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Signaling Pathway MEK1/2–ERK1/2 is Involved in the Cardioprotective Effect of Probiotic Strains in the Systemic Inflammatory Response in Rats

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ABSTRACT

Aim. To experimentally test the hypothesis of the participation of MEK1/2 and ERK1/2 kinases in the mechanism of the probiotic cardioprotection in the implementation of the signaling stage of the cardioprotective response to the administration of probiotic strains in the systemic inflammatory response in rats.

Materials and methods. The experiments were performed on male Wistar rats using a model of systemic inflammatory response syndrome, which includes obesity and chemically induced colitis. To provide probiotic cardioprotective effects, the animals were administered probiotic strains LA-5 and BB-12 orally. An inhibitor of MEK1/2 kinase and its associated ERK1/2 kinase PD98059 at a dose of 0.3 mg/kg were administered intravenously 20 minutes before the start of Langendorff perfusion of an isolated heart. The size of the necrosis zone (SNZ) was histochemically determined after 30 minutes of global ischemia and 90 minutes of reperfusion were simulated. Markers of the systemic inflammatory response (SIR) were detected in the blood.

Results. In the group of rats with a model of SIR in comparison with the control, a significant increase in the number of leukocytes and an increase in the level of proinflammatory cytokines in the blood, as well as a significant increase in SNZ were found (by 39% in relation to CTR, $p < 0.05$). In the group with probiotic correction, a significantly smaller SNZ was noted in relation to SIR, whereas in rats with the introduction of probiotics and the substance PD98059, SNZ was significantly bigger, i.e. the cancellation of the cardioprotective effect of probiotic therapy occurred.

Conclusion. Based on the conclusion that the cardioprotective effect was abolished by PD98059 administration, it can be assumed that the probiotic effect is provided by the MEK1/2 and ERK1/2 kinase pathways.

Keywords: myocardium, ischemia-reperfusion, cardioprotection, systemic inflammatory response, probiotics, MEK1/2 and ERK1/2 kinases, PD 98059

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Внутриклеточный сигнальный путь MEK1/2–ERK1/2 участвует в реализации кардиопротективного эффекта пробиотических штаммов при системном воспалительном ответе у крыс

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РЕЗЮМЕ

Цель. Экспериментально проверить гипотезу об участии киназ MEK1/2 и ERK1/2 в реализации сигнального этапа кардиопротективного ответа на введение смеси пробиотических штаммов *Lactobacillus acidophilus* (LA-5) и *Bifidobacterium animalis* subsp. *lactis* (BB-12) при системном воспалительном ответе у крыс.

Материалы и методы. Эксперименты выполнены на самцах крыс стока Вистар на модели синдрома системного воспалительного ответа, включающей ожирение и химически индуцированный колит. Для обеспечения пробиотической кардиопротекции животным внутрижелудочно вводили пробиотические штаммы LA-5 и BB-12. Ингибитор MEK1/2 киназы и сопряженной с ней ERK1/2 киназы PD98059 в дозе 0,3 мг/кг вводили внутрибрюшинно за 20 мин до начала перфузии изолированного сердца по Лангендорфу. Моделировали 30 мин глобальной ишемии и 90 мин реперфузии, после чего гистохимически определяли размер зоны некроза (РЗН). В крови определяли маркеры системного воспалительного ответа (СВО).

Результаты. В группе крыс на модели СВО по отношению к контролю отмечено значимое увеличение числа лейкоцитов и повышение уровня провоспалительных цитокинов в крови, а также значимое увеличение РЗН (на 39% по отношению к КТР, $p < 0,05$). В группе с пробиотичекой коррекцией отмечен значимо меньший РЗН по отношению к СВО, тогда как у крыс с введением пробиотиков и вещества PD98059 РЗН был значимо выше, т.е. произошла отмена кардиопротективного эффекта пробиотической терапии.

Заключение. На модели СВО пробиотик-индуцированная кардиопротекция обеспечивается при участии сигнального пути киназ, предотвращающих реперфузионное повреждение, включая MEK1/2 и ERK1/2 киназы.

Ключевые слова: миокард, ишемия-реперфузия, кардиопротекция, системный воспалительный ответ, пробиотики, киназы MEK1/2 И ERK1/2, PD 98059

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией данной статьи.

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INTRODUCTION

According to the Fourth Universal Definition of Myocardial Infarction (MI), most cases of the disease belong to type 1, which occurs spontaneously as a result of destabilization of an atherosclerotic plaque, the development of thrombosis, and atherothrombotic and atherothrombotic occlusion of a coronary artery. Early myocardial revascularization in MI is associated with a significant improvement in prognosis; however, restoration of blood flow through the infarct-related artery leads to the myocardial reperfusion injury. Under specific circumstances, reperfusion injury may be irreversible and result in a twofold increase in infarct size compared to the volume observed at the time of ischemia cessation [1]. The mechanisms of early ischemia – reperfusion injury (IRI) of the myocardium include oxidative stress, hypercontracture of cardiomyocytes, calcium overload, and opening of the mitochondrial permeability transition pore (mPTP). Experimental studies have demonstrated the effectiveness of numerous pharmacological and non-pharmacological interventions that reduce myocardial IRI. Nevertheless, the results of randomized clinical trials of different types of myocardial conditioning, as well as pharmacological cardioprotectors reproducing the effects of brief ischemia – reperfusion cycles, have been less convincing [2]. These facts encourage the ongoing search for new non-invasive and safe methods to induce a cardioprotective response. One such approach is targeted modulation of the intestinal microbiota composition – a new direction in non-pharmacological cardioprotection that has emerged over the past 10–15 years [3]. One possible explanation for the insufficiently effective transfer of experimental data on cardioprotection into clinical practice is the reduced efficacy of cardioprotective interventions with age and in the presence of comorbidities. In our previous studies, administration of the probiotic strains *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium animalis subsp. lactis* (BB-12) to animals with systemic inflammatory response (SIR) led to a reduction in infarct size, which was associated with specific qualitative changes in the intestinal microbiota composition and decreased plasma concentrations of proinflammatory cytokines [4]. Other authors have shown that administration of *Bifidobacterium animalis subsp. lactis 420* (B420) to mice with dysbiosis induced by a high-fat diet resulted in a significant decrease in infarct size caused by 30-minute coronary artery occlusion followed by reperfusion *in vivo* [5]. Investigation of the molecular mechanisms of probiotic-induced cardioprotection is at an early stage. It has been suggested that the reduction in MI size after probiotic administration may be due to decreased intestinal epithelial permeability accompanied by reduced microbial translocation, altered production of several gut microbial metabolites – primarily short-chain fatty acids – and increased bile acid levels [3]. At the same time, the similarity between the molecular mechanisms of intracellular protective signaling in the myocardium during classical cardioprotection (e.g., ischemic conditioning) and those during probiotic-induced protection remains poorly studied. It is known that classical cardioprotective stimuli activate the reperfusion injury salvage kinase (RISK) pathway, which includes phosphatidylinositol-3-OH kinase (PI3K) and extracellular signal-regulated kinases 1/2 (ERK1/2), as well as the survivor activating factor enhancement (SAFE) pathway [6]. Activated kinase cascades affect end effectors, such as ATP-sensitive potassium channels and the mitochondrial pore, thereby directly reducing IRI [7].

The present study is aimed at examining the dependence of the infarct-limiting effect of probiotic cardioprotection, induced by LA-5 and BB-12 administration to rats with SIR, on activation of one branch of the RISK signaling pathway – specifically, the MEK1/2 - ERK1/2 kinases. To achieve this goal, pharmacological inhibition of the interaction between phosphorylated MEK1/2 and inactive ERK1/2 was performed using the noncompetitive cyclic inhibitor PD98059 containing an amino group. The ERK1/2 inhibitor PD98059 was administered to animals exhibiting a previously formed probiotic cardioprotective response immediately before the start of isolated heart perfusion and simulation of global myocardial ischemia – reperfusion.

MATERIALS AND METHODS

The study was conducted on male Wistar rats obtained from the Nursery for Laboratory Animals, branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences (Pushchino, Russia), in compliance with the principles of humane treatment of laboratory animals. The protocol was approved by the Animal Care and Use Committee of the Almazov National Medical Research Center, Ministry of Healthcare of the Russian Federation (Minutes No. PZ23_9_V2 dated September 6, 2023). The animals were randomly divided into four groups: 1) control group (CTR, $n = 9$): rats kept under standard vivarium conditions with a regular laboratory diet

and free access to drinking water; 2) systemic inflammatory response (SIR, n = 9): after modeling of SIR [8], animals received 1 mL of normal saline orally once daily for 7 days. Twenty minutes before heart removal, 0.2 mL of water for injection was administered intravenously; 3) SIR + probiotic correction (SIR+PRC, n = 9) – after SIR modeling, the rats were intragastrically administered a a mixture of probiotic strains Lactobacillus acidophilus (LA-5) and Bifidobacterium animalis subsp. lactis (BB-12) at a dose of 10^8 CFU per animal for 7 days. Intravenous injection of 0.2 mL water was performed according to the previous protocol; 4) SIR + probiotic correction + PD98059 inhibitor (SIR+PRC+IPD, n = 9) – animals underwent the same procedures as the SIR+PRC group but received an intravenous injection of the MEK1/2 and ERK1/2 kinase inhibitor PD98059 at a dose of 0.3 mg/kg 20 minutes before heart removal in 0.2 mL of water for injection [9].

Global ischemia – reperfusion of the isolated heart was modeled using the Langendorff technique of isolated heart perfusion. One day before the completion of the experiment, under short-term anesthesia, 1.5 mL of whole blood was collected from the large subcutaneous vein for hematological and immunological analyses. A complete blood count was performed using an automated veterinary three-differential hematology analyzer (URIT-3000 Vet Plus, URIT Medical Electronic, China). The levels of tumor necrosis factor-alpha (TNFα), interleukin (IL)-1β, IL-6, and interferon-gamma (IFNγ) were determined by enzyme-linked immunosorbent assay (ELISA) (MR-96A, Mindray, China). Using the PhysExp hardware-software system (Cardioprotect LLC, Russia), the following parameters were recorded after 15 minutes of stabilization and at 15, 30, 45, 60, 75, and 90 minutes of reperfusion: left ventricular systolic pressure (LVSP, mm Hg), heart rate (HR, bpmin), and coronary flow (CF, mL/min). Infarct size was determined planimetrically after staining heart slices with 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37 °C. The 1.5-2.0-mm-thick slices were photographed from both sides. The unstained (TTC-negative) area was calculated as a percentage of the total slice area. The mean size of necrotic zone (SNZ) for each heart was expressed as a percentage of the total analyzed area.

Statistical analysis was performed using the STATISTICA 12.0 software package. The Shapiro–Wilk and Kolmogorov–Smirnov tests were used to assess normality. To address the issue of multiple comparisons, three initial groups were formed at the experimental planning stage: CTR (n = 9), SIR (n = 9), and SIR+PRC (n = 18). On the final day, after administration of water or the inhibitor, the SIR+PRC (n = 9) and SIR+PRC+IPD (n = 9) groups were formed. Considering the small sample size and the lack of normal distribution for several variables, blood parameters were analyzed using the nonparametric Kruskal–Wallis ANOVA by ranks, followed by post-hoc multiple comparisons (Kruskal–Wallis ANOVA and median test). The size of the necrosis zone (SNZ, %) was analyzed using the same statistical approach as for blood parameters. Data in tables and text were presented as median (Me) with interquartile range (25%; 75%). For hemodynamic measurements, Repeated Measures ANOVA followed by Tukey's post-hoc test was applied. Hemodynamic data were presented as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered statistically significant.

RESULTS

Animals in the SIR group had significantly lower body weight at the end of observation compared with the control group (326 ± 19 g vs. 350 ± 8 g, $p < 0.01$). The changes in body weight did not differ between the SIR, SIR+PRC (323 ± 13 g), and SIR+PRC+IPD (318 ± 12 g) groups. In the SIR group, the total leukocyte count was significantly higher than in the control group – by 43%, with lymphocytes increased by 46%, monocytes – by 56%, and granulocytes – by 39% ($p < 0.05$). In the SIR+PRC and SIR+PRC+IPD groups, no significant differences were observed compared with CTR and SIR, except for a reduction in total leukocyte count in the SIR+PRC+IPD group relative to SIR ($p < 0.05$).

In the SIR group compared with CTR, levels of TNFα increased by 48%, IL-1β – by 507%, IL-6 – by 75%, and IFNγ – by 342% ($p < 0.05$). In the SIR+PRC and SIR+PRC+IPD groups, cytokine levels were close to control values, showing significant decreases relative to SIR, except for persistently elevated IL-1β by 350% and 407%, respectively ($p < 0.05$, Table 2).

Hemodynamic parameters did not differ significantly between groups at baseline. During the entire reperfusion period, the SIR group showed a pronounced increase in LVSP and CF by the end of observation. In the probiotic-treated groups, LVSP remained at the control level. At the end of reperfusion, CF was higher in the SIR+PRC group relative to CTR, while in SIR+PRC+IPD, it was reduced compared with SIR (Table 3).

Table 1

Hematological Parameters, *Me* [25; 75]				
Group	CTR	SIR	SIR+PRC	SIR+PRC+IPD
Leukocytes, 10^9/L	12(7;13)	21(19;23)*	13(10;17)	13(9;14)#
Lymphocytes, 10^9/L	2.7(1.5;3.2)	5.0(4.1;5.5)*	3.7(2.6;4.1)	3.2(2.4;4.4)
Monocytes, 10^9/L	1.1(0.8;1.4)	2.5(1.5;5.7)*	2.1(1.1;2.4)	1.4(1.1;1.9)
Granulocytes, 10^9/L	7.3(5.9;8.0)	11.9(11.5;13.1)*	8.3(6.8;10.6)	8.1(6.3;8.3)
Erythrocytes, 10^{12}/L	4.0(3.7;4.3)	4.2(4.0;4.3)	4.2(3.6;4.4)	3.2(3.2;3.3)
Platelets, 10^9/L	305(218;373)	310(286;400)	433(238;469)	415(358;463)

* $p < 0.05$ vs. CTR; # $p < 0.05$ vs. SIR (Mann–Whitney *U* test).

Table 2

Levels of TNFα, IL-1β, IL-6, and IFNγ, *Me* [25; 75]				
Group Analyte	*CTR*	*SIR*	*SIR+PRC*	*SIR+PRC+IPD*
TNFα	9.8(8.1;11.3)	19.0(17.0;20.4)*	12.6(10.1;14.0)#	13.1(12.7;13.5)#
IL-1β	14(11;20)	85(57;119)*	63(30;81)*	76(48;85)*
IL-6	6.9(6.8;7.8)	12.1(8.3;14.0)*	7.8(7.3;9.0)#	6.3(5.7;6.6)#
IFNγ	6.1(5.3;9.8)	27(25;48) *	7.0(4.8;32)#	6.8(5.3;35.0)#

* $p < 0.05$ vs. CTR; # $p < 0.05$ vs. SIR (Mann–Whitney *U* test).

Table 3

Hemodynamic Parameters, $M \pm SEM$								
Group	Hemodynamic parameters	Basel.	reperfusion					
			15 min.	30 min.	45 min.	60 min.	75 min.	90 min.
CTR	LVSP	129±24	93±3	84±4	79±2	77±2	75±2	73±1.3
	HR (bpm)	291±10	394±39	377±22	367±11	290±19	304±37	371±23
	CF (mL/min)	11.0±1.6	6.0±0.9	5.0±0.5	4.0±0.1	3.5±0.6	3.1±0.4	2.3±0.3
SIR	LVSP	140±13	158±27*	149±25*	143±24*	145±21*	144±23*	141±23*
	HR (bpm)	244±43	280±29	274±23	320±32	310±27	318±15	345±29
	CF (mL/min)	8.6±0.6	4.9±0.5	4.6±0.5	4.4±0.4	4.1±0.4	3.8±0.3	3.4±0.3*
SIR+ PRC	LVSP	128±15	89±3#	83±3.5#	78±4#	75±3#	74±3#	72±3#
	HR (bpm)	232±20	461±51	214±38	353±31	341±22	338±24	313±17
	CF (mL/min)	10.1±1.2	5.4±0.5	4.9±0.4	5.0±0.5	4.4±0.4	4.4±0.4*	3.8±0.4*
SIR+ PRC+ IPD	LVSP	132±10	95±4#	88±5#	85±4#	83±5#	82±5#	79±5#
	HR (bpm)	259±25	314±51	373±22	343±27	356±27	344±21	337±24
	CF (mL/min)	9.3±0.9	4.8±0.5	3.6±0.4	3.2±0.2	2.3±0.5#	2.4±0.4#	2.3±0.4#

* $p < 0.05$ vs. CTR; # $p < 0.05$ vs. SIR (Tukey's post-hoc test).

In the CTR, SIR, SIR+PRC, and SIR+PRC+IPD groups, the necrotic zone occupied 37(37;45)%, 61(57;64)%, 49(45;53)%, and 56(53;60)% of the total slice area, respectively. This indicates a 39% increase in SNZ in the SIR group compared with CTR ($p < 0.05$) and a 20% reduction in the SIR+PRC group compared with SIR ($p < 0.05$).

DISCUSSION

In this study, a model of systemic inflammation was used as a comorbid background for the analysis of cardioprotection. This model was based on an increase in visceral adipose tissue mass due to feeding the animals with a high-fat diet in combination with acute inflammation of the large intestine, induced by chemical injury to the mucosa. In fact, such a combination of low-grade and acute inflammation is accompanied by the development of systemic inflammatory response syndrome (SIRS), the presence of which was confirmed by an increased leukocyte count in peripheral blood, negative body weight dynamics, and a marked elevation of proinflammatory cytokines (TNFα, IL-1β, IL-6, and IFNγ) in the blood. Our findings of more pronounced myocardial

damage during global ischemia – reperfusion under SIRS conditions are generally consistent with the literature. Thus, in a clinical study by J. Odeberg et al. (2016), it was shown that pre-existing inflammation, verified by elevated C-reactive protein levels and leukocyte count, was associated with higher incidence of myocardial infarction in patients with unstable angina and with a less favorable clinical course [10]. Experimental studies also indicate that the severity of myocardial IRI increases in the presence of systemic inflammation, for example in a model of dextran sulfate-induced inflammatory bowel disease in mice [11]. There is no doubt that a key role in reducing myocardial resistance to IRI under SIRS conditions belongs to the effects of proinflammatory cytokines and chemokines on cardiomyocytes. It is known that cytokines such as TNFα and IL-1β mediate receptor-dependent damaging effects on cardiomyocytes, which include the activation of programmed cell death pathways, enhanced production of reactive oxygen species, and other mechanisms [12]. This is supported by the fact that genetic or pharmacological blockade of proinflammatory cytokines is accompanied by a reduction in infarct size and leukocyte infiltration, as well as attenuation of left ventricular dilatation and dysfunction [13].

Probiotic therapy with *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium animalis* subsp. *lactis* (BB-12) administered for seven days to animals with SIRS was associated with a reduction in infarct size. At the same time, the use of probiotics led to a decrease in TNFα and IL-1β concentrations. The pathways by which the protective changes in gut microbiota composition are transferre from the intestine to the heart during IRI are of particular interest. In addition to attenuating the deleterious effects of proinflammatory cytokines, current literature considers mechanisms of direct influence of substances secreted by the intestinal microbiota and entering the circulation. Such humoral molecular signals may include short-chain fatty acids acting on free fatty acid receptor 3 (FFAR3), as well as bile acids, which affect target cells through the nuclear farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor 1 (TGR5) [14]. A neurogenic pathway of cardioprotective signal transmission resulting from activation of the microbiota – gut – brain axis is also not excluded [15]. However, in the context of the present work, the main focus was not on the mechanisms of signal transfer from the intestine with an altered microbiota composition to the heart or on the receptor systems of cardiomyocytes that perceive these stimuli, but rather on the intracellular signaling systems responsible for increasing cardiomyocyte resistance to IRI. Traditionally, the molecular mechanisms of cardioprotection are considered in terms of three successive stages: 1) the trigger stage, associated with receptor and non-receptor actions of signaling molecules on molecular targets in the cardiomyocyte; 2) the mediator stage, which includes activation of several intracellular signaling kinase cascades; and 3) the effector stage, involving changes in the activity of several terminal effectors of cardioprotection, such as mitochondrial and sarcolemmal ATP-sensitive potassium channels and the mitochondrial permeability transition pore. A pivotal role in the description of intracellular cardioprotective signaling pathways was played by the discovery of the RISK pathway by D.M. Yellon et al. [16]. This pathway is activated by many endogenous ligands, including adipokines, growth factors, hormones, and other biologically active substances. The circulating levels of some of these mediators may change upon probiotic administration or other interventions targeting gut microbiota composition, such as microbiota transplantation or metabolic surgery. The RISK signaling pathway is also activated during ischemic preconditioning of the myocardium; its activation persists throughout the ischemic phase of injury and exerts a cardioprotective effect in the reperfusion phase by limiting opening of the mitochondrial permeability transition pore [17]. This leads to reduced apoptosis, attenuation of oxidative stress, decreased mitochondrial calcium overload, and other effects that contribute to the mitigation of myocardial IRI [18]. The RISK pathway has two branches that converge on p70S6 kinase, phosphorylating and activating it; activated p70S6 kinase in turn suppresses glycogen synthase kinase-3β (GSK-3β) activity. One branch of the RISK pathway is represented by phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt), and the other – by the mitogen-activated protein kinases MEK1/2 and ERK1/2. There is a reciprocal relationship between these two branches, since inhibition of one cascade leads to activation of the other, and vice versa [19]. As active GSK-3β promotes opening of the mitochondrial permeability transition pore, suppression of its activity as a result of RISK pathway activation has pronounced cardioprotective consequences [20]. In recent years, it has been shown that inhibition of GSK-3β can elicit cardioprotection not only by suppressing opening of the mitochondrial pore but

also through other mechanisms, such as modulation of autophagy [21].

In the present study, we tested the hypothesis that the cardioprotective action of altered intestinal microbiota composition resulting from regular administration of probiotic strains is mediated at the level of intracellular signaling involving components of the RISK pathway, specifically through its cascade associated with MEK1/2 and ERK1/2 kinases. Blockade of signaling at the MEK1/2 - ERK1/2 level using PD98059 led to the loss of the infarct-limiting effect of probiotic cardioprotection under systemic inflammation, which indicates the key role of this signaling cascade in mediating the effects of probiotics on the heart. Apparently, the RISK pathway represents a non-specific, common terminal cell signaling pathway aimed at increasing myocardial resistance to IRI.

CONCLUSION

This study demonstrated that repeated gastric administration of probiotic bacterial strains to rats with systemic inflammatory response produced a cardioprotective effect manifested by a reduction in infarct size. Pharmacological inhibition of the MEK1/2 - ERK1/2 signaling pathway, belonging to the broader RISK pathway, abolished the probiotic-induced cardioprotection. These findings indicate that maintaining ERK1/2 activity during reperfusion is essential for protection against ischemia – reperfusion injury induced by gut microbiota modulation.

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