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脑啡肽和褪黑素对失血性休克心肌细胞 RIPK3 表达的影响

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[摘　要]　**目的：**观察失血性休克时脑啡肽(D-Ala^2-Leu^5-enkephalin，DADLE)和褪黑素对受体相互作用蛋白3(receptor-interacting protein kinase 3，RIPK3)、三磷酸肌醇受体/电压依赖性阴离子通道(1,4,5-trisphosphate receptor/voltage dependent anion channels，IP3R/VDAC)及氧自由基(reactive oxygen species，ROS)表达的影响，探讨其心肌保护作用的机制。**方法：**将30只雄性SD大鼠随机分为5组，分别为Shock组、NaCl组、Melatonin组、DADLE组、DM组，大鼠休克后分组复苏，复苏至MAP为55~60 mmHg并维持3 h。采用蛋白质印迹法检测心肌组织RIPK3及黄嘌呤氧化酶(xanthine oxidase，XO)表达水平；免疫组织化学观察心肌组织IP3R及VDAC表达水平；免疫荧光检测心肌ROS表达水平。**结果：**与Melatonin组、DADLE组相比，DM组RIPK3表达水平虽然无统计学意义(P>0.05)，但DM组RIPK3表达水平整体降低；与Melatonin组、DADLE组相比，DM组XO表达水平显著降低(P<0.05)。DM组IP3R表达水平与Melatonin组、DADLE组相比整体降低。DM组与Melatonin组、DADLE组相比，DM组VDAC表达水平显著降低(P<0.05)。ROS表达水平整体较其他组降低，DM组与Melatonin组、DADLE组相比差异无统计学意义(P>0.05)。**结论：**失血性休克早期复苏时应用DADLE、褪黑素可降低RIPK3及IP3R/VDAC表达，通过抑制XO减少ROS生成。

[关键词]　失血性休克；脑啡肽；褪黑素；心肌细胞；受体相互作用蛋白3

Effects of DADLE and melatonin on expression of RIPK3 in hemorrhagic shock cardiomyocytes

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Abstract　**Objective:** This study intends to observe the effects of D-Ala^2-Leu^5-enkephalin (DADLE) and melatonin on the expression of receptor-interacting protein kinase 3 (RIPK3), 1,4,5-trisphosphate receptor/voltage dependent anion channels (IP3R/VDAC) and reactive oxygen species (ROS) during hemorrhagic shock and to explore the mechanism of their protective effects on myocardium. **Methods:** Thirty male SD rats were randomly divided into a Shock group, a NaCl group, a Melatonin group, a DADLE group and a DM group. The rats were resuscitated after

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shock and were resuscitated to 55–60 mmHg for 3 h. The expression level of RIPK3 and xanthine oxidase (XO) in myocardium was detected by Western blotting, the expression level of IP3R and VDAC in myocardium was observed by immunohistochemistry, and the expression level of ROS in myocardium was detected by immunofluorescence. **Results:** Compared with Melatonin group and DADLE group, the expression level of RIPK3 in DM group was not statistically significant (P>0.05). However, the expression level of RIPK3 in DM group was significantly lower than that in Melatonin group and DADLE group, and the expression level of XO in DM group was significantly lower than that in Melatonin group and DADLE group (P<0.05). The expression level of IP3R in DM group was lower than that in Melatonin group and DADLE group as a whole. The expression of VDAC in DM group was significantly lower than that in Melatonin group and DADLE group (P<0.05). The expression level of ROS in DM group was lower than that in other groups. However, there was no significant difference between DM group, Melatonin group and DADLE group (P>0.05). **Conclusion:** DADLE and melatonin can reduce the expression of RIPK3 and IP3R/VDAC during early resuscitation of hemorrhagic shock, and reduce the production of ROS by inhibiting XO.

Keywords hemorrhagic shock; D-Ala^2-Leu^5-enkephalin; melatonin; cardiomyocytes; RIPK3

发生失血性休克时有效循环血容量减少，组织缺血、缺氧导致器官缺血性损伤，复苏时发生氧化应激性再灌注损伤，进一步加重损伤并导致较高的并发症及病死率[1-2]。早期研究[3-4]表明：一些药物如非选择性δ受体激动剂脑啡肽(D-Ala^2-Leu^5-enkephalin，DADLE)、褪黑素等，可增强机体对抗缺氧、缺血、再灌注损伤的能力，减轻炎症反应、细胞凋亡及器官损伤，提高动物生存率，是器官保护研究的热点方向。心肌缺血再灌注损伤的研究[5-6]发现：上调内质网-线粒体微区受体相互作用蛋白3(receptor-interacting protein kinase 3，RIPK3)可引起内质网应激并活化及三磷酸肌醇受体/电压依赖性阴离子通道(1,4,5-trisphosphate receptor/voltage dependent anion channels，IP3R/VDAC)，伴有细胞内黄嘌呤氧化酶(xanthine oxidase，XO)表达增加，是产生大量细胞氧自由基(reactive oxygen species，ROS)的关键环节。因此，本研究在大鼠失血性休克模型应用DADLE、褪黑素，观察RIPK3、IP3R/VDAC及ROS的表达变化，探讨保护心肌的作用机制。

1 对象与方法

1.1 动物

雄性SD大鼠(Sprague Dawley大鼠)30只，体重为250~300 g(购自空军军医大学动物中心)。空军军医大学动物保护与使用委员会批准该实验方案。

1.2 方法

1.2.1 试剂配制

DADLE(英国Abcam公司)溶解于蒸馏水后，用生理盐水稀释至0.1 mg/mL。褪黑素(美国Sigma Chemical公司)溶解于无水乙醇后，用生理盐水稀释至1 mg/mL。DM复苏液为DADLE、褪黑素溶解后混合，加生理盐水稀释至DADLE(0.1 mg/mL)、褪黑素(1 mg/mL)。

1.2.2 动物模型

实验前动物禁食8 h，可自由饮水。予腹腔注射10%水合氯醛诱导麻醉，右侧股动脉、股静脉放置聚乙烯导管(PE-50)，动脉端连接压力传感器与心电监测仪(飞利浦MP50)记录动脉血压和心率，静脉端给药、输液。股动脉插管处放血10 min将大鼠的平均动脉压(mean arterial pressure，MAP)降至35~40 mmHg(1 mmHg=0.133 kPa)[7]，维持低血压状态1 h后开始复苏，复苏至MAP为55~60 mmHg并维持3 h[8]。

1.2.3 实验分组及给药

大鼠随机分为5组：Shock组(n=6)，不给予液体复苏；NaCl组(n=6)，10 min内先按10 mL/kg给予生理盐水再继续给予生理盐水复苏；Melatonin组(n=6)，10 min内按10 mg/kg给予褪黑素溶液[9]复苏后再给予生理盐水；DADLE组(n=6)，10 min内按1 mg/kg用量给予DADLE溶液[10]复苏后再给予生理盐水；DM组(n=6)，10 min内先给予DM复苏液10 mL/kg再给予生理盐水。3 h后处死大鼠，取心肌组织。

1.3 蛋白质印迹法检测相关蛋白的表达

大鼠心肌组织匀浆后提取蛋白质，在10% SDS-PAGE凝胶上加入等量蛋白质电泳，PVDF转膜，封闭、4 ℃条件下与相应一抗孵育过夜：抗大鼠单克隆抗体RIPK3(1∶1 000，英国Abcam公司)、XO(1∶1 000，英国Abcam公司)。与第二抗体在室温下孵育后洗涤，通过增强化学发光底物试剂盒(美国Thermo公司)检测抗原-抗体复合物。采用Image Lab分

析系统(美国BIO-RAD公司)分析目标波段灰度值。

1.4 免疫组织化学方法检测相关蛋白表达水平

予大鼠心肌组织石蜡包埋、切片，抗原修复、血清封闭，与一抗在4 ℃孵育过夜(抗大鼠单克隆抗体VDAC、IP3R，英国Abcam公司)，再与二抗室温下孵育后用DAB显色，苏木精将细胞核染为蓝色，阳性表达为棕黄色。使用Image-Pro Plus 6.0分析软件，统一以像素面积pixel为标准单位，分别测量每张切片中5个视野阳性的累积光密度值(integrated optical density，IOD)以及对应的组织像素面积(area)，计算面密度(areal density)=IOD/area。

1.5 免疫荧光检测心肌 ROS 表达水平

予大鼠心肌组织石蜡包埋、切片后，ROS染色，阳性表达为相应荧光素标记的红光，荧光显微镜下观察并采集图像。使用Halo v3.0.311.314分析软件中Indica Labs - Area Quantification FL v2.1.2模块分别定量每张切片的目的区域的组织面积(μm^2)、阳性面积(μm^2)，计算平均强度。

1.6 统计学处理

采用SPSS 13.0软件分析数据。数据以均数±标准差($\bar{x}\pm s$)表示。组间比较采用单因素方差分析，组间两两比较采用SNK(Student-Newman-Keuls)检验。P<0.05为差异有统计学意义。

2 结果

2.1 心肌 RIPK3 及 XO 蛋白表达水平

在失血性休克大鼠心肌组织中，Shock组、NaCl组RIPK3及XO表达水平明显升高(P<0.05；图1A，表1)。DM组分别与Shock组、NaCl组相比，DM组RIPK3表达水平显著降低(P<0.05；图1B，表1)；与Melatonin组、DADLE组相比，DM组RIPK3表达水平差异无统计学意义(P>0.05；图1B，表1)，但DM组RIPK3平均表达水平与Melatonin组、DADLE组相比整体降低。与Shock组、NaCl组相比，DM组XO表达水平显著降低(P<0.05，图1C，表1)；与Melatonin组、DADLE组相比，DM组XO表达水平显著降低(P<0.05；图1C，表1)。

2.2 心肌 VDAC 及 IP3R 表达水平

在大鼠心肌组织中，Shock组、NaCl组VDAC、IP3R表达水平较高，与Shock组、NaCl组相比，DM组IP3R表达水平显著降低(P<0.05；图2C，表1)；与Melatonin组、DADLE组相比，DM组IP3R表达水平虽然无统计学意义(P>0.05；图2C，表1)，但DM组IP3R表达水平与Melatonin组、DADLE组相比是整体降低的(图2C，表1)。DM组VDAC表达水平与Shock组、NaCl组相比显著降低(P<0.05；图2D，表1)，与Melatonin组、DADLE组相比，DM组VDAC表达水平显著降低(P<0.05；图2D，表1)。

2.3 心肌 ROS 表达水平

Shock组、NaCl组ROS表达水平明显升高，尤其是NaCl组ROS表达水平显著升高；Melatonin组、DADLE组、DM组ROS表达水平较NaCl组降低(P<0.05)，DM组ROS表达水平整体较其他组别降低，DM组与Melatonin组、DADLE组相比差异无统计学意义(P>0.05；图3，表1)。

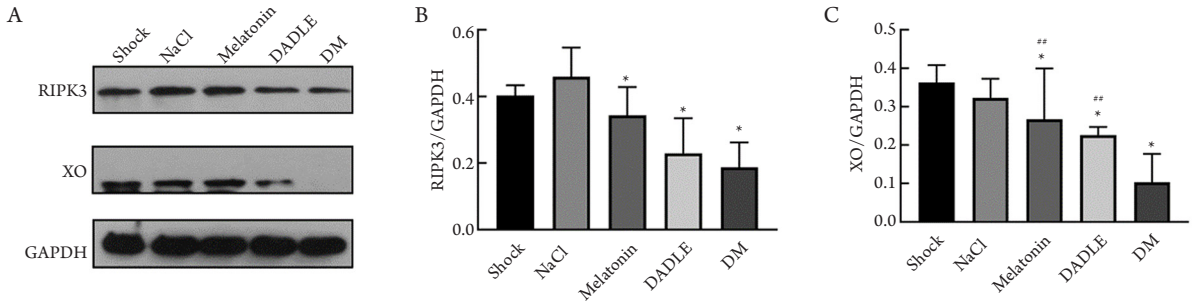

图1 各组RIPK3、XO蛋白表达水平比较

Figure 1 Comparison of protein level of RIPK3 and XO in each group

(A)RIPK3、XO蛋白质印迹电泳图；(B)各组RIPK3表达水平灰度值比较；(C)各组XO表达水平灰度值比较。与NaCl组相比，*P<0.05；与DM组相比，$^{\#\#}P$<0.05。

(A) Western blotting results of RIPK3 and XO; (B) Comparison of gray values of RIPK3 expression levels in each group; (C) Comparison of gray values of XO expression levels in each group. Compared with NaCl group, *P<0.05; compared with DM group, $^{\#\#}P$<0.05.

表1 各组RIPK3、XO、IP3R、VDAC、ROS表达水平比较($\bar{x} \pm s$)

Table 1 Data summary of RIPK3, XO, IP3R, VDAC and ROS in each group ($\bar{x} \pm s$)

指标	Shock组	NaCl组	Melatonin组	DADLE组	DM组	*P*
RIPK3(灰度值)	0.403 ± 0.03	0.460 ± 0.08	0.320 ± 0.053*	0.230 ± 0.104*	0.187 ± 0.074*	0.006
XO(灰度值)	0.363 ± 0.045	0.323 ± 0.049	0.267 ± 0.133*&	0.287 ± 0.075*&	0.103 ± 0.074*	0.025
IP3R(IOD/area)	40.62 ± 19.32	43.61 ± 20.73	10.32 ± 7.20*#	13.32 ± 4.89*#	10.77 ± 5.88*#	0.039
VDAC(IOD/area)	608.29 ± 15.51	1 180.84 ± 57.14	260.83 ± 10.47*#	282.39 ± 13.33*#	206.57 ± 6.34*#	<0.001
ROS(平均强度)	22.49 ± 1.96	23.29 ± 2.05	20.37 ± 1.34*	19.46 ± 1.54*	19.61 ± 1.46*	0.062

与NaCl组相比，*P<0.05；与Shock组相比，#P<0.05；与DM组相比，&P<0.05。

Compared with NaCl group, *P<0.05; compared with Shock group, #P<0.05; compared with DM group, &P<0.05.

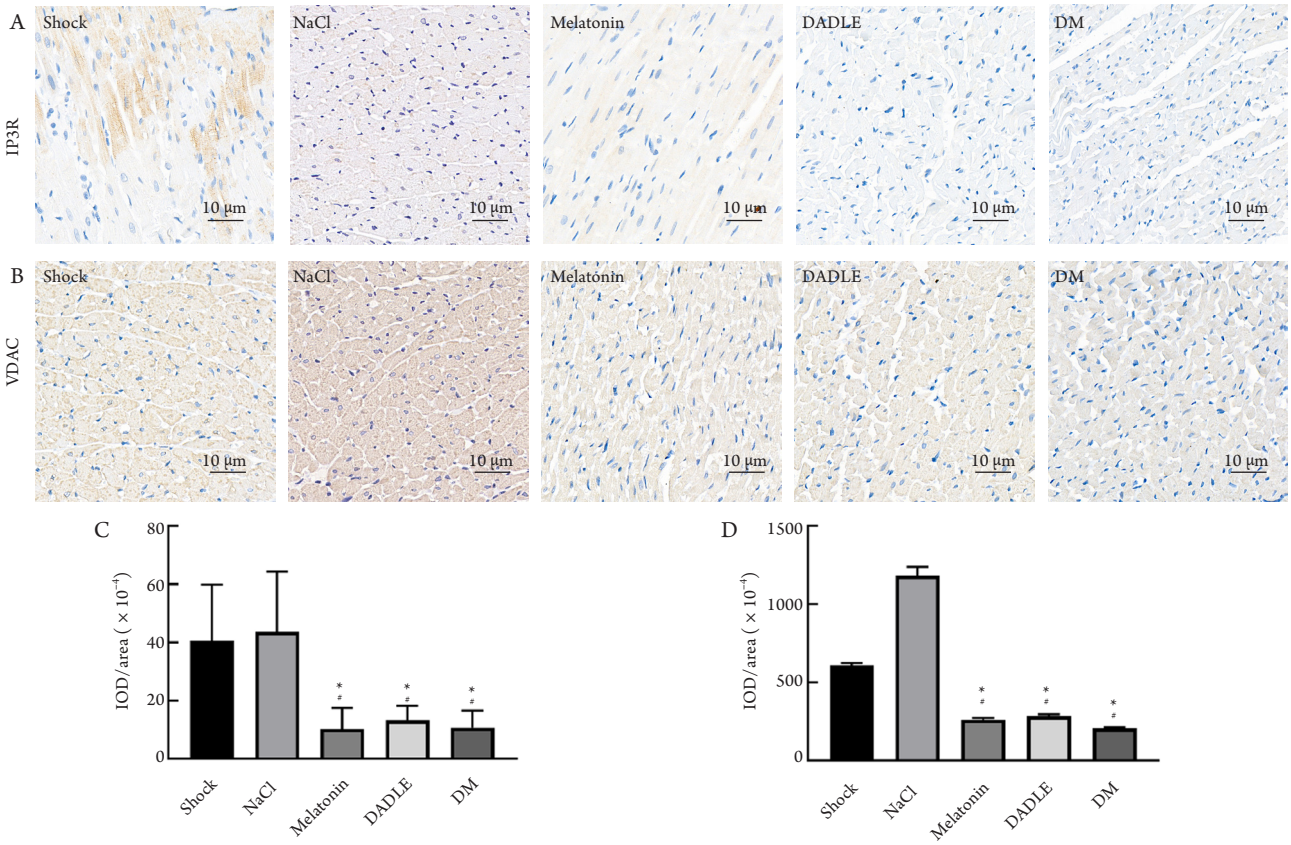

图2 各组IP3R、VDAC免疫组织化学及表达水平比较

Figure 2 Comparison of immunohistochemistry and expression levels of IP3R and VDAC in each group

(A)IP3R免疫组织化学(DAB染色，×400)；(B)VDAC免疫组织化学(DAB染色，×400)；(C)各组IP3R表达水平比较；(D)各组VDAC表达水平比较。与NaCl组相比，*P<0.05；与Shock组相比，#P<0.05。

(A) IP3R immunohistochemical results (DAB staining, ×400); (B) VDAC immunohistochemical results (DAB staining, ×400); (C) The expression levels of IP3R in each group was compared; (D) The expression levels of VDAC in each group were compared. Compared with NaCl group, *P<0.05; compared with Shock group, #P<0.05.

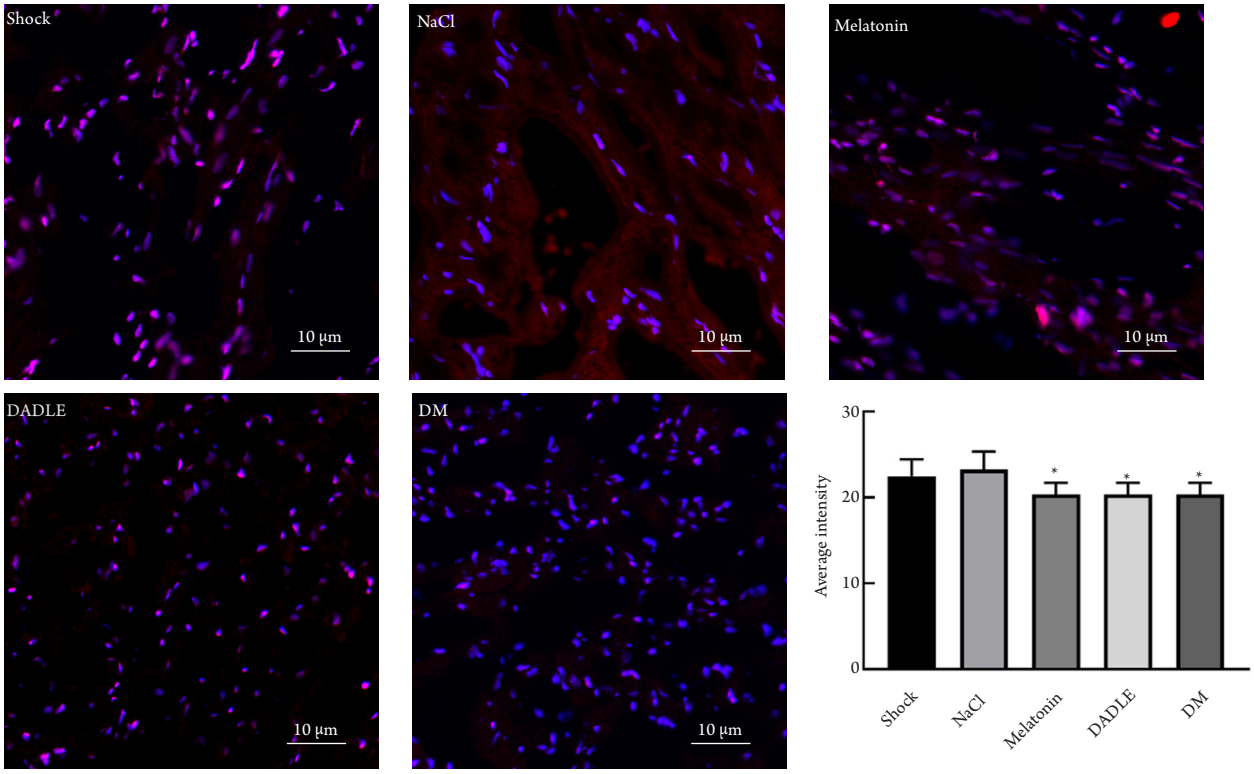

图3 各组ROS免疫荧光表达结果(免疫荧光染色，×400)

Figure 3 Results of ROS immunofluorescence expression in each group (immunofluorescence staining, ×400)

与NaCl组相比，*P<0.05。

Compared with the NaCl group, *P<0.05.

3 讨论

本研究发现：阿片δ受体激动剂DADLE、褪黑素在大鼠失血性休克模型可显著下调心肌细胞RIPK3表达、降低IP3R/VDAC水平，减少ROS生成；复合应用DADLE、褪黑素未能显示出进一步的保护效应。

失血性休克时组织灌注不足致三磷酸腺苷(adenosine-triphosphate，ATP)生成减少，各脏器缺血、缺氧导致细胞凋亡，和多脏器功能衰竭，早期、及时、正确地对失血性休克进行复苏对提高生存率及减少并发症发生及其重要，但战场环境及偏远地区的休克复苏存在诸多困难，如监护设施简陋、血源短缺、药品缺乏等等，因此在这种条件下早期复苏如何提高机体耐受力，为后期救治提供时间窗，显得尤为重要。课题组前期及多项研究[11-16]表明：液体复苏时使用一些药物如DADLE、褪黑素等，可减轻缺血、缺氧引起的器官损伤、炎症反应，增强机体多个脏器(心、肝、肺、脑等)对抗缺氧、缺血、再灌注损伤的能力，延长早期救治的时间窗。DADLE在缺血、缺氧条件下可通过调节自噬、关闭线粒体通透性转换孔(mitochondrial permeability transition pore，mPTP)或激活促生存激酶通路发挥对心肌的保护作用[11,13,17]。在关于心脏微血管内皮细胞的研究中，褪黑素通过MAPK/ERK修饰了IP3R/VDAC表达，阻止[Ca^{2+}]c/[Ca^{2+}]m超载，保护内皮细胞免受氧化应激损伤，并维持线粒体结构和功能的完整性，最终阻断线粒体介导的细胞死亡，褪黑素亦可通过抑制自噬来发挥心肌细胞保护作用[16,18-19]。由此可见，褪黑素及DADLE都可在缺血、缺氧、再灌注等条件下对心肌发挥保护作用，但是DADLE和褪黑素产生这些有益作用的机制仍未完全明确。

近期研究[20]发现：线粒体损伤和内质网应激是缺血再灌注损伤的关键环节，针对线粒体和内质网结构、功能的研究已成为近年来迅速发展的研究领域。针对心肌线粒体、内质网的研究[21]发现：心肌细胞内的内质网与线粒体之间存在由多种蛋白质构成的缔合区被称为“内质网-线粒体微区”或“线粒体相关的内质网膜”，参与细胞氧化应激、细胞凋亡、钙稳态调节等病理生理过

程，其中内质网上的肌醇IP3R通过GRP75位于线粒体上的VDAC在钙信号转导中起重要作用。IP3R的表达受到RIPK3的调节，RIPK3是受体相互作用蛋白家族中的一员，其高表达与心脏功能障碍和心肌细胞凋亡呈正相关，而敲除*RIPK3*基因可下调再灌注损伤诱发的IP3R表达和内质网应激[6,22]。此外，既往研究[23]证实RIPK3缺失还可抑制内皮细胞凋亡，减轻血管腔肿胀，维持微血管通畅，降低黏附分子表达，限制心肌炎症反应。而在失血性休克复苏时，DADLE、褪黑素对RIPK3和IP3R及其下游ROS产物的影响尚未研究。

本研究表明：DADLE、褪黑素均显著降低RIPK3活性和IP3R表达，减少ROS产生。内质网-线粒体微区可维持ROS的动态平衡，再灌注时ROS爆发不仅导致心肌细胞氧化应激反应，还诱发大量Ca^{2+}释放，放大Ca^{2+}振荡[24]。线粒体Ca^{2+}超载触发mPTP过度开放，引起线粒体依赖性细胞死亡，并通过触发Ca^{2+}-XO-线粒体-ROS轴放大心脏再灌注损伤[16,25]。研究[5,26]证实：若IP3R表达增强会导致线粒体钙超载，进而通过钙调蛋白激酶II(CaMKII，Calmodulin kinase II，CaMKII-mPTP)激活再灌注心脏细胞死亡信号通路或XO-ROS-mPTP通路。由此可见，内质网-线粒体微区的RIPK3和IP3R是缺血再灌注损伤中钙稳态和氧化应激的关键组分，DADLE、褪黑素的细胞保护作用与抑制RIPK3和IP3R表达密切相关。

单独使用DADLE或褪黑素降低RIPK3和IP3R的表达可能与二者抑制炎症因子产生相关。动物实验已证实，单独使用DADLE与褪黑素在失血性休克复苏过程中都可抑制炎症因子如IL-6、肿瘤坏死因子-α(tumor necrosis factor-α，TNF-α)等的产生，进而减轻机体炎症反应[9,27]。炎症介质TNF-α通过与肿瘤坏死因子受体(tumor necrosis factor receptor 1，TNFR1)相结合，可触发凋亡及坏死[28-29]。与坏死有关的信号引发RIPK3多个位点磷酸化，进而与混合家族激酶域样结构域(mixed series protein kinase-like domains，MLKL)结合形成RIPK3-MLKL，此为坏死驱动的关键因素[30-32]。在失血性休克复苏中，DADLE和褪黑素可能通过降低炎症因子TNF-α的产生，减少凋亡及坏死信号的转导，线粒体-内质网微区RIPK3磷酸化活化及表达减少，从而使得IP3R表达亦下调，减少ROS生成，并稳定心肌细胞中的钙稳态，减少线粒体钙超载，进而使得CaMKII-mPTP或XO-ROS-mPTP通路激活减少，mPTP开放时间减少，最终减少了心肌细胞的死亡。DADLE及褪黑素亦可能通过减少RIPK3-MLKL复合物的形成，抑制坏死通路的激活，发挥心肌保护作用，但二者具体的分子机制仍需深入研究。

但本研究中复合应用DADLE和褪黑素时虽然RIPK3和IP3R表达均比单独应用有降低，但未显示出统计学意义，ROS的生成更无明显变化，可能的原因如下：首先，此次实验中主要观察早期复苏即3 h复苏时间内的心肌损伤变化，观察时间较短，心肌在这3 h的时间内损伤程度有限，炎症介质产生有限，二者合用对其联合保护作用未充分显示；其次，在有限的心肌损伤程度下，单独应用DADLE或褪黑素炎症抑制作用已达上限，二者合用对TNF-α的表达已无下调空间，因此将二者合用并不能进一步下调RIPK3的表达；除此之外，内质网-线粒体微区的RIPK3和IP3R表达在失血性休克时可能受到多种途径的调节，因而在降低ROS的生成中没有协同效应。此研究证实了DADLE、褪黑素可通过下调RIPK3发挥心肌保护作用，但实验中仍有许多不足之处，对炎症因子、钙超载表达、心肌细胞凋亡或坏死、mPTP开放程度等都未进行深入研究。

综上所述，失血性休克早期复苏时应用DADLE、褪黑素可降低RIPK3及IP3R/VDAC表达，通过抑制XO减少ROS生成。

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