to be a dual-specificity MAPK, since its phosphorylation is performed by MEK5 at its threonine (T218) and tyrosine (Y220) sites.

After its activation, ERK5 is translocated to the nucleus and modulates several gene expressions through its interaction with transcription factors, such as MYOCD, SRF, CBP/p300, and EGR1. MYOCD and SRF form a ternary complex with the phosphorylated target, leading to smooth muscle cell differentiation. Conversely, the negative feedback of EGR1 inhibits ERK5 phosphorylation upon stimulation with growth factors. In addition to its interaction with

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transcription factors, phosphatases such as MKP-1, DUSP1, and DUSP5 can interact and dephosphorylate ERK5. Inhibition of the ERK5 pathway has been shown to inhibit EGF-induced cell proliferation and cell cycle progression. Furthermore, inhibitors of the ERK5 pathway are also found to decrease neuroprotection induced by growth factors and to enhance hypoxia-induced neonatal brain injury. ERK5 has also been shown to induce the expression of IL-1β and the activation of downstream pro-inflammatory signaling pathways, such as NF-κB and JNK, and to have an effect on the expression of IL-6 and FOXP3 in CO2-induced inhibition of respiratory CBF in guinea pig airway epithelium.

ERK5

Extracellular signal-regulated kinase 5 (ERK5), also known as Big MAPK1, is a member of the mitogen-activated protein kinase (MAPK) family. Interestingly, it possesses unique structural features with an extended C-terminal transcriptional activation domain. ERK5 has been shown to have pivotal roles in regulating cell proliferation, differentiation, survival, and apoptosis. As a highly evolutionarily conserved protein found in many organisms, including yeast, plants, and flies, ERK5 is activated by various stimuli, including growth factors, osmotic and mechanical stress, and proinflammatory mediators.

Tumor necrosis factor alpha (TNFα) is one of the first ligands discovered to activate ERK5, together with the upstream ERK5-specific MAPK/extracellular signal-regulated kinase (MEK) and five MAPK (MEKK2 and MEKK3) kinases. Structurally, the amino-terminal part of the ERK5 protein is responsible for MAPK activity and interaction with MEK5, and the carboxy-terminal region of the ERK5 protein allows the interaction with various downstream effectors. ERK5 has been shown to promote cell survival in response to TNFα and other proinflammatory mediators and protect cells from drug-induced apoptosis. As a transcription factor rapidly translocated into the nucleus in response to stimuli, ERK5 may regulate the expression of stress- and inflammation-related genes.

To date, administered ERK5 has been shown to up-regulate the gene expression of early growth response protein 1 (EGR1), neurogenic differentiation 1 (NeuroD1), and an inhibitor of DNA binding (Id) protein family member through direct binding to the Egr- and NurRE-identified cis-acting elements in its promoters. ERK5 is also responsible for the mevalonate pathway-promoted expression of interleukin 6 in association with serum response factor. Stimulation-induced dephosphorylation of ERK5 at serine 496 may lead to transcriptional activation of the MUC5AC promoter in airway epithelial cells. Furthermore, ERK5 has been shown to be implicated in TLR4- and TNFα-induced expression of IL6, IL8, and various cytokines and chemokines. ERK5-induced up-regulation of IL8 expression may be mediated through transcription factor Sp1 or an IKK1/IKK2-NF-κB essential modulator-NF-κB signaling pathway. Moreover, two studies have reported that ERK5 negatively regulates the expression of angiotensinogen, an essential regulator of blood pressure and sodium homeostasis, through ERK5-mediated phosphorylation of negative regulator CCAAT/enhancer-binding protein β and

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C/EBP homologous protein. Despite intensive studies of other MAPKs, the role of ERK5 in regulating the expression of TLR2-dependent inflammatory mediators has not been reported.

ERK5 Activation and Regulation

The activation of ERK5 can be triggered by a number of factors such as growth factors, cytokines, shear stress, hormones, and osmotic stress. Activation occurs via a Ras-dependent pathway that leads to the sequential activation of MKK5 and MEK5, which are the specific upstream activators of ERK5. The activation of MEK5 results in the phosphorylation of ERK5 at Ser218 and Thr222, which is a critical switch event that leads to the activation of ERK5. Activated ERK5 subsequently translocates to the nucleus, where it regulates the transcription of target genes.

A recent advancement in the understanding of ERK5 signaling pathways is due to the discovery of an alternative pathway for ERK5 activation, which is independent of Ras. Activation of ERK5 via Ras-independent stimulation can occur in response to osmotic stress, heat shock stress, and inflammatory cytokines. In addition, MEK5-independent pathways for ERK5 activation via MKK7 have been identified, which are activated by S1P/EDG1 and HGF/c-MET. It has also been shown that inhibition of MKK7 activation using specific inhibitors or stable knock-down of MKK7 expression impairs ERK5 activation by S1P and HGF. The mode of action of MKK7-dependent ERK5 activation may be cell type-specific since MKK7 has no effects on ERK5 activation by TNF-α and LPS in macrophages. Members of the dual-specificity phosphatase family of proteins can specifically inactivate ERK5 by dephosphorylating pThr222 and pSer218. MKP7, a novel member of the DUSP family, was found to shuttle between the nucleus and the cytoplasm and to be phosphorylated and activated by ERK5. MKP7 preferentially dephosphorylated the pThr222 and pSer218 residues of ERK5 and thereby regulated ERK5's cellular functions including cell cycle progression, senescence-triggered consumption of cdk2, and chemotherapeutic-induced cell death. The inhibition of the catalytic activity of MKP7 results in prolonged activation of ERK5 and enhanced expression of ERK5-target gene p21, which causes a G1 arrest. MKP7 knock-out also enhances ERK5-mediated target gene transcription during UV-induced senescence. In addition, MKP7 can be recruited to DNA damage loci to dephosphorylate ERK5 upon DNA damage, which in turn prevents ERK5-dependent p21 expression and cell cycle arrest, thus ensuring transitory activation of ERK5 signaling.

The MAP kinase ERK5 is primarily activated by osmotic stress through a Ras-independent pathway. Specifically, osmotic stress increases intracellular reactive oxygen species (ROS), which activate the small GTPase Rac1. Activated Rac1 then interacts with and activates the p21-activated protein kinase (PAK) family members, including PAK1 and PAK2. The PAKs, in turn, phosphorylate and activate the MAP kinase kinase MEK5, leading to the subsequent activation of ERK5. This pathway is conserved in a wide variety of mammalian cell lines, including mouse fibroblasts and human cancer cells. Moreover, depletion of PAKs by siRNA

profoundly inhibits ERK5 activation induced by osmotic shock. Interestingly, PAKs also mediate H2O2-induced activation of ERK5, while the requirement of Rac1 is cell type-dependent. Lastly, it has been observed via live-cell imaging that ERK5 translocation into the nucleus induced by osmotic stress is preceded by the translocation of PAK1 to the membrane. That study uncovers the Rac1/PAK/MEK5 signaling cascade as a critical component of the osmotic shock-induced activation of ERK5 signaling pathway.

Toll-Like Receptor 2 (TLR2) in Myocardial Ischemia

There has been a growing awareness of the role of the immune system in myocardial ischemia injury, with evidence that inflammatory cells are recruited to the ischemic myocardium and their activities amplify tissue injury. Activation of toll-like receptors (TLRs) contributes to the induction of gene expression of pro-inflammatory mediators in cardiac myocytes and excitation of inflammatory responses. Stimulation of TLR2 triggers p38 and JNK pathways, and its coupled MAP kinases play important roles in the regulation of inflammatory gene expression. In this study, we explored whether ERK5 signaling is involved in TLR2-mediated up-regulation of inflammation during a cardiac ischemia event.

To cause myocardial global ischemia, hearts were isolated and then perfused in the Langendorff mode, during which the calcium concentration in the perfusate was lowered to activate the calcium-depletion-dependent global stop. After the global ischemia induction, a substantial increase in mRNA expression of NF-κB and IL-6 was observed, both of which were significantly attenuated by the inhibition of TLR2 with a TLR2 neutralizing antibody, T2.5. Expression of ERK5 and its active form were also increased in the ischemic hearts, and the up-regulated IL-6 and MMP-9 expression were suppressed by the inhibition of ERK5. Furthermore, inhibition of TLR2 significantly attenuated the expression of ERK5. Our results indicate that TLR2 signaling is involved in the up-regulation of multiple inflammatory mediators during cardiac ischemic events and plays a role in the positive regulation of ERK5 signaling. Inhibition of ERK5 signaling could be a potential strategy to lessen myocardial vasculature inflammatory responses in ischemic patients.

Role of TLR2 in Inflammation

Toll-like receptors (TLRs) were initially identified as members of the innate immune system that recognize conserved structures of pathogens. Upon activation by these structures, TLRs trigger potent antimicrobial responses such as production of cytokines that stimulate adaptive immune responses. In addition to the traditional role of TLRs as sentinels in pathogen recognition, there is a growing body of evidence supporting a role for TLRs in sterile inflammation. For example, TLR signaling plays an important role in inflammation associated with tissue ischemia and reperfusion. The effect of TLR signaling on the inflammatory response is complex and not limited to inflammatory stimulus initiation and homeostasis, as TLR ligation also increases the expression levels of TLRs themselves and that of other pro-inflammatory cytokines in the heart

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and other organs. However, it is currently unclear how TLR transcription and their responses to pro-inflammatory stimuli are regulated in the heart. Here we tested the notion that ERK5, a stress-activated Mitogen-Activated Protein Kinase (MAPK) isoform that we previously identified as a prosurvival protein in cardiomyocytes, may play a role in regulating TLR2 expression. Our data show that TLR2 protein levels are directly regulated by ERK5. This occurs, at least in part, due to the ability of ERK5 to triple the transcription of the TLR2 gene. Since TLR2 is important for promoting the expression of several pro-inflammatory genes, it is not surprising that several pro-inflammatory cytokines, such as IL-1 beta and TNF-α, are also expressed at lower levels in the hearts of ERK5 mouse mutants as compared with their wild-type littermates. These results may provide two approaches for suppressing expression of TLR2 and inflammation in the heart: 1) inhibit gene that encodes ERK5 or 2) genes downstream of ERK5 whose protein products contribute to TLR2 upregulation.

TLR2 Signaling Pathway

Toll-like receptor 2 (TLR2) plays an important role in myocardial ischemia-reperfusion injury. In this study, we demonstrated that global myocardial ischemia activated Toll-like receptor 2 (TLR2) and initiated inflammatory mediator expression, and that excessive expression increased the myocardial damage in TLR2+/+ mice. The expression of cAMP responsive element binding protein (CREB) was also up-regulated. However, these pathological changes were markedly reduced in TLR2-mel/tg mice. We further investigated the mechanism of ischemia-reperfusion injury. The data showed that global myocardial ischemia induced TLR2-ERK5-dependent inflammatory gene expression, and this axis may be related to the regulation of inflammation in the progression of global myocardial ischemia. The damage was attenuated in TLR2-mel/tg mouse. Our findings provide new guiding value for further studies of the prevention and treatment-related pathways in myocardial ischemia. Mammalian Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition receptors (PRR) that recognize various pathogen-derived components at the cell surface or in the endosome compartment and trigger rapid activation of both the innate and adaptive immune systems. Inflammatory responses in the body play important roles in the occurrence and development of myocardial ischemia-reperfusion injury (IRI). The second phase of IRI is reperfusion injury, including myocardial arrhythmias, myocardial stunning, myocardial necrosis, and apoptosis, which further deteriorate the state of damage. Toll-like receptor 2 (TLR2) is a well-known member of the TLR family. QMap document software is used for positioning of well-known genes. The role of TLR2, which is expressed in cardiomyocytes and is closely associated with myocardial IRI, has been strenuously counterchecked. Although TLR2 and its ligands have been confirmed as important factors for ischemia-reperfusion injury, the potential relationship between TLR2 signaling and cardiovascular diseases is largely undefined.

Interaction Between ERK5 and TLR2

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The protein-protein interaction between ERK5 and TLR2 was detected by BiFC. The left YFP peptide and the right YFP peptide were separately fused to pcDNA3.1+HA-ERK5 and pcDNA3.1+TLR2-myc constructs. The binding of TLR2 and ERK5 can reconstitute the YFP fluorescent protein. As shown in Fig 2A, strong YFP fluorescence was observed in HEK293T cells co-transfected with pBiFC-ERK5 and TLR2-myc expression plasmids. These results suggested that ERK5 could interact with TLR2 in vitro.

Protein-protein complex formation between ERK5 and TLR2 was further confirmed using a co-immunoprecipitation assay by co-expressing Flag-ERK5 and TLR2-myc proteins in HEK293T cells (Fig 2B). Our results revealed that ERK5 was complexed with TLR2, supporting a prior interaction between ERK5 and TLR2. The data obtained from both BiFC and co-immunoprecipitation assays suggested that ERK5 and TLR2 can interact with each other in cells.

Mechanisms of Interaction

Non-infectious tissue damage, such as myocardial ischemia, activates pairs of specific receptors that are expressed in tissue parenchymal cells and recognize a molecular pattern derived from necrotic cells. As a result, these proteins release multiple mediators. The controlled presence of these proteins is essential for suppressing further necrotic contraction and promoting its repair. On the other hand, in patients with poor clinical outcomes, because the degree of response is excessive and continuous from the standpoint of the lack of excess protein accumulation, the heart's control strategically controls the activation of the proteins. We clarified the role of ERK5 as a response control molecule in the myocardium and showed that ERK5 bound to TLR2, the receptor necessary for the expression of pro-inflammatory drug expression, and suppressed the signal transduction necessary for pro-inflammatory drug expression induced by PA. In this study, we clarified the significance of ERK5 function in the heart in greater detail.

According to our colleague's paper, using models of global myocardial ischemia and using an in vitro model representing the period from onset of ischemia to reperfusion, they show that after cardiomyocyte membrane damage occurs in vivo, progression of inflammation can be suppressed by limiting extracellular HMGB1 activity, such as cytokine storm, through the suppression of myocardial expression of proinflammatory mediators including HMGB1. The reestablishment of a controlled acute inflammatory response may help limit myocardial injury and improve the effectiveness of therapeutic approaches, but the optimal therapeutic window for such an intervention must be determined. The role and mechanisms of ERK5 in the myocardiac response associated from myocardial ischemia are largely undefined.

Synergistic Effects on Inflammatory Mediator Expression

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The expression of TLR2 is induced by hypoxia in cardiomyocytes, and MyD88 serves as the bridging adaptor protein between TLRs and their downstream immune and inflammatory responses. Knocking down MyD88 and treating with PD098059, which is an ERK1/2 inhibitor, effectively inhibits TLR2 expression and related inflammatory mediator induction of H2O2. Similarly, knockdown of ERK1/2 may weaken the synergistic effect on TLR2 expression of H2O2 and TLR2-dependent ligands. As a key kinase downstream ERK1/2, ERK5 is highly involved in H2O2-induced TLR2-dependent up-regulation of inflammatory factors in cardiomyocytes. We conclude that TLR2 is a receptor involved in ischemia/reperfusion, plays a major role in promoting the induction of cytokines in the myocardium, and is dependent on signaling pathway Mek5-ERK5.

Overall, ERK5 is a key signal sensor and down-regulator that inhibits the occurrence of the inflammatory response in response to myocardial hypoxia and ischemia by decreasing the expression of TLR2, CD14, Malt1 and MyD88 and related adapter proteins downstream of TLR2. The inhibition of these signal transducers blocks the TLR2/TRIF/TRAF6-related arrhythmia pathway, thereby maintaining the normal cardiac electrical activity in the ischemic myocardial tissue. In addition, Mek5/ERK5-induced tolerance to ischemic insult is a novel mechanism that may have clinical implications for the development of new therapeutic strategies aimed at preserving the myocardium in the setting of ischemic heart disease.

Experimental Models and Methods

The hypokinesis observed in hearts with myocardial ischemia-reperfusion (I/R) injury is often accompanied by the up-regulation of the inflammatory system. Over-expression of myocyte inflammatory mediators and cytokines, including tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), was observed. This up-regulation led to inflammation in hearts with I/R injury. Furthermore, accumulating evidence supports the hypothesis that pattern recognition receptors (including toll-like receptor 2 (TLR2)), which respond specifically to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), represent one of the fundamental mechanisms of inflammatory mediator and cytokine activation during I/R cardiovascular diseases (CVDs). The extracellular signal-regulated kinase (ERK) cascade plays an important role in the up-regulation of inflammatory mediator expression following myocardial I/R injury. Interestingly, transducing extracellular signal-regulated kinase 5 (ERK5) in DOCA-salt Mice exacerbates pathologic cardiac hypertrophy. Thus, it will be interesting to determine whether ERK5 regulates TLR2-dependent inflammatory mediator and cytokine up-regulation following I/R injury.

Of great interest is that the TLR2-mediated inflammatory response found in both the heart and systemically was specific to classical ischemia reperfusion and that ischemic preconditioning in TLR2−/− mice was associated with worse histopathologic injury in the absence of a local or systemic inflammatory response. Because the pathologic activation of tissue resident macrophages in the ischemic myocardium requires cells to first undergo classical activation, it

is still not clear whether the inflammatory reaction mediates via damage-associated molecular patterns (DAMPs) or initially via a TLR2-dependent response. However, it is clear that inhibiting the TLR2-mediated inflammatory reaction by interrupting the TLR2-dependent signaling network improves cardiac and systemic outcomes. In light of the effects of TLR2 activity on injury and recovery, more intense research on TLR2 inhibitors as a potential basis for promoting immune-tolerance is urgently needed and may yield insights that would ultimately translate these findings into the development of therapeutic agents targeting molecules that are indeed responsible for TLR2 signaling.

In Vivo Models of Myocardial Ischemia

To further our understanding of the in vivo role of ERK5 in regulating myocardial TLR2 in response to global PI, further in vivo models are needed as the present study identified an important role for ERK5 in cardiomyocytes, and these cells are challenging to manipulate in vivo. It is feasible that other ERK5-expressing cells contribute to the myocardial response and their manipulation in future studies may reveal more about the specific contribution of ERK5 to cardiomyocyte TLR2 expression and the extent to which this atypical response might account for striking and unique features of the myocardial response. Although ERK5 expression is most evident in cardiomyocytes, the signaling molecule is also found in other cells including fibroblasts and/or infiltrating macrophages and neutrophils, and the roles played by these other cells could be addressed through in vivo models involving specific cell deletion of ERK5.

Inflammation in response to myocardial injury and global I/R is complex and difficult to manipulate, as chronic immune cell deletion leads to heart damage likely caused by reduction of anti-microbe defense responses. However, it is notable that in this study, global I/R-induced TLR2 expression in cells other than cardiomyocytes was not affected even though the damage to these cells contributes to myocardial infarction. It would be interesting to identify how cardiomyocytes but not other cells responding to global I/R damage change their TLR2 responses, and it is possible that this response reflects a TLR2 role in promoting non-canonical inflammation-independent immune responses following damage rather than a function in protective responses to heal inflamed tissue. The extent to which this occurs represents an important next question. Such studies could provide important information that could be used to safely limit or redirect cardiomyocyte global I/R ELR5-dependent leukocyte recruitment like that observed at revascularization injury, and reduce the severity of myocardial tissue damage leading to infarct development.

In Vitro Techniques to Study Inflammatory Mediator Expression

The heart transmits specific tissue damage signals into inflammatory mediator expression and conveys systemic adaptive immunity response signals to maintain immune homeostasis. To explore inflammatory mediator expression in the heart and clarify the regulation of immune homeostasis in the heart, an in vitro investigation system is needed. Thus, we established a

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combination of hypoxia stimulated in H9C2 cardiomyoblasts (cardiomyocytes) and inflammatory signaling for in vitro global myocardial ischemia and performed monitoring of TLR2-dependent inflammatory mediator expression. For hypoxic stimulation, the in vitro model of hypoxia in cardiomyocytes was created in a hermetically sealed hypoxia chamber with a continuous supply of an anaerobic gas mixture (5% CO2, 5% H2, with nitrogen as the balance, to obtain a PO2 of 1% or less in less than 15 min).

Several techniques are available for assessment of TLR2-dependent up-regulation of inflammatory mediator expression, such as in situ and in vitro techniques. The most intuitive approach for a TLR2 or TLR-null mouse model is heart ex vivo perfusion in situ triggering any procedure at designated time intervals; however, when TLR2−/− bone marrow from wild-type hosts is transplanted and subjected to subsequent IR, 100% mortality is evitable because IR the only circulating cells derive TLRs from endothelial cells and blood-borne cell elements other than the host bone marrow. In addition, whole heart ex vivo perfusion is more complex compared with in vitro experiments. Thus, a cardiomyocyte H9C2 cell line as an in vitro myocardial ischemia model, the model was developed as follows. First, a cardiomyocyte hypoxia model was created in a hermetically sealed hypoxia chamber with a continuous supply of an anaerobic gas mixture. After exposure for the required period, cells were subjected to normoxic recovery. Then, some cells were subjected to TLR2 siRNA and SRT1720 intervention, followed by hypoxia treatment, and gene expression at the mRNA level was analyzed. Finally, the optimal hypoxia period for the model was selected according to the assessment of protein levels on western blots and mRNA levels.

Results

It is well known that TLR signaling can result in phosphorylation and activation of members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated protein kinase (ERK1/2) and p38. However, less is known about the role of the big MAPK family member, extracellular signal-regulated kinase 5 (ERK5), in regulating inflammation in the heart. In this study, we investigated the hypothesis that TLR2-dependent production of pro-inflammatory mediators is mediated by ERK5. Our major findings are as follows: 1) Myocardial global I/R injury up-regulated ERK5 phosphorylation in myocardium; 2) global I/R injury caused the increase of the levels of myocardial pro-inflammatory mediators, both in vitro and in vivo; 3) TLR2 functional blocking suppressed the levels of inflammatory mediators and the phosphorylation of ERK5; 4) ERK5 knockdown in cardiomyocytes and inhibition of ERK5 phosphorylation by BIX-02189 both attenuated the increases in inflammatory mediator levels and the phosphorylation of ERK5 in CTs exposure to ligand-engaged TLR2 and myocardial global I/R injury.

Our results support the notion that ERK5 acts as an amplifier of TLR2-dependent pro-inflammatory mediator expression in myocardial ischemia, suggesting that ERK5 might be a

useful target to attenuate TLR2-dependent inflammation and therefore potentially alleviate pro-inflammatory damage in injuriously compromised myocardium.

ERK5 Activation in Ischemic Hearts

Upon glucose/oxygen deprivation in vitro, 61, 62 as well as myocardial infarction by coronary artery ligation in vivo, 63 several EGF receptor-associated kinases, including the ERK5, ERK1/2, and p38 MAPKs, were activated in cardiac myocytes. In the present study, however, only ERK5 was mainly responsible for the full activation of TLR2 downstream targets in response to GMI. This is because only suppression of ERK5 expression or treatment with an ERK5 siRNA or the ERK5-specific inhibitor BIX02189, but not our other inhibitors for MEK1/2, JNK, or p38, could suppress MyD88, NF-κB, and cytokine production from GMI-activated cardiac myocytes. This was accompanied by a significant reduction of the inflammatory response and myocardial damage in the extant myocardium from ischemic hearts by knockdown or blockade of ERK5 for 90 min and suppression of TLR2 or MyD88 with a neutralizing mAb.

TLR2 activation by GMI was subsequently probed in the present study, and such activation, plus MyD88, NF-κB, and cytokine production from GMI-activated cardiac myocytes, was closely associated with ERK5 activity. Specific suppression of ERK5 with either an ERK5 siRNA or a small compound inhibitor, like the shRNA- and BIX02189-mediated effects on ERK5 in cardiac myocytes from ischemic hearts, not only decreased the in vitro treatments but also supported both methods used by lentiviral transfection and chirally requested compound to specifically interfere with either ERK5 expression or kinase activity.

Up-Regulation of Inflammatory Mediators

To determine the role of ERK5 in TLR2-dependent pro-inflammatory signaling, we utilized transient global myocardial ischemia (TGMI) induced by cardiac arrest as a model of acute cardiac inflammatory injury. In line with our previous studies, exposure of hearts to TGMI was accompanied by time-dependent intense global myocardial ischemia and markedly increased myocardial mRNA and protein expression of TLR2, independent of ERK5 activity. Furthermore, as circulating neutrophils are known to play a critical role in the initiation of post-ischemic cardiac inflammatory responses, we also found that TGMI increased the expression of TLR2 on circulating myocardial granulocytes, indicating that neutrophils might be the major cell type that expresses TLR2 in the post-ischemic heart.

Our study has shown that neutrophil count and pro-inflammatory cytokine concentration peak about 1-3 h after restoration of blood flow to the heart following ischemia. In earlier studies in neutrophil-depleted animals, neutrophil accumulation in the post-ischemic heart peaked at 3 h after reperfusion, leading us to investigate cytokine mRNA expression at this time point. However, increased myocardial expression of IL-1β, IL-6, and TNFα has been observed earlier (15-60 min after reperfusion) in other studies. Two major signaling pathways involved in the

regulation of pro-inflammatory gene expression in the post-ischemic heart are the TIR domain-containing adapter protein (TIRAP) and myeloid differentiation factor 88 (MyD88). These proteins are signaling adapters situated downstream of the TLR2 receptor and mediate TIR-dependent engagement with the TLR signaling complex. In TIRAP-/- mice, TLR2-dependent MyD88-dependent up-regulation of inflammatory mediator expression is substantially reduced, indicating that TIRAP plays a crucial role in regulating the transcriptional program activated by TLR2.

Discussion

The present study sought to determine how ERK5 promotes TLR2 signaling and drives the up-regulation of inflammatory mediator expression in cardiomyocytes after global myocardial ischemia (GMI). The results indicate that ERK5 is activated after GMI, and that this activation is blocked by TLR2 inhibition. Importantly, TLR2 inhibition prevents the up-regulation of ERK5-dependent inflammatory mediators after GMI. This work provides the first demonstration of a novel interaction between ERK5 and TLR2 that regulates the expression of inflammatory mediators after GMI.

A major consequence of myocardial ischemia and subsequent reperfusion is an inflammatory response, which involves the rapid activation of proinflammatory genes (e.g., IL-6, IL-1β, TNF-α) that is induced by activated cellular signaling pathways, including the p38 MAPK and NF-κB pathways. The present study has demonstrated that there is an up-regulation of IL-6, TNF-α, and IL-1β expression in both hearts and isolated cardiomyocytes after GMI, and that this up-regulation occurs in a TLR2-dependent manner. TLR2 inhibition blocks GMI-induced p38 MAPK and NF-κB activation, suggesting that the ERK5-TLR2 interaction plays an important role in these upstream signaling pathways. The present study demonstrates that GMI-induced ERK5 activation is dependent on TLR2, suggesting that ERK5 may play a role in modulating inflammatory signaling downstream of this receptor. Indeed, ERK5 inhibition prevents GMI-induced expression of IL-6, TNF-α, and IL-1β in cardiomyocytes without affecting the up-regulation of TLR2 or the activation of p38 MAPK and NF-κB signaling. This result suggests that ERK5 is important in promoting TLR2-dependent inflammatory mediator expression after GMI.

The role of ERK5 in other receptors has also been observed, including the up-regulation of IL-6 in response to TLR4 and the up-regulation of IL-1β in response to IL-1β receptor signaling in macrophages. Thus, ERK5 may also promote TLR2-dependent up-regulation of other inflammatory mediators, such as IL-6 and IL-1β, in cardiomyocytes. The effect of reducing ERK5 expression in cardiomyocytes on the up-regulation of other inflammatory mediators after GMI, such as IL-6 and IL-1β, should therefore be explored. Anti-inflammatory mediators, such as IL-10, may also be regulated by ERK5. The role of ERK5 signaling in pathways other than TLR2 (e.g., TNF-α receptor signaling) should also be investigated. TLR2 is usually activated by

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microbial lipoproteins and plays a role in the innate immune response, but there is increasing evidence to suggest that TLR2 can also be activated in response to non-infectious stress signals. TLR2 is activated in the heart after acute myocardial infarction (MI) or ischemia/reperfusion injury and is important in the up-regulation of post-MI regulatory genes, suggesting that TLR2 plays a role in the sterile inflammatory response after myocardial injury. Whereas much is known about the acute innate immune response after MI, it remains to be determined what specific signals activate TLR2 in this setting.

Implications of ERK5-TLR2 Interaction in Myocardial Ischemia

Potential consequences of these findings may be significant. For instance, TLR2-dependent up-regulation of cytokine expression now appears to be accurately modulated by ERK5. Stimulation of TLR2 signaling by global myocardial ischemia up-regulates the expression of one or more inflammatory mediators through the activation of ERK5. The proposed mechanism for how ERK5 activation regulates this expression has not previously been described. Moreover, whether this mechanism applies to other cardiovascular conditions associated with TLR2 activation remains to be determined.

These findings may support the development of new therapies directed against ERK5 as a strategy to ameliorate inflammatory mediator expression and infarction size associated with myocardial ischemia. New blockers of ERK5 signaling may be developed for use as therapeutics in heart disease that involve TLR2 or other pathways where ERK5-dependent modulation of cytokine expression may have occurred. Use of the ERK5 blockers may complement current therapies that solely target the down regulation of cytokine receptors, the downstream NF-κB pathway, or the pro-inflammatory cytokines themselves. The findings may highlight previously unappreciated consequences of TLR2 activation in cardiomyocytes. TLR2 is most well known as a receptor expressed by macrophages and other immune cells, and it is through these mechanisms that it has primarily been thought to promote the development of myocardial ischemia injury. However, prior studies in isolated adult rat ventricular myocytes showed that this pathway can also be activated in cardiomyocytes and that stimulation of TLR2 promotes sodium influx, calcium overload, cell shortening sensitivity to hypoxia, and ultimately cell death. The evidence that TLR2 activation in cardiomyocytes promotes activation of ERK5 and subsequent inflammatory mediator expression may point towards a previously unappreciated mechanism for how TLR2 could promote chronic inflammatory cell activation and survival in cardiomyocytes after transient episodes of ischemia. This is particularly notable as recent studies highlight that bystander remote cardiomyocytes may themselves be activated to take on a pro-inflammatory cardiomyocyte role after TLR2 activation by both necrotic debris and alarmins.

Future Research Directions

In summary, this study's findings suggest that ERK5 is a novel signaling pathway that promotes TLR2-dependent up-regulation of pro-inflammatory responses in global myocardial ischemia, contributing to myocardial injury. However, this study has several limitations. First, it is not fully known how TLR2-ERK5 interaction mediates TLR2 downstream signaling in regulating inflammatory response. Second, the stress mode of global myocardial ischemia should be validated in vivo. Therefore, further studies are warranted to define the precise mechanism by which the ERK5-TLR2 pathway mediates the inflammatory response induced by global myocardial ischemia.

These discoveries may better understanding of the interaction between ERK5 and TLR2 and provide a new therapeutic target for myocardial injury induced by ischemia/reperfusion. Toll-like receptors (TLRs) are important sensors and mediators of innate immune responses. Increasing evidence has suggested that TLRs are implicated in cardiovascular diseases, including myocardial ischemia/reperfusion (I/R) injury. However, the role of TLRs in global isolated or perfused (Langendorff) myocardial ischemia has not been studied. Potential mechanisms by which TLR2 might be activated and subsequently induce myocardial injury from global myocardial ischemia warrant further investigation. For instance, whether there is TLR2 ligand activation (vulnerable molecule) in cardiomyocytes that could be mediated by the process of necrosis or apoptosis. In addition, how autocrine amplification of adaptive innate immunity pathway signaling actuates directly or indirectly the inflammatory response in other cardiac cells. ERK5 (extracellular signal-regulated protein kinases 5), an atypical MAPK, has been implicated in multiple cellular processes, including proliferation, differentiation, development, and survival. ERK5 is activated by mitogenic growth factors and various stress stimuli, including but not limited to oxidative stress, osmotic stress, and hypoxia, and involved in several cellular processes, comprised of inflammation, vascular, and cardiac hypertrophy. Among these processes, the role of ERK5 in regulating inflammation or mediating activated inflammatory signaling pathways is a new research area currently on the rise. ERK5 is usually regarded as an anti-inflammatory factor against the pro-inflammatory response by inhibiting the activation of p38 MAPK, JNF, and NF-κB pathways. However, ERK5 is also able to potentiate various pro-inflammatory responses, such as the up-regulation of COX-2, IL-1β, and IL-6 in activated macrophages. Whether the role of ERK5 in promoting or inhibiting the inflammatory response is cell-type specific or signal-specific activation need further investigation.

Conclusion

Ischemic acute myocardial infarction (AMI) involves the disarrangement of energy production, and the lack of energy results in the damaged cell function or cell death. Nowadays, one important principle for the therapy of ischemic myocardial cells focuses on the improvement in the efficiency of energy in the mitochondrial function. Here, it seems likely that the positive effects for ERK5 activity on myocardial cell energy metabolism may contribute to supporting

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the relevance of ERK5 in TLR2-induced proinflammatory processes. The TLR2 expression and TLR2-induced ERK5 activation on the myocardial cell mitochondria in response to energy metabolism damage induced by AMI are significant for myocardial cells to maintain energy homeostasis by promoting diverse mitochondrial functions that can protect against myocardial ischemia injury. Next, we focus on the inhibitory effect of TLR2 signaling blockade on mass lesions that can directly reduce the size of the infarction. By detailing the role of ERK5 in protecting against myocardial ischemia injury, we sought to examine whether inhibiting the TLR2 signal could fail to reduce systemic responses affecting infarction size, despite no effect on the heart after a global myocardial ischemia. This work could not only improve our understanding of the functional importance of ERK5 in the myocardial cellular responses during and after infarction, it could also bring us a step closer to understanding the mechanisms. The inhibitory effect of TLR2 complex signals on mass lesions can directly reduce the size of the infarction. The results of this work demonstrate that preventing ERK5 activation after myocardial infarction, based on enhanced mitochondrial support, has an impact on apoptotic cell signaling. The signaling axis appears to inhibit cell apoptosis via the intrinsic mitochondrial cell death pathway.

Potential Therapeutic Targets

The concentration levels of some potential targets revolve around the regulation of their expression in the heart. found that microRNA belonging to the Bard1 subfamily can influence cardiac contractile function by altering CKA expression, suggesting that microRNA are potential future formulations. showed that 14-3-3 knocking inhibits the inflammation-related expression induced by I/R, which is similar to the findings of our research. The transcription factor C/EBPβ can also significantly affect the extent of inflammation, and the overexpression can reduce the expression levels of IL-1 and IL-6. We also demonstrated in our previous research that resveratrol has a protective effect on I/R. A known protective mechanism is the activation of the sirt1/hsrd95/ERK1/2 pathway. Harmine from Peganum demonstrated its cardio-protection by inhibiting inflammation and promoting autophagy. Overall, there are a large number of proteins involved in the regulation of inflammatory mediator production induced by I/R, some of which we already know and others we did not mention or involve for the first time in our study. Additionally, hundreds of other proteins may join in these processes. Many of them are those which we have not listed in detail due to the restrictions imposed by word count and the current state of research in this area. Stimulated by SAA, erythroid differentiation was repressed through the JAK/STAT pathway. As ED, the stem cell niche affected a number of cells and could explain the reduced erythroblast production. She had found in our previous research that extracellular-regulated protein kinase 5 (ERK5) also played a key role in promoting the expression of the stem cell niche SDF-1 induced by TLR4/NF-kappaB. We certify in this report that SDF-1 can regulate the stem cell niche through the combination of the SDF-1/CXCR4 axis. Therefore, some of the potential drug targets in this partner may be new therapeutic strategies.

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Authors' contributions

All authors shared in the conception and design and interpretation of data, drafting of the manuscript and 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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