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microRNA-21 在大鼠心肌缺氧复氧损伤中的作用及其对细胞自噬的影响

李 辉* 赵 锴 王 磊
(商丘市第一人民医院心血管内科，商丘 476000)

摘要 本文应用大鼠心肌细胞缺氧 / 复氧损伤模型，探讨 microRNA-21(miR-21)在大鼠心肌缺氧复氧损伤中的作用及其对细胞自噬的影响．缺氧复氧后，RT-PCR 检测发现心肌 miR-21 表达上调($P < 0.05$)，流式细胞术检测表明细胞凋亡增加，RT-PCR 及蛋白质印迹(Western blot)检测发现 p62 显著下调而 beclin-1 显著上调($P < 0.05$)，提示缺氧复氧诱导心肌细胞凋亡和自噬异常．脂质体转染 miR-21 mimic 后，细胞凋亡显著增加($P < 0.05$)，p62 显著上调而 beclin-1 显著下调($P < 0.05$)，而转染 miR-21 抑制剂引起相反结果，提示 miR-21 在心肌缺氧复氧损伤中具有促进细胞凋亡、抑制细胞自噬的作用．生物信息学预测显示，Rab11a 的 3′-UTR 含有 miR-21 的结合位点，双荧光素酶基因报告系统及 Rab11a 过表达实验表明 Rab11a 是 miR-21 的靶基因之一．心肌过表达 Rab11a 能减少缺氧复氧后 miR-21 介导的细胞凋亡及自噬．由此表明，在大鼠心肌缺氧复氧损伤中，miR-21 可能通过负调控 Rab11a 促进心肌细胞凋亡，抑制心肌细胞自噬．本研究可能为预防和治疗心肌缺血再灌注损伤提供新策略．

关键词 心肌细胞，缺氧 / 复氧，miR-21，凋亡，自噬，Rab11a
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心肌缺血 / 再灌注损伤是缺血性心脏病临床治疗中最常见的一种病理损害，可加重心肌结构破坏和心功能障碍，是影响缺血性心脏病临床治疗及预后的重要因素[1]．随着缺血性心脏病的高发，如何预防或减轻心肌缺血 / 再灌注损伤已成为研究热点．microRNAs 是一类高度保守的内源小分子 RNA，可以通过结合靶基因的 3′非翻译区(3′-UTR)降解靶基因或抑制其翻译，进而负调控靶基因转录后的表达水平[2]．研究表明，microRNA-21 (miR-21)不仅参与心血管系统的正常发育，还调控着心肌肥厚、心律失常等多个病理生理过程[3-4]．已证实 miR-21 可通过调控细胞凋亡影响心肌细胞的损伤[5]，然而 miR-21 在心肌缺血 / 再灌注损伤中的作用及其靶标机制还有待进一步的阐明．

心肌细胞的缺氧 / 复氧(hypoxia/reoxygen，H/R)是心肌缺血 / 再灌注损伤主要的病变基础，心肌细胞的 H/R 模型可用于模拟心肌缺血 / 再灌注损伤．本研究通过建立大鼠心肌细胞 H/R 模型模拟心肌缺血 / 再灌注损伤，探究心肌细胞缺氧 / 复氧损伤中 miR-21 的作用及其对心肌细胞自噬的影响，以期为治疗心肌缺血 / 再灌注损伤提供新方向．

1 材料与方法

1.1 主要材料与试剂

清洁级 SD 大鼠乳鼠(河南省实验动物中心，许可证号：SCXK(豫) 2010-0002)；大鼠心肌 H9c2 细胞、人胚肾 HEK293 细胞(中国科学院上海细胞生物学研究所)；DMEM 培养基、胎牛血清 FBS 和青链霉素双抗溶液(Gibco 公司)；Ⅱ型胶原酶、胰蛋白酶、牛血清白蛋白 BSA (Sigma 公司)；

* 通讯联系人．
Tel: 0370-3255637，E-mail: shidasda@163.com
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Lipofectamine™ 2000 转染试剂、Trizol 试剂(Invitrogen 公司)；miR-21 模拟物(mimics)、miR-21 抑制剂(inhibitors)和 PCR 引物(上海吉玛制药技术有限公司)；Annexin Ⅴ-EGFP/PI 双染细胞凋亡检测试剂盒(上海宸凛生物科技有限公司)；TaqMan miRNA 反转录试剂盒、TaqMan 基因表达试剂盒(Applied Biosystems 公司)；RIPA 裂解液、BCA 蛋白质定量试剂盒(碧云天生物技术有限公司)；PVDF 膜(Millipore 公司)；兔抗大鼠 Rab11a、beclin-1、p62 多克隆抗体，小鼠抗大鼠 β-actin 单克隆抗体、辣根过氧化物酶标记的山羊抗兔二抗、兔抗小鼠二抗(Abcam 公司)；双荧光素酶报告基因检测试剂盒(Promega 公司).

1.2　方法

1.2.1　心肌细胞缺氧 / 复氧模型的建立

无菌条件下取 SD 乳鼠心室肌，Ⅱ型胶原酶和胰蛋白酶反复消化，差速贴壁法获得大鼠原代心肌细胞，接种于含 10% FBS 和 100 mg/L 青链霉素双抗的 DMEM 培养基，37℃、5% CO_2 培养约 48 h，待增殖良好，进入后续实验．用预充有 5% CO_2 和 95% N_2 的缺氧液(无糖无血清的 DMEM 培养基)置换大鼠 H9c2 心肌细胞与大鼠原代心肌细胞原培养基，在含 5% CO_2 和 95% N_2 的密闭培养箱中，37℃缺氧培养 12 h．再用预充有 5% CO_2 和 95% O_2 的含糖复氧液(含 10% FBS 的 DMEM 培养基)置换缺氧液，于含 5% CO_2 和 95% O_2 的密闭培养箱中，37℃培养 4 h，建立 H/R 模型.

1.2.2　脂质体转染

胰蛋白酶消化心肌细胞，取约 1×10^6 个相应细胞接种于 12 孔板，无血清 DMEM 培养基继续培养过夜，以保证转染前细胞汇合度能够达到 50%左右．采用 Lipofectamine™ 2000 分别将浓度为 50 nmol/L 的 miR-21 mimics 和 miR-21 inhibitors 转染至心肌细胞，并以 control miRNA 作对照．37℃、5% CO_2 孵育 6 h 后更换新鲜的含 10% FBS 的 DMEM 培养基，继续培养 24 h，RT-PCR 检测 miR-21．实验可分组为：Control，正常心肌细胞组；H/R，缺氧复氧损伤处理组；H/R +control miRNA，转染 control miRNA 后再 H/R 组；H/R+ miR-21 mimics，转染 miR-21 mimics 后再 H/R 组；H/R+miR-21 inhibitors，转染 miR-21 inhibitors 后再 H/R 组.

1.2.3　Annexin-Ⅴ-EGFP/PI 双染法流式细胞术

胰蛋白酶消化心肌细胞，离心收集后经冷 PBS 洗涤 2 次，加入 1×结合缓冲液(binding buffer)，制成终浓度约为 1×10^9/L 的细胞悬液 500 μl．向细胞悬液中先加入 5 μl Annexin Ⅴ-EGFP，温和混匀，4℃避光反应 15 min，再加入 10 μl PI，温和混匀，4℃避光孵育 5 min，1 h 内流式细胞仪检测细胞凋亡.

1.2.4　实时定量 RT-PCR

Trizol 法提取细胞总 RNA，TaqMan miRNA 反转录试剂盒合成模板 cDNA．用 TaqMan 基因表达试剂盒检测各组细胞中 miR-21 及 *Rab11a* mRNA 的表达．反应在 ABI 7500 荧光定量 PCR 仪上进行，条件为 95℃ 3 min，95℃ 12 s，58℃ 30 s，共 40 次循环．以 β-actin 为内参，据 $2^{-\Delta\Delta CT}$ 法计算 mRNA 相对表达量．miR-21 反转录引物序列为 5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCA-GTTGAGTCAACATC 3′．用于检测 miR-21 的引物序列为：上游 5′ ACACTCCAGCTGGGTAGCT-TATCAGACTGA 3′，下游 5′ GTGTCGTGGAGT-CGGCAATTC 3′；用于检测 *Rab11a* 引物序列为：上游 5′ CCTGGTCCCACAGATACCAC 3′，下游 5′ CTCAGAC CTGGGAAATGGAC 3′；用于检测 beclin-1 引物序列为：上游 5′ TGGATCACCCAC-TCTGTGAG 3′，下游 5′ TTATTGGCCAGAGCA-TGGAG 3′；用于检测 p62 引物序列为：上游 5′ TATTACAGCCAGAGTCAAGG 3′，下游 5′ CT-ACATACAGAAGCCAGAATG 3′；用于检测 β-actin 的引物序列为：上游 5′ ATTGCCGACAG-GATGCAGAA 3′，下游 5′ CAAGATCATTGCTC-CTCCTGAG CGCA 3′.

1.2.5　蛋白质印迹

RIPA 裂解液裂解细胞，离心提取总蛋白，BCA 试剂盒测定蛋白质浓度．10% SDS-PAGE 电泳结束后，半干法将蛋白转移至 PVDF 膜，5%脱脂奶粉和 1×TBST 混合液封闭 2 h，漂洗后分别加入用 5% BSA 和 TBS 稀释好的相关一抗(兔抗大鼠 Rab11a、beclin-1、p62 多克隆抗体，小鼠抗大鼠 β-actin 单克隆抗体)，4℃孵育过夜，漂洗后分别加入用 5%脱脂奶粉和 1×TBS 稀释过的相应二抗(HRP 标记的山羊抗兔二抗，兔抗小鼠二抗)，常温孵育 1 h，漂洗后置于发光液中显影扫描，软件 Quantity One V4.62 分析.

1.2.6　双荧光素酶基因报告系统

构建 *Rab11a* 野生型(pGL3-*Rab11a* 3′UTR-WT)和突变型(pGL3-*Rab11a* 3′UTR-MU)报告基因载体，脂质体法将报告基因载体和 miR-21mimics 分别共

转染至含海肾素表达质粒(pRL-TK vector)的HEK293细胞，双荧光素酶报告基因检测试剂盒检测．实验可分为3组：Control，仅转染control miRNA；Rab11a 3′ UTR (WT)，共转染miR-21 mimics与pGL3-Rab11a 3′ UTR-WT；Rab11a 3′ UTR (MU)，共转染 miR-21 mimics与pGL3-Rab11a 3′UTR-MU．

1.2.7 *Rab11a*过表达载体的构建及转染

构建不含miR-21结合位点的*Rab11a*过表达载体，与miR-21 mimics共转染至心肌细胞，37℃、5% CO_2培养48 h后，检测Rab11a在mRNA和蛋白质水平的表达及细胞凋亡和自噬变化．

1.2.8 统计学分析

应用SPSS 19.0进行统计学分析，每个实验重复3次，数据以均数±标准差($\bar{x} \pm s$)表示，采用*t*检验进行组间比较．

2 结　果

2.1 H/R前后心肌细胞miR-21的表达

实时定量RT-PCR检测miR-21在H/R损伤的心肌细胞中的表达，结果显示，与Control组相比，H/R损伤的大鼠原代心肌细胞与H9c2心肌细胞中，miR-21表达均显著上调($P < 0.05$，图1)，表明miR-21在心肌细胞缺血再灌注可能发挥重要作用．应用大鼠心肌H9c2细胞做后续实验．

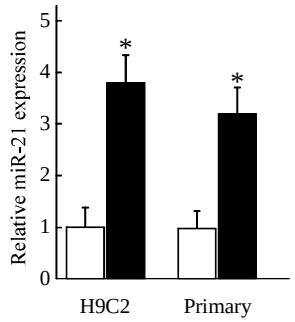

Fig. 1　The expression of miR-21 in cardiomyocytes treated with H/R injury

H9C2 cardiomyocytes and primary cardiomyocytes from rats were exposed to hypoxia/reoxy genation injury (H/R) or not (Control), qRT-PCR was used to determine miR-21 expression. *$P < 0.05$ *vs*. Control. □: Control; ■: H/R.

2.2 miR-21对H/R损伤诱导的心肌细胞凋亡的影响

细胞经H/R处理后，与Control组相比，H/R组心肌细胞凋亡显著增加($P < 0.05$)．转染miR-21 mimics或miR-21 inhibitors，进一步探讨miR-21对心肌细胞凋亡的影响．结果显示，与H/R组相比，H/R+miR-21 mimics组心肌细胞凋亡显著增加($P < 0.05$)，而H/R+miR-21 inhibitors组心肌细胞凋亡显著减少($P < 0.05$，图2)，表明miR-21能够促进H/R损伤心肌细胞的凋亡．

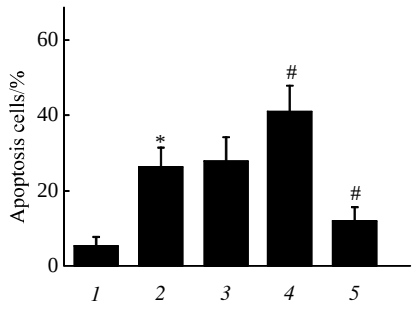

Fig. 2　The effect of miR-21 on myocardial apoptosis induced by H/R injury

H9C2 cardiomyocytes were exposed to hypoxia/reoxy genation injury (H/R) or not (Control) after respective transfection of Control miRNA (H/R + Control miRNA), miR-21 mimics (H/R + miR-21 mimics) or miR-21 inhibitors (H/R+miR-21 inhibitors), flow cytometry analysis was used to detect cardiomyocytes apoptosis. *$P < 0.05$ *vs*. Control. #$P < 0.05$ *vs*. H/R. *1*: Control; *2*: H/R; *3*: H/R + Control miRNA; *4*: H/R + miR-21 mimics; *5*: H/R+miR-21 inhibitors.

2.3 miR-21对H/R损伤诱导的心肌细胞自噬的影响

蛋白质印迹分析显示，细胞经H/R处理后，与Control组相比，H/R组p62表达显著下调而beclin-1表达显著上调($P < 0.05$，图3)，表明H/R诱发了心肌细胞自噬异常．转染miR-21 mimics或miR-21 inhibitors，进一步探讨miR-21对心肌细胞自噬的影响．结果显示，与H/R组相比，H/R+miR-21 mimics组p62表达显著上调而beclin-1表达显著下调($P < 0.05$)，H/R+miR-21 inhibitors组p62表达显著下调而beclin-1表达显著上调($P < 0.05$，图3)，表明miR-21能够抑制H/R损伤心肌细胞的自噬．

2.4 miR-21对H/R损伤心肌细胞Rab11a表达的影响

实时定量RT-PCR和蛋白质印迹检测过表达miR-21对Rab11a表达的影响．结果显示，与

Control 组相比，H/R 组心肌细胞中 Rab11a 在 mRNA 和蛋白水平均表达下调($P < 0.05$，图 4)，与 H/R 组相比，H/R +miR-21 mimics 组 Rab11a 在 mRNA 水平表达无显著变化，但在蛋白质水平显著下调($P < 0.05$).

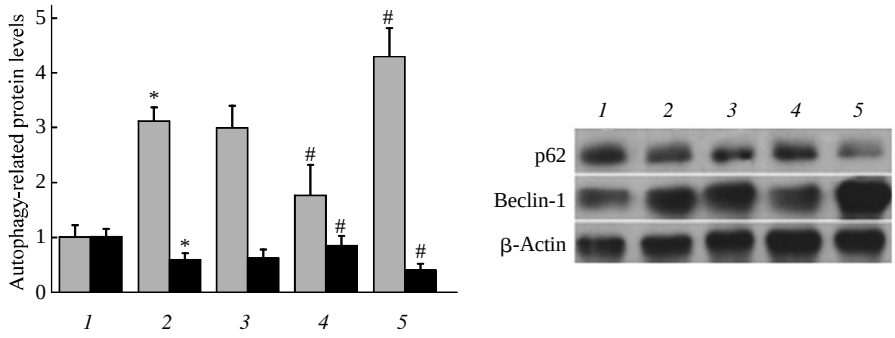

Fig. 3　The effect of miR-21 on myocardial autophagy induced by H/R injury

H9C2 cardiomyocytes were exposed to hypoxia/reoxy genation injury (H/R) or not (Control) after respective transfection of control miRNA (H/R + Control miRNA), miR-21 mimics (H/R+miR-21 mimics) or miR-21 inhibitors (H/R+miR-21 inhibitors), Western blot was employed to determine the expression of beclin-1 and p62. H/R.*$P < 0.05$ *vs*. Control. #$P < 0.05$ *vs*. H/R. □: Beclin-1; ■ : p62. *1*: Control; *2*: H/R; *3*: H/R+ control miRNA; *4*: H/R+ miR-21 mimics; *5*: H/R+miR-21 inhibitors.

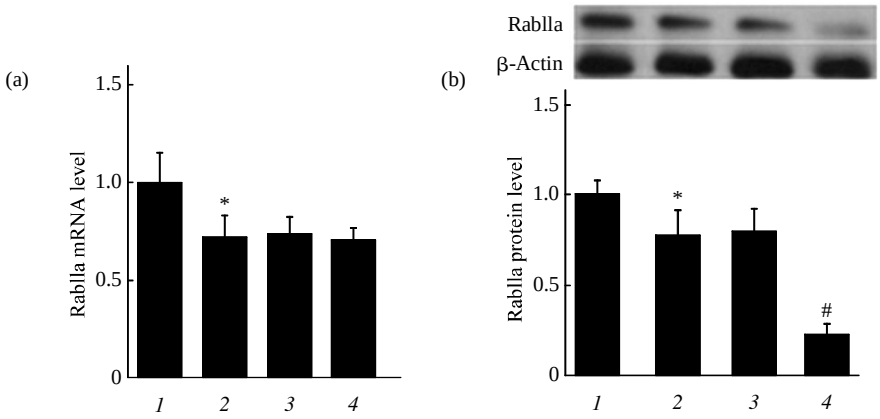

Fig. 4　The influence of miR-21 on expression of Rab11a

H9C2 cardiomyocytes were exposed to hypoxia/reoxy genation injury (H/R) or not (Control) after respective transfection of Control miRNA (H/R + Control miRNA) or miR-21 mimics (H/R+miR-21 mimics), qRT-PCR (a) and Western blot (b) was employed to determine the expression of Rab11a. *$P < 0.05$ *vs*. Control. #$P < 0.05$ *vs*. H/R. *1*: Control; *2*: H/R; *3*: H/R+control miRNA; *4*: H/R+miR-21 mimics.

2.5　双荧光素酶报告基因系统验证 miR-21 的靶基因

生物信息学方法预测到 Rab11a 的 3′-UTR 含有 miR-21 的结合位点(图 5a)，Rab11a 可能是 miR-21 的靶基因之一．双荧光素酶基因报告实验显示，与 Control 组相比，携带野生型报告基因载体的 Rab11a 3′-UTR(WT)组荧光素酶活性显著下调($P < 0.05$)，而携带突变型报告基因载体的 Rab11a 3′-UTR(MU)组荧光素酶活性无明显变化(图 5b).

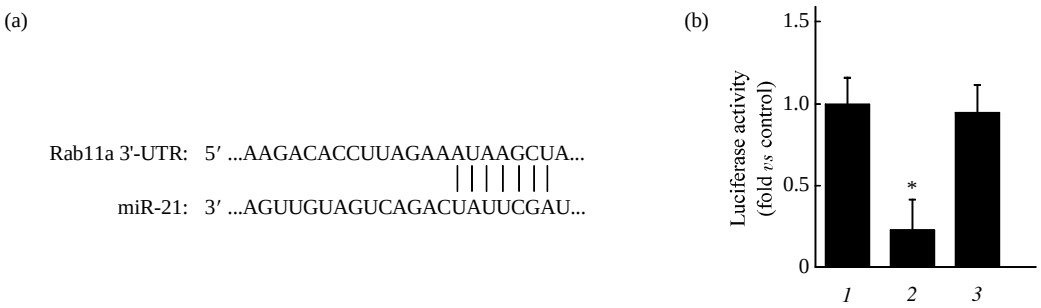

Fig. 5　Dual luciferase report gene assay

Bioinformatics predictionsuggest that 3′-UTR of Rab11a contains predicted binding site for miR-21(a). Recombinant plasmids containing wild-type 3′ UTR of Rab11a (Rab11a 3′UTR-WT) or mutant 3′UTR of Rab11a (Rab11a 3′UTR-MU) were constructed and co-transfected with miR-21 mimics into HEK293 cells containing pRL-TKvector, control miRNA co-transfected with pRL-TK vector as internal control reporter (Control), dual luciferase reporter assay (b) was used to determine luciferase activity.*P < 0. 05 *vs*. Control. *1*: Control; *2*: Rab11a 3′-UTR(WT); *3*: Rab11a 3′-UTR(MU).

2.6　Rab11a 过表达对 miR-21 介导的细胞自噬和凋亡的影响

与 Control 组相比，转染 Rab11a 过表达载体后 Rab11a 在 mRNA 和蛋白质水平均表达上调(P < 0.05，图 6a, b). 与 H/R+miR-21 mimics 组相比，H/R+miR-21 mimics+Rab11a 组细胞凋亡显著减少(P < 0.05，图 6c). 与 H/R+miR-21 mimics 组相比，H/R+miR-21 mimics+Rab11a 组 beclin-1 表达显著上调(P<0.05)，p62 表达显著下调(P<0.05，图 6d, e).

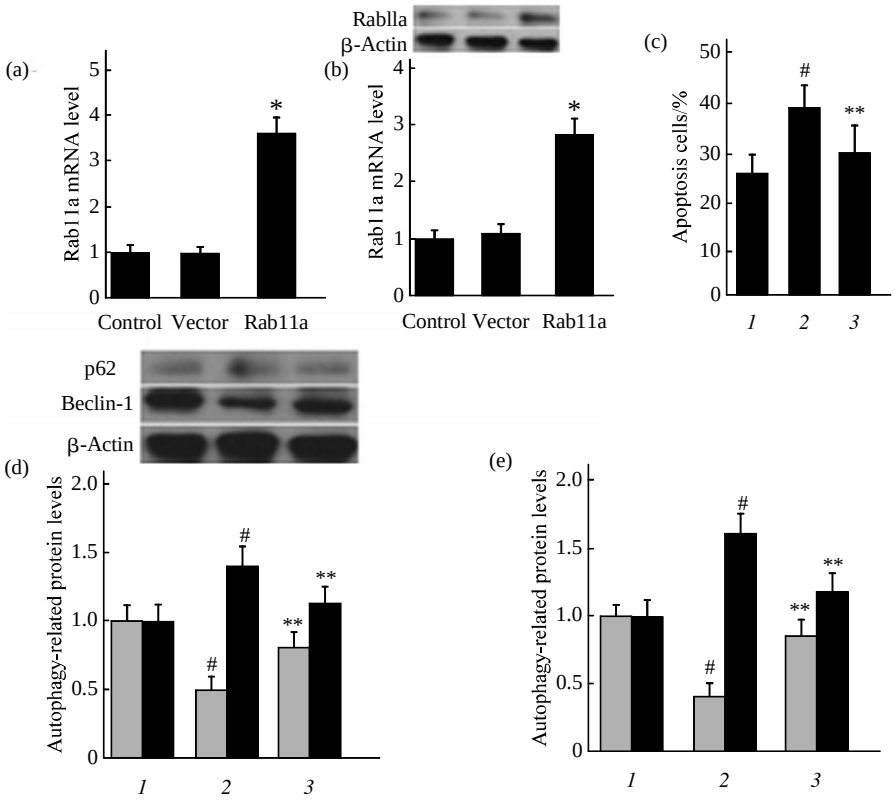

Fig. 6　Overexpression of Rab11a changes miR-21 mediated autophagy and apoptosis

Rab11a overexpressed plasmid which have no miR-21 binding site was constructed and transfected into H9C2 cardiomyocytes (Rab11a) or not (Control). H9C2 cardiomyocytes were exposed to hypoxia/reoxygenation injury (H/R) after transfection of miR-21 mimics (H/R+miR-21 mimics), or cotransfection of miR-21 mimics and Rab11a overexpressed plasmid (H/R+miR-21 mimics+Rab11a). qRT-PCR and Western blot was employed to determine the expression of Rab11a (a, b), beclin-1 and P62 (e, d), flow cytometryanalysis was used to detect cardiomyocytes apoptosis(c). *P < 0. 05 *vs*. Control and vector. #P < 0. 05 *vs*. H/R. **P < 0. 05 *vs*. H/R+miR-21 mimics. *1*: H/R; *2*: H/R+ miR-21 mimics; *3*: H/R+ miR-21 mimics+Rab11a. (d, e) □: beclin-1; ■: p62.

3 讨 论

缺血性心脏病已成为当今世界严重威胁人类健康和生命安全的重大疾病之一，尽快恢复缺血区的血流灌注是其最直接有效的治疗方法[6]. 然而研究发现，缺血心肌再次恢复血流灌注后，会出现心律失常、心肌梗死面积扩大、心功能显著下降等现象，即发生心肌缺血 / 再灌注损伤. 心肌缺血 / 再灌注损伤是一种复杂的多因素病理生理过程，往往伴随着心肌细胞的坏死、凋亡以及自噬[7]，也会引起 microRNA 的异常表达[8]. 凋亡是细胞为了更好地生存而主动死亡的过程，而自噬是胞内受损或老化的蛋白质和细胞器代谢更新的方式，过度或不足的凋亡或自噬都可能引起细胞受损，导致疾病发生[9]. 研究已证实通过 microRNA 调控心肌细胞的凋亡或自噬有助于减轻心肌缺血再灌注损伤[10-11].

本研究建立了大鼠心肌细胞缺氧 / 复氧模型模拟心肌缺血再灌注损伤，发现在大鼠心肌 H/R 损伤中 miR-21 表达升高，且过表达 miR-21 能够促进心肌细胞凋亡. 这与心肌缺血再灌注损伤 microRNA 表达谱中 miR-21 表达上调一致[12]. 此外，也有研究发现心肌梗死早期梗死区 miR-21 显著下调，但梗死周边区域 miR-21 上调[13]. miR-21 可以通过调控 PDCD4、PTEN/Akt 等调控缺血再灌注诱发的细胞凋亡[14]. beclin-1 和 p62 是两种常见的自噬相关蛋白，beclin-1 与自噬的诱导发生有关[15]，而 P62 往往随着自噬发生降解[16]. 本研究发现，H/R 损伤可诱使大鼠心肌细胞 beclin-1 上调而 p62 下调，证实 H/R 可诱发细胞自噬，若过表达 miR-21，则 beclin-1 下调而 p62 上调，表明 H/R 中 miR-21 能够抑制心肌细胞自噬. 然而，miR-21 在缺血再灌注损伤心肌中的表达变化及其作用仍存在争议，被视作“双刃剑”[17]，与缺血和再灌注的时间长短、损伤程度、病理机制等密切相关.

Rab11a 是一种小分子 GTP 酶，主要参与调节细胞内吞再循环过程，还与细胞自噬[18]、胞外分泌、信号传递等生命活动相关. 本研究发现 Rab11a 的 3′-UTR 含有 miR-21 的结合位点，通过双荧光素酶报告基因检测系统和 Rab11a 过表达证实 Rab11a 是 miR-21 的靶基因之一. 在 H/R 损伤中 Rab11a 蛋白表达下调，若过表达 miR-21，Rab11a 蛋白表达减少；若过表达 Rab11a，可减弱 miR-21 对心肌细胞的促凋亡抑自噬作用. 这表明 miR-21 可能通过调控 Rab11a 的表达影响心肌细胞的凋亡和自噬水平.

综上所述，本研究表明，在大鼠心肌细胞缺氧复氧损伤中，miR-21 表达增加，Rab11a 表达异常，miR-21 可能通过负调控 Rab11a 的表达，参与心肌细胞自噬与凋亡的调控. 然而，miR-21 靶基因众多，涉及多种信号通路，其发挥功能所涉及的具体分子机制和调控网络，尚待今后进一步的探讨.

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The Role of microRNA-21 in Rat Cardiomyocytes Exposed to Hypoxia/Reoxygenation Injury and Its Influence on Myocardial Autophagy

LI Hui*, ZHAO Kai, WANG Lei

(*Department of Cardiovascular Medicine, The First People's Hospital of Shangqiu, Shangqiu* 476000, China)

Abstract The hypoxia/reoxygenation injury model was established in rat cardiomyocytes to investigate the influence of microRNA-21 on apoptosis and autophagy in rat cardiomyocytes exposed to hypoxia/reoxygenation injury. According to RT-PCR, miR-21 was up-regulated($P < 0.05$) in myocardial cells after hypoxia/reoxygenation injury. Moreover, myocardial apoptosis was aggravated according to flow cytometry. According to RT-PCR and Western blot, p62 was down-regulated whereas beclin-1 was up-regulated in cardiomyocytes ($P < 0.05$) after hypoxia/reoxygenation injury. These consequences inditicated that hypoxia reoxygenation induced abnormal myocardial apoptosis and autophagy. miR-21 mimic or inhibitor were transfected into cardiomyocytes *via* liposome. MiR-21 mimic transfection significantly enhanced myocardial apoptosis ($P < 0.05$), up-regulated expression of p62, and down-regulated of expression of beclin-1in myocardial cells in rat cardiomyocytes($P < 0.05$), while miR-21 inhibitor transfection caused opposite effects. These data suggested that miR-21 in rat cardiomyocytes exposed to myocardial hypoxia reoxygenation injury can accelerate cell apoptosis and inhibit cell autophagy. Bioinformatics projections shown that Rab11a 3'-UTR contains a binding site for miR-21. Dual luciferase report gene assay system, coupled with the overexpression of Rab11a assay validated that Rab11a is one of miR-21 target genes. Overexpression of Rab11a significantly attenuated myocardial apoptosis，up-regulation of p62 and down-regulation of beclin-1 induced by miR-21in hypoxia/reoxygenation injury. In conclusion, miR-21 can promote myocardial apoptosis and inhibit myocardial autophagy by negative regulating Rab11a in rat cardiomyocytes exposed to hypoxia/reoxygenation injury.This research proposes a new strategy for the prevention and treatment of myocardial ischemia-reperfusion injury.

Key words cardiomyocytes, hypoxia/reoxygenation, microRNA-21,apoptosis; autophagy,Rab11a

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*Corresponding author.

Tel: 86-370-3255637, E-mail: shidasda@163.com

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