doi: 10.13241/j.cnki.pmb.2018.02.005

孤儿核受体 Nur77 对缺 / 复氧损伤中心肌细胞自噬的调节 *

游晓华 董斐斐 李松华 刘夙璇 赵仙先△

(第二军医大学长海医院心血管内科 上海 200433)

摘要 目的:研究孤儿核受体 Nur77 对缺 / 复氧损伤中心肌细胞自噬的调节作用。**方法:**差速贴壁法分离乳鼠心肌细胞,经免疫荧光染色鉴定纯度。缺氧(1 %O_2、5 %CO_2 和 94 %N_2)培养 12 h 后,常氧培养 2 h 构建心肌细胞缺 / 复氧损伤。实时定量 PCR 和 western blot 的方法检测 Nur77 的表达变化。通过 siRNA 转染抑制心肌细胞 nur77 表达,通过自噬标志蛋白表达改变作为细胞自噬水平的变化。**结果:**原代分离的心肌细胞纯度 95 %以上。缺氧 12 h 和缺 / 复氧(12 h/2 h)刺激后,心肌细胞中 Nur77 表达都明显升高(P<0.01)。与缺氧组相比,缺 / 复氧组细胞质中的水平明显增加(P<0.01),细胞核中 Nur77 水平无明显变化。抑制 Nur77 后,缺 / 复氧组自噬水平明显降低,缺氧组心肌细胞自噬水平无明显变化。**结论:**Nur77 参与缺 / 复氧损伤中心肌细胞自噬水平的调节。

关键词:孤儿核受体 77;心肌细胞;缺复氧损伤;自噬

中图分类号:R-33; R541 **文献标识码:**A **文章编号:**1673-6273(2018)02-219-03

Regulatory Role of Orphan Nuclear Receptor Nur77 in Cardiomyocytes Autophagy during Hypoxia/Reoxygenation Injury*

YOU Xiao-hua, DONG Fei-fei, LI Song-hua, LIU Su-xuan, ZHAO Xian-xian△

(Department of Cardiology, Second Military Medical University, Shanghai, 200433, China)

ABSTRACT Objective: To study regulation of orphan nuclear receptor Nur77 on cardiomyocytes autophagy during hypoxia/reoxygenation injury. **Methods:** Primary neonatal rat cardiomyocytes were obtained by differential adherence method, and were identified purity by immunofluorescence staining. Cardiomyocytes hypoxia/reoxygenation injury was established by culturing in hypoxic condition (1% O_2, 5% CO_2 and 94% N_2) for 12 h, followed by reoxygenation for 2 h. Expression of nur77 was determined by real-time PCR. Cardiomyocytes Nur77 expression was inhibited by siRNA transfection. Autophagy level was determined according to the expression of autophagy marker. **Results:** The purity of separated cardiomyocytes reached by more than 95 %. The expression of Nur77 was significantly increased in cardiomyocytes after hypoxia (12 h) and hypoxia/reoxygenation (12 h/2 h) treatment (P<0.01). Compared with hypoxia group, Nur77 was detected significantly increase in cytoplasm from hypoxia/reoxygenation treated cardiomyocytes, but no changes in nucleus. By Nur77 inhibition, the autophagy level was significantly decreased in hypoxia/reoxygenation treated cardiomyocytes, but no significantly change was found in hypoxia treated cardiomyocytes. **Conclusion:** Nur77 was involved in regulation of cardiomyocytes autophagy during hypoxia/reoxygenation injury.

Key words: Nur77; Cardiomyocytes; Hypoxia/reoxygenation injury; Autophagy

Chinese Library Classification(CLC): R-33; R541 Document code: A

Article ID:1673-6273(2018)02-219-03

前言

心肌缺血再灌注损伤是临床上心血管疾病手术治疗后常见的心脏损害,如何减轻缺血再灌注(ischemia/reperfusion, I/R)对心脏的损伤一直是临床研究的重点[1,2]。自噬是真核细胞内普遍存在的一种降解机制,基础状态下的自噬可通过清除损伤细胞器或错误折叠蛋白质,维持细胞稳态[3]。研究发现,心肌细胞自噬是缺血再灌注损伤的重要机制。心肌缺血再灌注过程中,各种刺激可能通过不同信号转导途径激活超出生理限度的自噬,将加重心肌损伤[4,5]。孤儿核受体 Nur77 是一种立刻早期基因,是核受体超家族的重要成员之一[6,7]。近期研究发现,Nur77 可通过与线粒体外膜蛋白 Nix 作用并移位至线粒体,引发线粒体过度清除,造成黑色素瘤细胞自噬性凋亡[8]。本研究通过心肌细胞缺复氧损伤的细胞模型,研究 Nur77 的表达变化及心肌细胞自噬的调节作用。

1 材料和方法

1.1 动物与试剂

* 基金项目:上海市自然科学基金项目(14ZR1408100)

作者简介:游晓华(1975-),女,主治医师,主要研究方向:心血管疾病发病机制与诊断治疗,电话:021-31161266,E-mail: youxiaohua@medmail.com.cn

△ 通讯作者:赵仙先,电话:021-31161266,E-mail: xianxianz2010@163.com

(收稿日期:2017-06-16 接受日期:2017-07-12)

新生 1-2 天的 SD 乳鼠通过本校实验动物中心购买。BrdU 和胶原酶(II 型)购于 Sigma 公司。RNA 提取试剂盒、逆转录试剂盒和定量 PCR 试剂盒均购于 TARAKA 公司。α-actinin、Nur77 及 β-actin 抗体均购于 Abcam 公司。

1.2 心肌细胞分离

将 SD 乳鼠经酒精消毒皮肤后，无菌条件下分离出心室，迅速置入预冷的 D-Hanks 缓冲液中，清洗干净后将组织剪成碎块。加入 II 型胶原酶(0.1%)，4℃条件下内放置过夜。离心 2 次去除消化酶后，以 DMEM 细胞培养液(含 10%小牛血清)重悬后，37℃，5%CO_2 条件下静置培养 2.5 h。利用差速贴壁的方法分离出心肌细胞，经血球计数板计数后，以 3× 10^5/ 孔的密度接种于 12 孔培养板。随后更换为含 BrdU(0.1 mmol/L)的 DMEM 培养液继续培养 2d，用于进一步实验。

1.3 心肌细胞免疫荧光

以多聚甲醛(4%，PBS 配制)将心肌细胞在 4℃条件下固定过夜，加入 Triton X-100(0.5%)进行渗透化处理，马血清(5%)常温封闭 1 h 后，加入 α-actinin 抗体(1∶1000)，4℃条件下杂交过夜。经 PBST 缓冲液洗涤后，加入荧光二抗(1:2000)，室温杂交 1.5 h。经 PBST 缓冲液洗涤后，用 DAPI 进行细胞核染色，并在荧光显微镜下观察鉴定。

1.4 心肌细胞缺 / 复氧培养

将分离的心肌细胞在三气培养箱中，以 1%O_2、5%CO_2 和 94%N_2 的条件缺氧培养 12 h，随后转移至 5%CO_2 的常规条件继续培养 2 h，造成心肌细胞缺 / 复氧损伤。以细胞生长活力(CCK-8 法检测)和培养液上清中乳酸脱氢酶(Lactate dehydrogenase，LDH)水平(ELISA 法检测)作为心肌细胞损伤标准。

1.5 实时定量 PCR

将提取的细胞总 RNA 经紫外分光光度计检测质量后，以 200 ng RNA 样品为模板，按试剂盒说明进行逆转录反应，反应条件为 37℃(15 min)；85℃(5 s)。以适当倍数稀释后的逆转录产物为模板，进行实时定量 PCR 反应。反应条件为 95℃(5 s)，55℃(15 s)，，72℃(15 s)，设置 40 个 PCR 循环。β-actin 为内参基因。引物序列如下：Nur77-F(5'-3'): AGCTTGGGTGTTGATGTTCC；Nur77-R(5'-3'): GGAGGCCATGTCGATCAG。β-actin-F(5'-3')：CCCGCGAGTACAACCTTCT；β-actin-R(5'-3')：CGTCATCCATGGCGAACT。

1.6 蛋白质免疫印迹

利用 SDS 裂解液提取心肌细胞总蛋白，通过 BCA 定量后取 30 μg 总蛋白，与上样缓冲液混匀，沸水浴处理 15 min 后迅速置于冰上 5 min。经 10% SDS-PAGE 凝胶电泳进行蛋白分离，转膜后以 5%脱脂牛奶进行封闭，并加入一抗(1:1000)，于 4℃条件下杂交过夜。用 TBS-T 缓冲液清洗后加入二抗(1:1000)，常温下杂交 2 h。用 TBS-T 缓冲液清洗后，通过 ECL 试剂盒显影。

1.7 统计学处理

所有数据采用 $\bar{x}\pm s$ 表示，应用 SPSS 统计学软件进行分析。两组间比较利用 Student-t 检验，多组间比较利用 one-way ANOVA 方差分析。P<0.05 差异认定有统计学意义。

2 结果

2.1 心肌细胞的免疫荧光鉴定

差速贴壁分离的心肌细胞培养 72h 后，形态上主要以多边形为主，具有自发性搏动行为。免疫荧光染色结果(图 1)显示，呈绿色荧光信号的心肌细胞约在 95%以上，符合实验所需。

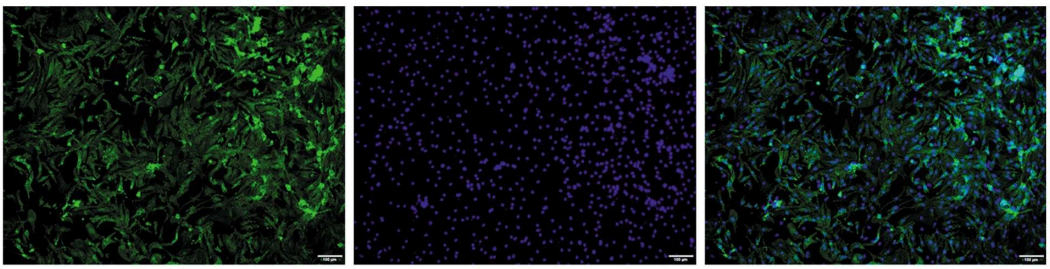

图 1 乳鼠心肌细胞的 α-actinin 免疫荧光鉴定

Fig.1 Identification of cultured neonatal rat cardiomyocytes by immunofluorescence with α-actinin

2.2 缺 / 复氧刺激对心肌细胞 Nur77 表达和定位的影响

蛋白质免疫印迹(图 2A)结果发现，缺氧组和缺 / 复氧组中 Nur77 的表达，较常规培养组均明显升高(P<0.01)，但两组间无明显差异。分离细胞核和细胞质后提取 RNA，定量 PCR 结果(图 2B)，细胞核中 Nur-77 在缺氧组及缺 / 复氧组均明显升高，两组间无明显差异。但细胞质中，缺 / 复氧组细胞质中 Nur77 的表达较缺氧组和常规培养组均明显增加(P<0.01)。

2.3 Nur77 对缺 / 复氧刺激心肌细胞自噬水平的影响

通过 siRNA 转染干预 Nur77 的表达，定量 PCR(图 3A)结果显示，24 h 后，Nur77 的表达水平明显降低(P<0.01)，是对照组的 0.39± 0.06 倍。蛋白质免疫印迹(图 3B)结果发现，未抑制

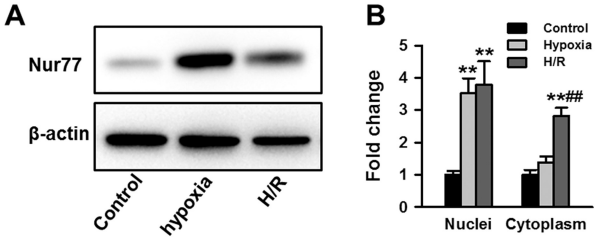

图 2 缺 / 复氧刺激对心肌细胞 Nur77 表达和定位的影响

Fig.2 Role of hypoxia /reoxygenation on expression and location of Nur77 in cardiomyocytes

Nur77 的条件下，自噬标志蛋白 Beclin 1 在缺氧组和缺 / 复氧组的表达均明显高于对照组。但抑制 Nur77 后，Beclin 1 在缺氧组无明显变化，在缺 / 复氧组却明显降低。

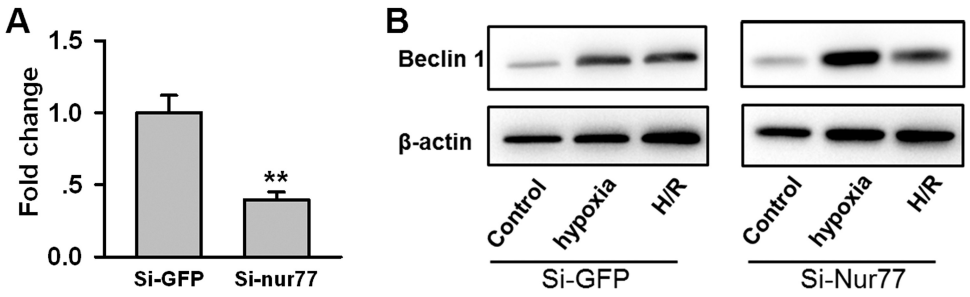

图 3 抑制 Nur77 对缺 / 复氧刺激心肌细胞自噬水平的影响

Fig.3 Role of Nur77 inhibition on cardiomyocytes autophagy induced by hypoxia /reoxygenation

3 讨论

细胞自噬是缺血再灌注过程中心肌细胞死亡的重要机制之一，持续缺氧培养后，心肌细胞中自噬体出现，而复氧后自噬体的数量明显增加。在急性或慢性缺血条件下自噬均可被激活，而再灌注阶段自噬体的形成又进一步增加[9,10]。目前一致认为，在心肌缺血阶段，自噬可以降解无功能的胞浆蛋白和为细胞器提供营养物质，也可以抑制凋亡，从而发挥细胞保护作用[11,12]。但在再灌注阶段，自噬对心肌细胞存活的作用目前尚未阐明。自噬在再灌注阶段的作用具有两面性，造成这些差异的机制可能在于，缺血和再灌注时激活自噬的信号转导通路可能存在差别，缺血时主要由 AMPK 介导，而再灌注时则由心肌细胞内质网应激，自由基超载以及 Beclin1 的活化，使自噬过度激活，从而加速细胞死亡[13,14]。心肌缺血再灌注过程中，不同的应激水平和刺激可能通过不同信号转导途径激活自噬，超出生理限度的自噬，如果得不到有效的控制，将加重心肌损伤[15,16]。

Nur77 作为核受体超家族的重要成员之一，可被多种活性因子诱导表达，对细胞增殖、分化发育和凋亡等过程具有重要的生物学功能[17,18]。在心肌细胞的生命活动中，Nur77 也具有重要的调节作用。Yan 等发现 Nur77 能抑制对 β- 肾上腺素诱导的心肌肥大[19]。Cheng 等发现 Nur77 的线粒体易位介导心肌细胞的凋亡[20]。我们的研究发现缺 / 复氧过程中，Nur77 可能通过线粒体易位，激发心肌细胞过度自噬，从而增加细胞凋亡。

本研究通过原代分离的乳鼠心肌细胞模型，结果确定了 Nur77 在心肌细胞缺复氧培养过程中的表达变化及分布，发现抑制 Nur77 能够显著降低心肌细胞自噬水平，提示 Nur77 对再灌注损伤过程中心肌缺血自噬水平具有调控作用。因此，进一步阐明 Nur77 对自噬的调控机制将为心肌缺血再灌注损伤的防治研究提供新的方向。

参 考 文 献(References)

[1] Weigt SS, Palchevskiy V, Belperio JA. Inflammasomes and IL-1 biology in the pathogenesis of allograft dysfunction [J]. J Clin Invest, 2017, 127(6): 2022-2029

[2] Jennings RB. Historical perspective on the pathology of myocardial ischemia/reperfusion injury[J]. Circ Res, 2013, 113(4): 428-438

[3] Lekli I, Haines DD, Balla G, Tosaki A. Autophagy: an adaptive physiological countermeasure to cellular senescence and ischaemia/reperfusion-associated cardiac arrhythmias [J]. J Cell Mol Med, 2017, 21(6): 1058-1072

[4] Hariharan N, Zhai P, Sadoshima J.Oxidative stress stimulates autophagic flux during ischemia/reperfusion [J]. Antioxid Redox Signal, 2011, 14(11): 2179-2190

[5] Zhang Y, Ren J. Targeting autophagy for the therapeutic application of histone deacetylase inhibitors in ischemia/reperfusion heart injury[J]. Circulation, 2014, 129(10): 1088-1091

[6] Niu G, Lu L, Gan J. Dual roles of orphan nuclear receptor TR3/Nur77/NGFI-B in mediating cell survival and apoptosis [J]. Int Rev Cell Mol Biol, 2014, 313: 219-258

[7] Ranhotra HS. The NR4A orphan nuclear receptors: mediators in metabolism and diseases [J]. J Recept Signal Transduct Res, 2015, 35 (2): 184-188

[8] Wang WJ, Wang Y, Chen HZ, et al. Orphan nuclear receptor TR3 acts in autophagic cell death via mitochondrial signaling pathway [J]. Nat Chem Biol, 2014, 10(2): 133-140

[9] Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes[J]. J Biol Chem, 2006, 281(40): 29776-29787

[10] Matsui Y, Kyoi S, Takagi H, et al. Molecular mechanisms and physiological significance of autophagy during myocardial ischemia and reperfusion[J]. Autophagy, 2008, 4(4): 409-415

[11] Kanamori H, Takemura G, Goto K, et al. Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion[J]. Am J Physiol Heart Circ Physiol, 2011, 300(6): H2261-2271

[12] Yan L, Vatner DE, Kim SJ, et al. Autophagy in chronically ischemic myocardium[J]. Proc Natl Acad Sci U S A, 2005, 102(39): 13807-13812

[13] Ravikumar B, Sarkar S, Davies JE, et al. Regulation of mammalian autophagy in physiology and pathophysiology [J]. Physiol Rev, 2010, 90(4): 1383-1435

[14] Matsui Y, Takagi H, Qu X, et al. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy [J]. Circ Res, 2007, 100 (6): 914-922

（下转第 258 页）

toxicity[J]. Pak J Pharm Sci, 2017, 30(6(Supplementary)): 2363-2368

[5] Wang Yu, Zhou Man-hong, Lu Yuan-lan, et al. Protective effect of 5-aminosalicylic acid on the kidney of paraquat poisoning rats by Nrf2-ARE signal pathway [J]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue, 2017, 29(11): 961-966

[6] Schroder M, Kaufman RJ. The mammalian unfolded protein response [J]. Annu Rev Biochem, 2005, 74: 739-789

[7] Lu M, Lawrence DA, Marsters S, et al. Opposing unfolded-protein-response signals converge on death receptor 5 to control apoptosis[J]. Science, 2014, 345(6192): 98-101

[8] Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in metazoans: cell survival in the land of plenty [J]. Nat Rev Mol Cell Biol, 2005, 6 (6): 439-448

[9] Szegezdi E, Logue SE, Gorman AM, et al. Mediators of endoplasmic reticulum stress-induced apoptosis [J]. EMBO reports, 2006, 7 (9): 880-885

[10] Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress[J]. Cell Death Differ, 2004, 11: 381-389

[11] Nakagawa T, Zhu H, Morishima N, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyliod-beta[J]. Nature, 2000, 403: 98-103

[12] Chinta SJ, Rane A, Poksay KS, et al. Coupling endoplasmic reticulum stress to the cell death program in dopaminergic cells:effect of paraquat[J]. Neuromolecular Med, 2008, 10(4): 333-342

[13] Meng Xiao-xiao, Liu Kan, Tan Jiu-ling, et al. The relationship of endoplasmic reticulum stress with paraquat induced lung fibrosis in rats[J]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue, 2013, 25(6): 331-334

[14] Zhang Zhi-jian, Dong Yao-yao, Li Xiao-ping, et al. Total flavonoids from Astragalus complanatus attenuates lung injury following paraquat poisonging in rats through inhibiting excessive endoplasmic reticulum stress and c-Jun N-terminal kinase pathway [J]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue, 2014, 26(6): 383-387

[15] Li Hai-feng, Zhao Shi-xing, Xing Bao-peng, et al. Ulinastatin suppresses endoplasmic reticulum stress and apoptosis in the hippocampus of rats with acute paraquat poisoning [J]. Neural Regen Res, 2015, 10(3): 467-472

[16] Chen Ya-wen, Yang Yuan-ting, Hung Dong-zong, et al. Paraquat induces lung alveolar epithelial cell apoptosis via Nrf-2-regulated mitochondrial dysfunction and ER stress [J]. Arch Toxicol, 2012, 86 (10): 1547-1558

[17] Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy[J]. Developmental Cell, 2004, 6(4): 463-477

[18] Henry Shih, Brian Lee, Randall J, et al. Boyle.The Aging Heart and Post-Infarction Left Ventricular Remodeling [J]. J Am Coll Cardiol, 2010, 57(1): 9-17

[19] Katlshiks S, Cuervo AM. Autophagy as a cell-repair mechanism: activation of chaperone-mediated autophagy during oxidative stress [J]. Mol Aspects Med, 2006, 27(5-6): 444-454

[20] Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in metazoans: cell survival in the land of plenty [J]. Nat Rev Mol Cell Biol, 2005, 6 (6): 439-448

[21] Hariharan N, Zhai P, Sadoshima J. Oxidative stress stimulates autophagic flux during ischemia/reperfusion [J]. Antioxid Redox Signal, 2011, 14(11): 2179-2190

[22] Xu Ling-jie, Wang Zhong. Chloroquine rescues A549 cells from paraquat-induced death[J]. Drug Chem Toxicol, 2016, 39(2): 167-173

[23] Li Hai-feng, Xing Bao-peng, Quan Yu-lan, et al. The effect of selective phosphatase inhibitors Salubrinal on autophagy and apoptosis in the lungtissue of rats with acute paraquat poisoning[J]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue, 2014, 26(9): 671-675

[24] Ding Wen-xing, Ni Hong-min, Gao Wen-tao, et al. Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival[J]. J Biol Chem, 2007, 282(7): 4702-4710

[25] Ishida Y, Nagata K. Autophagy eliminates a specific species of misfolded procollagen and plays a protective role in cell survival against ER stress[J]. Autophagy, 2009, 5(8): 1217-1219

[26] Niso-Santano M, Bravo-San Pedro JM, Gómez-Sánchez R, et al. ASK1 overexpression accelerates paraquat-induced autophagy via endoplasmic reticulum stress[J]. Toxicol Sci, 2011, 119(1): 156-168

(上接第 221 页)

[15] Xia Y, Liu Y, Xia T, et al. Activation of volume-sensitive Cl-channel mediates autophagy-related cell death in myocardial ischaemia/ reperfusion injury[J]. Oncotarget, 2016, 7(26): 39345-39362

[16] Huang Z, Wu S, Kong F, et al. MicroRNA-21 protects against cardiac hypoxia/reoxygenation injury by inhibiting excessive autophagy in H9c2 cells via the Akt/mTOR pathway [J]. J Cell Mol Med, 2017, 21 (3): 467-474

[17] Zhao Y, Bruemmer D. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology [J]. Arterioscler Thromb Vasc Biol, 2010, 30 (8): 1535-1541

[18] Levesque D, Rouillard C. Nur77 and retinoid X receptors: crucial factors in dopamine-related neuroadaptation [J]. Trends Neurosci, 2007, 30(1): 22-30

[19] Yan G, Zhu N, Huang S, et al. Orphan Nuclear Receptor Nur77 Inhibits Cardiac Hypertrophic Response to Beta-Adrenergic Stimulation[J]. Mol Cell Biol, 2015, 35(19): 3312-3323

[20] Cheng Z, Volkers M, Din S, et al. Mitochondrial translocation of Nur77 mediates cardiomyocyte apoptosis [J]. Eur Heart J, 2011, 32 (17): 2179-2188