C2C12细胞和RAW264.7细胞共培养后细胞内钙成像及棕榈酸钠诱导后的改变

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【摘要】　目的　观察RAW264.7细胞对棕榈酸钠诱导的C2C12细胞胰岛素抵抗模型中钙流的影响。**方法**　采用C2C12细胞和RAW264.7细胞共培养模拟在体骨骼肌状态。用含2%马血清的高糖培养基培养C2C12细胞，诱导其分化为成熟的肌管细胞后分为5组：对照组（RAW264.7单独培养组）、C2C12和RAW264.7共培养组、C2C12单独培养组，C2C12和RAW264.7共培养+棕榈酸钠（sodium palmitate，PA）组和C2C12单独培养+PA组。用PA（5 mmol/L）诱导培养24 h建立C2C12细胞胰岛素抵抗模型。将复苏和扩增的RAW264.7细胞等量均匀加入到C2C12细胞中共培养2天，改良台式液培养，钙离子荧光探针Fluo-4 AM装载两种细胞，1,7-二甲基黄嘌呤诱发细胞内的钙流，在激光共聚焦显微镜下拍摄和记录活体细胞的钙成像。**结果**　对照组未见明显的钙信号变化，共培养的C2C12细胞内有快速且明显的钙信号变化。单独培养的C2C12细胞内钙信号在整个观察期间持续缓慢增加且未出现明显下降，共培养的C2C12细胞内钙信号达峰速度明显快于单独培养的C2C12细胞（$P<0.001$）；加入PA后C2C12细胞内钙信号变化不明显，但PA诱导后共培养组C2C12细胞内仍会出现明显的钙信号变化，且钙信号达峰速度明显快于PA诱导后单独培养的C2C12细胞（$P<0.001$）。**结论**　RAW264.7细胞共培养可提升正常C2C12细胞和PA诱导后C2C12细胞内钙信号动态响应性。

【关键词】　C2C12细胞；　RAW264.7细胞；　共培养；　钙成像；　棕榈酸钠（PA）；　1,7-二甲基黄嘌呤

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Calcium imaging in C2C12 cells and RAW264.7 cells post co-culture and changes induced by sodium palmitate

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【Abstract】　Objective　To observe the effect of RAW264.7 cells on calcium sparks in a insulin resistance model of C2C12 cells induced by sodium palmitate.　**Methods**　C2C12 cells and RAW264.7 cells were co-cultured to simulate the *in vivo* state of skeletal muscle. C2C12 cells were cultured in high-glucose medium containing 2% horse serum to induce differentiation into mature myotubes, and then divided into 5 groups: control (RAW264.7 cells), co-culture of C2C12 with RAW264.7, C2C12 alone, co-culture of C2C12 with RAW264.7 plus sodium palmitate (PA), and C2C12 alone with PA. PA of 5 mmol/L was used to induce insulin resistance in C2C12 cells for 24 hours. Revived and expanded RAW264.7 cells were evenly added to C2C12 cells and co-cultured for two days. Subsequently, cells were maintained in modified suspension culture, and both cell types were loaded with the calcium ion fluorescent

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probe Fluo-4 AM. Finally, Paraxanthine was used to induce intracellular calcium sparks, which was captured and recorded under a laser confocal microscope. **Results** No significant calcium signal change was observed in the control group. Co-cultured C2C12 cells exhibited rapid and pronounced calcium signal changes, whereas calcium signals in C2C12 cells cultured alone increased slowly throughout the observation period without a sharp decline. The peak calcium signal was reached significantly faster in co-cultured C2C12 cells than that in C2C12 cells cultured alone ($P<0.001$). With PA induction, calcium signal changes in C2C12 cells were not markedly altered, while distinct calcium fluctuations were still observed in co-cultured C2C12 cells, and the peak calcium signal was reached significantly faster in co-cultured C2C12 cells than that in C2C12 cells cultured alone ($P<0.001$). **Conclusion** RAW264.7 cells enhance the dynamic responsiveness of calcium signaling in both normal and PA-stimulated C2C12 cells.

【Key words】 C2C12 cell; RAW264.7 cell; co-culture; calcium imaging; sodium palmitate (PA); Paraxanthine

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骨骼肌是参与机体葡萄糖代谢的重要外周组织,对于维持葡萄糖稳态和外周组织的胰岛素敏感性具有关键作用[1-2]。巨噬细胞是保护生物体免受病原体侵害的吞噬细胞[3]。巨噬细胞分布在所有组织中,并具有器官特异性功能,有助于脂肪组织的产热调节[4]、脾脏和肝脏的铁循环[5]、大脑中的突触修剪[6]及心脏的电传导[7]。骨骼肌中除了大量的肌纤维,还存在大量巨噬细胞,这些巨噬细胞在胚胎早期就已经被分配到各个组织和肌肉中,发挥着免疫监视、免疫应答和组织修复的作用[8-10]。骨骼肌中的巨噬细胞是否具有器官特异性功能目前尚无报道。

C2C12是一种小鼠成肌细胞系,来源于C3H小鼠的大腿骨骼肌,具有快速增殖和高效分化为肌管的能力,是研究骨骼肌分化、再生和肌肉相关疾病的经典模型[11]。RAW264.7是一种小鼠单核巨噬细胞白血病细胞系,源自BALB/c小鼠,具有巨噬细胞的典型功能[12-13]。建立C2C12与RAW264.7的共培养体系,能够直观研究两种细胞在近距离接触下生物学行为的相互影响。

骨骼肌收缩与钙流动密切相关,在骨骼肌收缩过程中,神经信号会激活肌质网Ryanodine钙离子通道,释放肌质网中的钙离子,并与肌球蛋白结合,从而引起肌肉纤维收缩,钙离子的流动是调控骨骼肌收缩的关键因素之一[14-15]。研究骨骼肌细胞内钙动力学是揭示巨噬细胞与骨骼肌细胞相互作用机制的关键切入点。棕榈酸钠(sodium palmitate,PA)能够诱导骨骼肌细胞产生胰岛素抵抗[16-19],但目前尚不清楚巨噬细胞对PA诱导的骨骼肌细胞胰岛素抵抗模型中钙信号的影响。

本研究利用离体的细胞实验模拟骨骼肌和巨噬细胞共存的环境,对RAW264.7细胞与C2C12细胞进行共培养,使用Fluo-4 AM检测肌纤维中的钙流动,探讨RAW264.7细胞对正常C2C12细胞和PA诱导的C2C12细胞胰岛素抵抗模型中钙流的影响,以期为研究代谢性疾病中骨骼肌微环境紊乱的病理生理过程提供实验依据和理论参考。

材料和方法

试剂和仪器 C2C12小鼠成肌细胞、RAW264.7小鼠单核巨噬细胞白血病细胞购自武汉普诺赛生命科技有限公司;钙离子荧光探针(Fluo4-AM)、BSA购自Beyotime公司;1,7-二甲基黄嘌呤(Paraxanthine)购自美国MCE公司;特级胎牛血清、马血清购自美国GIBCO公司;DMEM高糖培养基购自美国Hyclone公司;PS(青霉素+链霉素)购自美国Invitrogen公司;PA、Paraxanthine、Pluronic® F-127、二甲基亚砜(DMSO)、Hank's平衡盐溶液(HBSS)购自美国Thermo Fisher Scientific公司;改良台式液(一种平衡盐溶液,主要由氯化钠、氯化钾、氯化镁、氯化钙、磷酸盐、HEPES等组成,不含葡萄糖)购自北京索莱宝公司(货号:T1420)。激光共聚焦显微镜(型号:Leica TCS SP8,德国Leica Microsystems GmbH公司)。

细胞培养 配制含10% FBS+DMEM培养基

+1% PS的完全培养液。于-80 ℃中取出冻存管，迅速放入37 ℃水浴箱中复温解冻。吸取10 mL培养液加入离心管中，待C2C12细胞解冻后，用吸管吸出解冻液，加入离心管中吹打混匀，300×g离心5 min，弃上清，加入10 ml培养液重悬细胞沉淀，移入10 cm培养皿中，于37 ℃、5%CO_2培养箱中培养。隔天换液，当细胞生长至铺满85%的培养皿时，PBS洗涤3遍，用0.25%胰酶消化传代。

诱导成熟肌细胞 待细胞融合度>85%，换为2%马血清DMEM培养基，隔天换液，持续5~7天，至90%诱导分化为肌管细胞（即成熟骨骼肌细胞）后进行分组实验。

PA溶液配制 配置40% BSA：称取1.2 g脱脂BSA粉末置于50 mL离心管中，加入2 mL PBS溶液，室温下7 104×g离心15 min，BSA完全溶解，PBS定容至3 mL。称取0.033 4 g PA至15 mL离心管中，加入2 mL ddH_2O，置于75 ℃水浴至完全溶解，定容至3 mL，迅速与40% BSA溶液混合，得到PA-BSA贮存液（20% BSA，20 mmol/L PA）。0.22 μm过滤器过滤后，于4 ℃冰箱储存备用。使用时用DMEM将PA-BSA贮存液稀释4倍，得到PA-BSA（5% BSA，5 mmol/L PA）工作液。

实验分组 将细胞分为5组，分别为对照组（RAW264.7单独培养组）、C2C12和RAW264.7共培养组、C2C12单独培养组、C2C12和RAW264.7共培养+PA组和C2C12单独培养+PA组。+PA组加入PA-BSA工作液诱导培养24 h，其他组继续用完全培养基培养24 h，将复苏和扩增的RAW264.7细胞等量均匀加入到C2C12细胞中，共培养2天。

钙成像 配置Fluo-4 AM工作液：50 μg Fluo-4 AM加入44 μL DMSO中，加入Pluronic® F-127使其终浓度为0.1 %，用改良台式液稀释至3 μmol/L。弃去培养皿中原培养基，PBS清洗3遍，加入Fluo-4 AM工作液，避光室温孵育60 min；弃去Fluo-4 AM溶液，HBSS清洗3遍，每次2~3 min；加入改良台式液，Paraxanthine诱发钙流并在激光共聚焦显微镜下进行拍摄，拍摄间隔为1 s。所有实验重复3次。

统计学分析 使用Image J软件计算荧光强度。使用Graph Pad Prism 8.0软件进行作图和统计分析，钙信号达峰速度（ΔF/t）=（荧光峰值-基础荧光值）/达峰时间，数据以$\bar{x} \pm s$表示，采用非配对t检验进行组间比较。$P<0.05$为差异有统计学意义。

结　　果

C2C12细胞与RAW264.7共培养 经过2%马血清诱导成熟的C2C12细胞融合成一个长肌纤维，表现为多核。与RAW264.7共培养后，C2C12细胞进一步增殖分化，肌纤维改变为更加扁平和延伸的形态。PA刺激后C2C12细胞体积增加，细胞间连接减少，与RAW264.7共培养后，C2C12细胞形态也变为扁平和延伸（图1）。

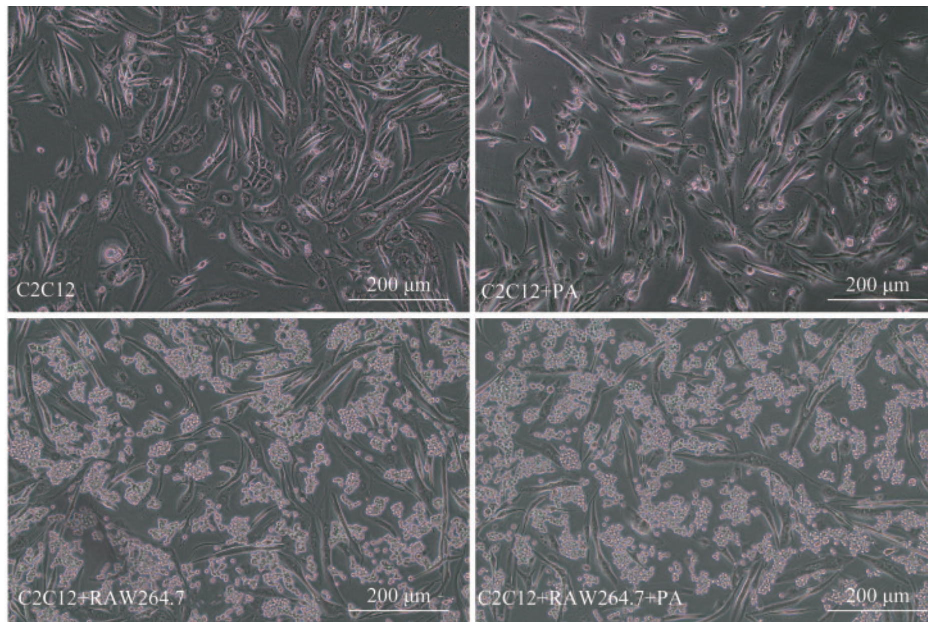
共培养和单独培养的C2C12细胞内钙流 Fluo-4 AM工作液孵育60 min后，在激光共聚焦显微镜下寻找合适视野开始记录，加入Paraxanthine诱导C2C12细胞肌质网释放钙离子。对照组未见明显的钙信号变化；共培养的C2C12细胞有快速且明显的钙信号变化，单独培养的C2C12细胞内钙信号持续缓慢增加，共培养的C2C12细胞内钙信号达峰速度明显快于单独培养的C2C12细胞（$P<0.001$，图2）。

PA诱导后单独培养和共培养的C2C12细胞内钙流 PA诱导后，通过Fluo-4AM监测C2C12细胞内钙离子发现，在加入Paraxanthine后单独培养的C2C12细胞内未见明显的钙信号改变；共培养的C2C12细胞内仍可见明显的钙信号变化，且钙信号达峰速度明显快于单独培养的C2C12细胞（$P<0.001$，图3）。

讨　　论

本研究旨在探讨巨噬细胞对骨骼肌细胞在正常状态及PA诱导的胰岛素抵抗状态下细胞内钙离子的动态调节作用。研究结果表明，与RAW264.7细胞共培养，能够显著增强C2C12肌管细胞在Paraxanthine激发下的钙流响应。由此提示，巨噬细胞可能通过某种相互作用，增强了C2C12细胞肌质网通过Ryanodine受体释放钙离子的能力，也可能直接或间接地调节了钙离子通道的活性。

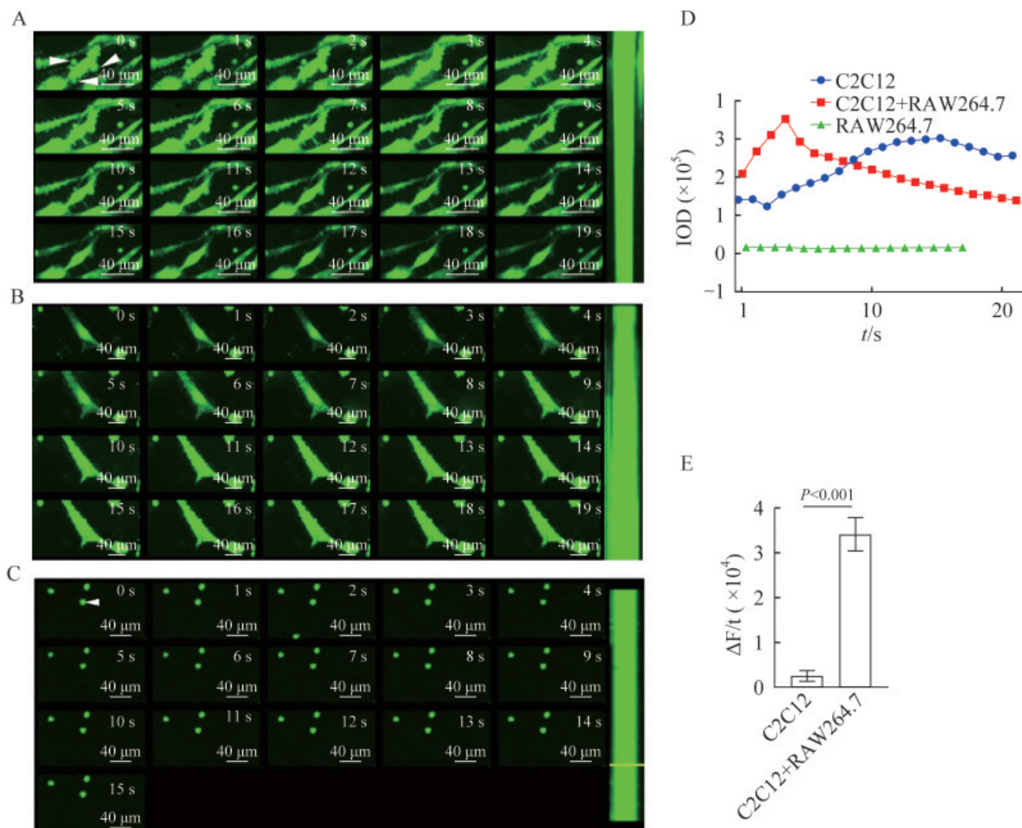
在PA诱导的C2C12细胞胰岛素抵抗模型中，单独培养的C2C12细胞内钙流响应明显减弱，这与既往研究中关于代谢应激下Ryanodine受体功能紊乱和钙稳态失调的报道一致[20-21]；C2C12细胞与RAW264.7细胞共培养后，胞内仍可见明显的钙流



PA：Sodium palmitate.

图1 光镜下各组细胞形态变化

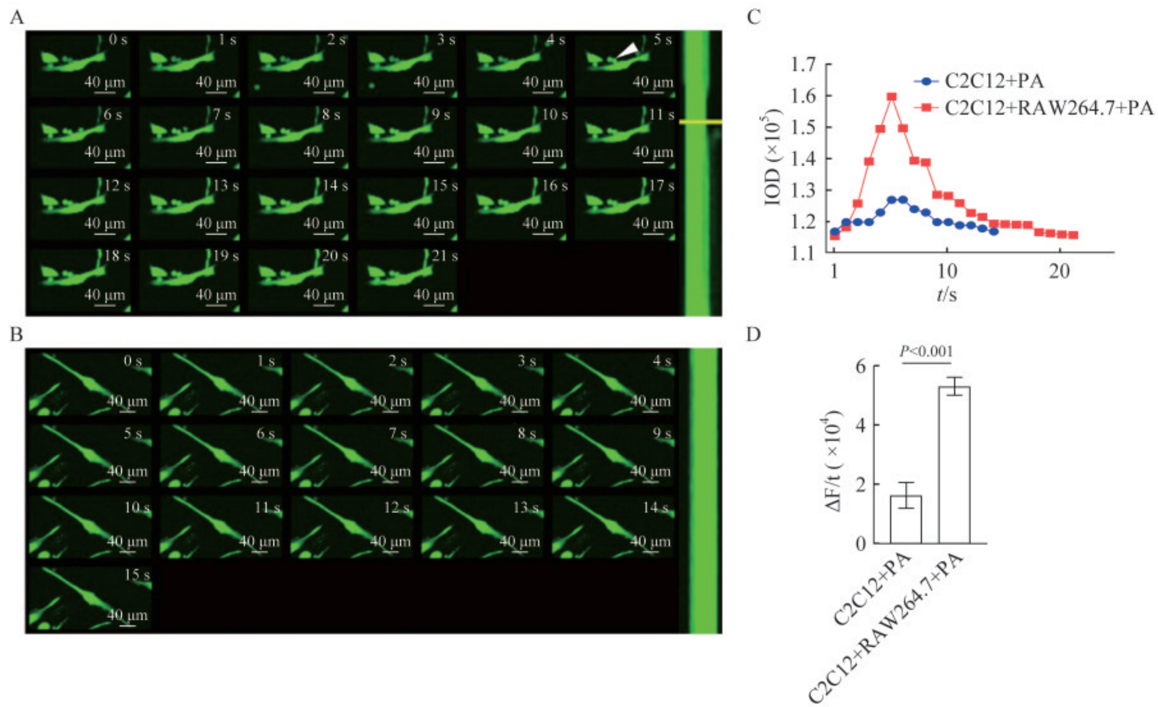
Fig 1 Morphological changes of cells in each group under light microscope



A-C：Calcium sparks in C2C12 cells co-cultured with RAW264.7 (A), mono-cultured C2C12 cells (B) and RAW264.7 cells (C); D：Quantitative analysis chart of calcium sparks. E：Comparison of calcium flux rate ($\Delta F/t$). Intracellular Fluo-4 fluorescence signals were recorded over time with a sampling interval of 1 s between frames. The arrows indicated RAW264.7 cells connected to C2C12 cells.

图2 C2C12细胞、RAW264.7细胞及共培养的C2C12细胞的钙成像

Fig 2 Calcium imaging of C2C12 cells, RAW264.7 cells and co-cultured C2C12 cells



A: Calcium sparks in C2C12+PA; B: Calcium sparks in C2C12+RAW264.7+PA; C: Quantitative analysis of calcium sparks; D: Comparison of calcium flux rate (ΔF/t). Intracellular Fluo-4 fluorescence signal was recorded over time with a sampling interval of 1 s between frames. The arrows indicated RAW264.7 cells connected to C2C12 cells. PA: Sodium palmitate.

图3 PA诱导后C2C12细胞钙成像

Fig 3 Calcium imaging of C2C12 cells induced by PA

信号。提示在模拟糖尿病病理状态的胰岛素抵抗条件下,巨噬细胞可能部分拮抗了PA对钙信号通路的抑制效应,在一定程度上恢复或改善了骨骼肌细胞的钙响应功能。近年来,关于巨噬细胞的研究取得了显著进展,巨噬细胞有助于脂肪组织的产热调节、脾脏和肝脏的铁循环、大脑的突触修剪以及心脏的电传导[22-25]。鉴于骨骼肌中也存在大量巨噬细胞,并发挥免疫监视作用,本研究结果为阐释其在骨骼肌这一重要代谢器官中的非免疫特异性功能提供了新的细胞学证据。

本研究的局限性在于:(1)使用的是小鼠永生化细胞系,研究结果需在原代细胞及动物模型中进一步验证。(2)简化的体外共培养体系无法完全模拟体内骨骼肌复杂的微环境,且巨噬细胞调控钙信号的具体分子机制尚未阐明,这也是未来研究的重点。

综上所述,本研究在细胞层面证实,巨噬细胞不仅能增强骨骼肌细胞在生理状态下的钙流响应,也在胰岛素抵抗条件下表现出功能调节潜力,使巨噬细胞超越了传统的免疫角色,为理解免疫细胞与骨骼肌功能间的精细协作提供了新的视角,也为探究相关肌肉代谢疾病的病理机制提供了新思路。

作者贡献声明 宋丽君,吴爽 文献复习,实验实施,论文撰写。沙勤,杨传信 实验指导,论文修订。童兴瑜 论文修订。蒋晖 实验设计和指导。

利益冲突声明 所有作者均声明不存在利益冲突。

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