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胞外酸化经ASIC1/RIP1途径抑制TFEB介导的巨噬细胞脂噬*

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摘要 目的 探讨胞外酸化对巨噬细胞脂噬的影响及其作用机制。**方法** 采用RAW264.7巨噬细胞，以pH 6.5培养液与25 mg/L氧化低密度脂蛋白（ox-LDL）共孵育24 h构建胞外酸化诱导的泡沫细胞模型。分别以ASIC1特异性阻断剂PcTx-1和RIP1抑制剂Nec-1干预胞外酸化诱导的RAW264.7巨噬细胞24 h，油红O染色检测细胞内脂质蓄积；蛋白质印迹（Western blot）检测总ASIC1、膜ASIC1、p-RIP1 Ser166、p-TFEB Ser142、LC3和p62蛋白的表达；激光共聚焦显微镜观察脂滴（Bodipy示踪）与自噬标志物LC3II和LAMP1共定位；透射电镜观察细胞内脂滴和脂噬泡的数量变化；胆固醇荧光试剂盒检测ABCA1介导的胆固醇流出。**结果** 与pH 7.4组相比较，pH 6.5胞外酸化组胞内的脂质蓄积和细胞质膜上的ASIC1蛋白表达显著增加，p-RIP1Ser166、p-TFEB Ser142水平升高，LC3II蛋白减少和p62蛋白增加，脂滴与LC3II和LAMP1的共定位都分别减少，细胞内的脂滴数量显著增加，自噬体和脂噬泡的数量则明显减少，ABCA1介导的巨噬细胞内胆固醇流出显著减少。然而，胞外酸化对RAW264.7巨噬细胞的上述效应能被ASIC1特异性阻断剂PcTx-1和RIP1抑制剂Nec-1所取消。**结论** 胞外酸化经激活ASIC1/RIP1途径促进TFEB磷酸化抑制巨噬细胞脂噬，ASIC1可能是防治动脉粥样硬化等脂质蓄积疾病的新靶点。

关键词 酸敏感离子通道1，胞外酸化，动脉粥样硬化，脂噬，受体相互作用蛋白1，转录因子EB
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动脉粥样硬化（atherosclerosis，AS）是以脂质在动脉血管壁蓄积为主要病理特征的慢性炎症性疾病。在AS病理进程中，因其病变处血管内膜组织液中H^+浓度升高而常发生微环境酸化（pH<7.0）[1-3]。越来越多的研究表明，这种酸化微环境与AS的发生、发展和转归密切相关，但其作用机制尚不清楚[4-6]。酸敏感离子通道1（acid-sensing ion channel 1，ASIC1）是微环境酸化的关键感受器[7]。其广泛表达于心血管等系统，细胞外升高的H^+通过激活ASIC1，将微环境酸化信号转导至细胞内，从而介导微环境酸化相关的心血管生理和病理功能[8-9]。新近研究发现，胞外酸化通过激活ASIC1抑制巨噬细胞ATP结合盒转运蛋白A1（ABCA1）介导的胆固醇流出，从而促进细胞内脂质蓄积和泡沫细胞形成[10]。这表明ASIC1在胞外酸化诱导的巨噬细胞源性泡沫细胞形成中起着重要作用。

受体相互作用蛋白1（receptor-interacting protein 1，RIP1）是一种具有丝氨酸/苏氨酸激酶活性的蛋白激酶，属于RIPs家族[11]。研究表明，ASIC1被H^+等配体激活后，其胞内的羧基端发生变构募集RIP1，促使后者发生自身磷酸化，磷酸化激活的RIP1则进一步募集下游信号分子，从而引起一系列胞外酸化诱导的病理生理过程[12]。转录因子EB（transcription factor EB，TFEB）是调控自噬的关键核转录因子，特别对自噬标志物微管相关蛋白轻链3（microtubule-associated proteinlight chain 3，LC3）和溶酶体相关膜蛋白1（lysosome-associated membrane protein 1，LAMP1）的生成至

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关重要[13]。TFEB启动自噬相关基因转录的核转位受其丝氨酸位点磷酸化调控。研究表明，当TFEB的142位点丝氨酸发生磷酸化（p-TFEB Ser142）时，该转录因子核转位受抑制，致使上述自噬相关基因的表达减少，从而抑制自噬[14]。上述资料提示，胞外酸化可能通过ASIC1促进RIP1和TFEB磷酸化抑制巨噬细胞自噬。

脂噬为选择性自噬，是指将包含脂滴的脂噬泡转运至溶酶体、经溶酶体酸脂酶降解为脂肪酸（经β氧化后清除）和游离胆固醇（经ABCA1转运至细胞外）的生物学过程[15-16]。因此，脂噬是通过自噬-溶酶体途径促进脂质代谢，防止细胞内脂质蓄积的重要机制。研究表明，激活脂噬减少氧化低密度脂蛋白（ox-LDL）诱导的巨噬细胞脂质蓄积；抑制脂噬则减少胆固醇流出，促进AS发生发展[17-18]。这表明脂噬抑制在致巨噬细胞脂质蓄积中发挥着重要作用。前期研究虽已证实胞外酸化激活巨噬细胞ASIC1和抑制ABCA1介导的胆固醇流出[10]，但胞外酸化对巨噬细胞脂噬的影响及其作用机制目前尚不清楚。

本研究将在细胞外酸化诱导的RAW264.7巨噬细胞模型，分别采用ASIC1和RIP1特异抑制剂干预，探讨胞外酸化是否通过激活ASIC1/RIP1途径促进TFEB磷酸化抑制巨噬细胞脂噬，从而减少细胞内胆固醇流出和促进脂质蓄积的作用机制，为以ASIC1为靶点的AS性疾病防治提供实验依据。

1 材料与方法

1.1 细胞株及其来源

鼠源RAW 264.7巨噬细胞（小鼠单核巨噬细胞白血病细胞）株由南华大学心血管病研究所赠送。

1.2 主要试剂

狼蛛毒素（PcTx-1）购自美国MCE公司；ox-LDL购自广州奕源公司；BI特级胎牛血清（FBS）购自以色列BI公司；Trypsin-EDTA、1640培养液购自美国Gibco公司；DAPI、饱和油红O染色液购自北京索莱宝科技有限公司；ECL发光液购自武汉博士德生物工程有限公司；RIP1抑制剂（Nec-1）购自美国Selleck公司；BCA蛋白定量试剂盒、RIPA裂解液、SDS-PAGE凝胶制备试剂盒、SDS-PAGE Loading Buffer、蛋白marker购自碧云天公司；膜蛋白提取试剂盒购自Abbkine公司；微管相关轻链蛋白3（LC3）、p62抗体、RIP1抗体、p-RIP1（Ser166）抗体购自英国Abcam公司；ASIC1抗体购自以色列Alonome公司；Na^+/K^+-ATPase抗体购自Abmart公司；p-TFEB（Ser142）抗体购自Affinity生物公司、Bodipy558/568 C12购自Thermo Fisher Scientific公司；LAMP1购自中国Abclone公司；HRP标记的山羊抗兔/鼠二抗购自美国Cell Signaling Technology公司；荧光二抗Alexa Fluor®488山羊抗兔购自美国Jackson TmmunoResearch。

1.3 酸性培养基配置

用微量移液器向分装的含10%胎牛血清DMEM高糖培养基中滴加pH 4.4 MES缓冲液，调整培养基pH值为6.5。酸度计监测培养液中的pH变化，每12 h换液一次，以维持培养基中pH值的相对稳定。

1.4 细胞培养与处理

RAW264.7巨噬细胞用含10% FBS的DMEM培养基在5% CO_2培养箱（37℃）中培养，待细胞融合度达80%~90%时进行传代接种。在探讨胞外酸化对细胞脂质蓄积和ASIC1/RIP1/TFEB信号途径介导脂噬影响的实验中，细胞分为pH 7.4、pH 7.4+25 mg/L ox-LDL、pH 6.5、pH 6.5+25 mg/L ox-LDL组。在探讨胞外酸化是否通过激活ASIC1/RIP1途径抑制巨噬细胞脂噬的实验中，细胞分为pH 7.4+25 mg/L ox-LDL组、pH 6.5+25 mg/L ox-LDL组、pH 6.5+25 mg/L ox-LDL+PcTx-1（10 μg/L）组、25 mg/L ox-LDL+Nec-1（20 μmol/L）组。24 h后收集细胞进行分子生物学和病理形态学等检测。

1.5 油红O染色

当细胞密度生长达到80%~90%时，将细胞接种于有爬片的6孔板内（2×10^5个/孔）。达到处理时间后，加入2 ml PBS缓冲液冲洗3次。用4%的多聚甲醛固定爬片30 min，每孔加入1 ml过滤的饱和油红O染色液，37℃染色25 min；用60%异丙醇漂洗1 min，加入1 ml苏木素染核30 s，随后立即用PBS缓冲液漂洗3次；盐酸酒精分化3 s，稀氨水反蓝15 s；甘油明胶封片保存，显微镜观察拍照，ImageJ proplus软件分析数据分析实验结果。

1.6 细胞膜蛋白分离和蛋白质印迹（Western blot）实验

收集细胞，弃上清，预冷PBS洗2次，加入1 ml 2% Triton X-100溶液冰浴15 min后，4℃，10 000 *g*离心5 min，收集上清液。预留100 μl上清液（总蛋白质）做下一步分析，剩余上清液37℃水浴

10 min，以分离亲水性蛋白的水相和疏水膜蛋白的去污剂相。用4℃ 500 μl缓冲液C（10 mmol/L Tris HCl、150 mmol/L NaCl、5 mmol/L EDTA）溶解去污剂相沉淀，冰浴2 min后，37℃水浴10 min，然后将溶液37℃，2 000 *g*离心5 min。以4℃ 500 μl缓冲液C再次抽提污剂相疏水膜蛋白。用BCA法测定蛋白质浓度；加入5×上样缓冲液，煮沸5 min使蛋白质变性。变性的蛋白质经10% SDS聚丙烯酰胺凝胶电泳分离后，湿转（90 min）至PVDF膜。5%脱脂奶粉封闭膜4 h后，分别加入一抗β-actin（1∶2 000）、Na^+/K^+-ATPase（1∶5 000）、ASIC1（1∶1 000）、RIP1（1∶1 000）、p-RIP1Ser166（1∶1 000）、p-TFEB Ser142（1∶1 000）、LC3（1∶1 000）和p62（1∶1 000），4℃孵育12 h。TBST洗膜后换二抗室温孵育2 h。洗膜后使用ECL化学发光法进行显影，凝胶成像系统拍照，Image J软件分析蛋白质条带灰度值。

1.7 激光共聚焦观察

将RAW264.7巨噬细胞接种于有爬片的24孔板内（5×10⁴个/孔），以相应药物或溶剂干预24 h后，吸出培养基，PBS清洗3次。4%多聚甲醛固定细胞20 min，PBS清洗3次，0.1% Triton X-100室温处理细胞30 min，PBS清洗3次。然后分别加入一抗LC3II（1∶200），LAMP1（1∶200），4℃孵育过夜；吸净一抗后加入荧光二抗，室温避光孵育2 h。PBS清洗后加入Bodipy，室温避光孵育20 min。最后滴加DAPI，避光孵育15 min，取出爬片用抗荧光猝灭剂封片，激光共聚焦显微镜分别观察Bodipy（脂滴）与LC3II（自噬体）和LAMP1（溶酶体）共定位，Image J proplus软件分析数据。

1.8 胆固醇流出实验

NBD-胆固醇荧光试剂盒检测细胞内ABCA1介导的胆固醇流出。RAW264.7巨噬细胞接种于含25 mg/L ox-LDL不同pH培养基的12孔培养板（细胞密度1×10⁵/孔），孵育24 h建立巨噬细胞源性泡沫细胞。然后加入不同pH值含5 μmol/L NBD-胆固醇的无酚红DMEM培养基，孵育4 h。PBS洗涤细胞3次，然后以含apoA-1（20 mg/L）的上述不同pH无酚红DMEM培养基孵育4 h。收集培养基；12孔板加入0.1% Triton X-100裂解细胞，静置30 min后收集细胞裂解液，将收集到的培养基或细胞裂解液加入至黑色聚苯乙烯96孔板（200 μl/孔），检测NBD-胆固醇的荧光强度（激发波长为469 nm，发射波长537 nm）。胆固醇流出率=培养基荧光强度/总荧光强度（培养基荧光强度+细胞裂解液荧光轻度）×100%。

1.9 透射电镜（TEM）观察

RAW264.7巨噬细胞以相应试剂处理24 h后，收集细胞；预冷PBS清洗细胞2次，2.5%戊二醛4℃前固定30 min，1%锇酸4℃后固定3 h。细胞经乙醇丙酮梯度脱水后以环氧树脂包埋，超薄切片（70 nm），醋酸双氧铀/枸橼酸铅双重染色，Jeol JEM SX 100透射电子显微镜下观察细胞内脂滴、自噬体、溶酶体和脂噬体（包含脂滴的溶酶体）等超微结构的变化。

1.10 统计分析

数据均以平均值±SEM表示，GraphPad Prism 9软件进行统计学分析。组间比较采用单因素方差分析（one-way ANOVA），$P<0.05$差异有统计学意义。

2 结　果

2.1 胞外酸化促进RAW264.7巨噬细胞的脂质蓄积

为了探讨胞外酸化促进细胞脂质蓄积的作用机制，以pH 6.5培养基（模拟AS病灶处的酸化微环境）孵育RAW264.7巨噬细胞24 h，油红O染色检测细胞内的脂质蓄积。结果表明，与pH 7.4组相比较，pH 6.5组内的脂质蓄积（红色区域）略有增加（图1a，b），但无统计学意义。然而，与pH 7.4+ox-LDL组相比，pH 6.5+ox-LDL组巨噬细胞内的脂质蓄积明显增加。这表明胞外酸化显著促进RAW264.7巨噬细胞的脂质蓄积。

2.2 胞外酸化促进RAW264.7细胞ASIC1膜转位和RIP1/TFEB磷酸化

接下来，以Western blot检测了胞外酸化对RAW264.7巨噬细胞ASIC1的膜转位，以及RIP1和TFEB磷酸化水平的影响（图2a，c）。结果表明，与相应pH 7.4组相比较，胞外酸化组细胞质膜ASIC1/总ASIC1蛋白的比值显著升高（图2b），这表明胞外酸化促进RAW264.7巨噬细胞的ASIC1向质膜转位。此外，与相应pH 7.4组相比较，胞外酸化组RAW264.7细胞的p-RIP1Ser166/总RIP1（图2d）比值和p-TFEBSer142蛋白（图2e）都分别显著增加，这表明胞外酸化促进RIP1和TFEB蛋白的磷酸化。以上研究结果提示，ASIC1激活和RIP1和TFEB磷酸化可能在胞外酸化促巨噬细胞脂质蓄积中起着重要作用。

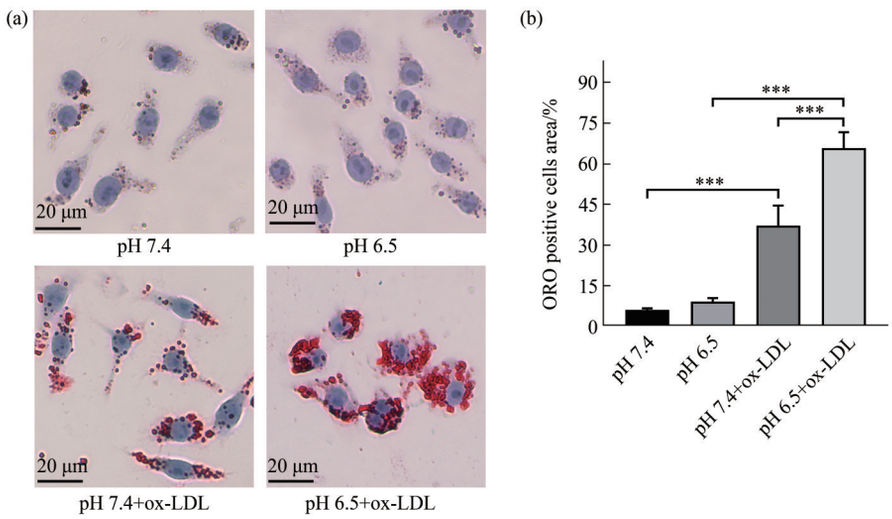

Fig. 1　Extracellular acidification accelerates lipid accumulation in macrophages

RAW 264.7 cells were cultured in pH 7.4 or 6.5 media with or without 25 mg/L ox-LDL for 24 h. (a) Lipid deposition was detected by oil red O (ORO) staining. (b) ORO staining positive areas were quantified. Results are expressed as the *means*±*SEM*. Statistical analysis was performed by one-way analysis of variance. ***P<0.001.

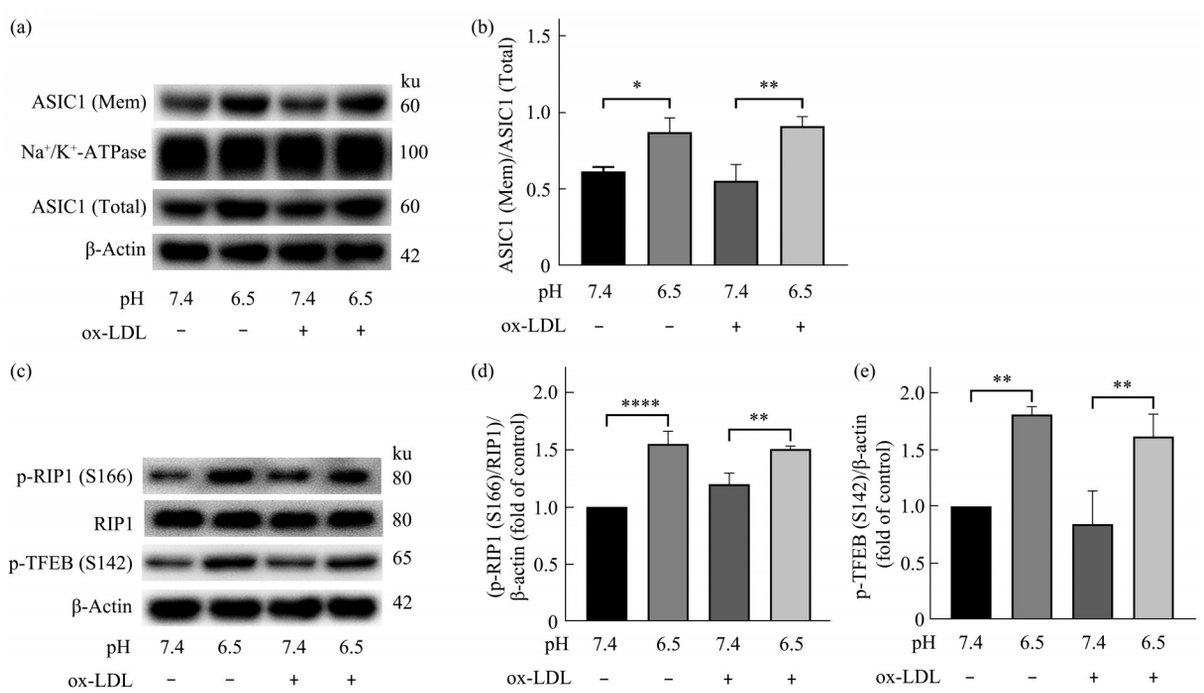

Fig. 2　Extracellular acidification promotes membrane translocation of ASIC1 and phosphorylation of RIP1 and TFEB in RAW 264.7 macrophages

RAW 264.7 macrophages were incubated in medium of indicated pH in the presence or absence of 25 mg/L ox-LDL for 24 h, and then the expressions of ASIC1, RIP1, p-RIP1 (Ser166), and p-TFEB Ser 142 proteins were measured by Western blot. (a) Representative Western blots of ASIC1 (Total) and ASIC1 (Mem). (b) The ratio of ASIC1 (Mem) to ASIC1 (Total) was calculated by relative densitometric values of ASIC1 (Mem) normalized to Na^+/K^+-ATPase that of ASIC1 (Total) normalized to β-actin. Mem: membrane. (c) Representative Western blots of total RIP1, p-RIP1 Ser166, and p-TFEB Ser142. (d, e) Quantification of p-RIP1 and p-TFEB protein levels by the NIH Image J software. Values were expressed as *means*±*SEM* (n=3). Statistical analysis was performed by one-way analysis of variance. *P<0.05, **P<0.01, ****P<0.000 1.

2.3　胞外酸化抑制RAW264.7巨噬细胞的自噬流

鉴于RIP1/TFEB通路是调控细胞自噬的关键信号途径，本文通过Western blot检测RAW264.7巨噬细胞LC3和p62蛋白的表达水平（图3a），以明确胞外酸化对巨噬细胞自噬流的影响。研究结果表明，与相应正常pH组相比较，胞外酸化组RAW264.7巨噬细胞的LC3II/LC3I蛋白比值显著降低（图3b），而p62蛋白的表达则明显增加（图3c）。上述结果表明，胞外酸化抑制RAW264.7巨噬细胞的自噬流，其机制可能与激活ASIC1促进RIP1和TFEB磷酸化有关。

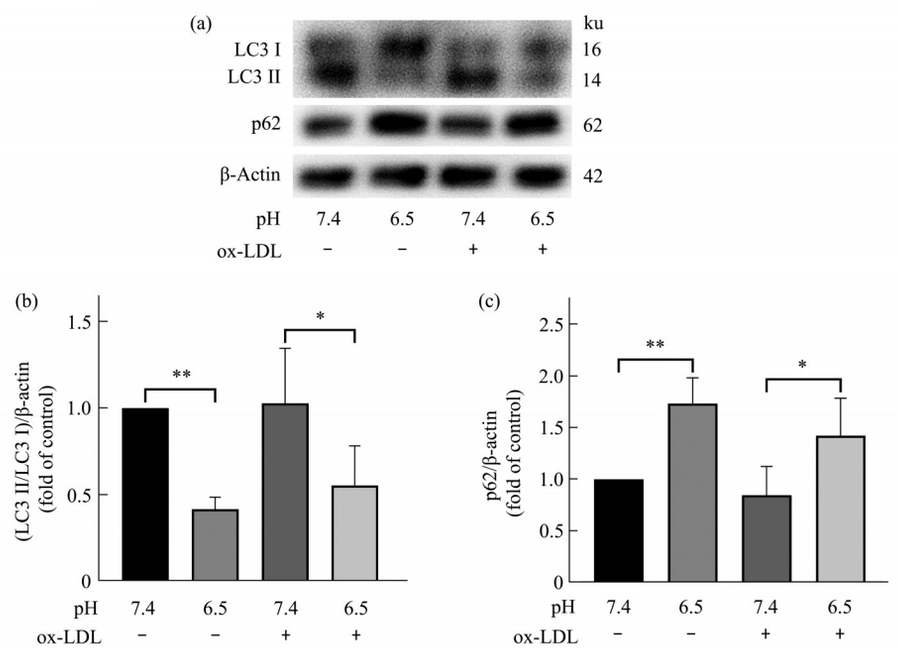

Fig. 3　Extracellular acidification impedes autophagy in RAW264.7 macrophages

RAW 264.7 cells were cultured in pH 7.4 or 6.5 media with or without 25 mg/L ox-LDL for 24 h. The expressions of LC3 and p62 proteins in the cells were determined by Western blot. (a) Representative Western blots of LC3I, LC3II, and p62. (b, c) Quantification of LC3I, LC3II, and p62 protein levels by the NIH Image J software. Values were expressed as means±*SEM* (*n*=4). Differences were statistically analyzed using one-way analysis of variance. **P*<0.05, ***P*<0.01.

2.4　胞外酸化通过激活ASIC1/RIP1途径促进TFEB磷酸化

为了明确胞外酸化促进TFEB磷酸化是否通过激活ASIC1/RIP1信号途径，分别使用ASIC1特异性阻断剂PcTx-1和RIP1抑制剂Nec-1干预胞外酸化诱导的RAW264.7细胞24 h。Western blot检测ASIC1、RIP1、p-RIP1、p-TFEB水平。结果显示，与pH 7.4组相比较，胞外酸化组RAW264.7细胞的ASIC1表达显著增加（图4a，b），p-RIP1Ser 166水平明显升高，p-RIP1Ser 166/RIP1比值显著升高（图4a，c），且p-TFEB Ser 142的水平也显著升高（图4a，d）。然而，胞外酸化对RAW 264.7细胞的上述效应能被ASIC1的特异阻断剂PcTx-1所取消。此外，胞外酸化促进巨噬细胞TFEB磷酸化的作用能被RIP1抑制剂Nec-1所拮抗。这表明胞外酸化通过激活RAW264.7细胞ASIC1，从而促进RIP1第166位点丝氨酸和TFEB第142位点丝氨酸的磷酸化。

2.5　胞外酸化通过ASIC1/RIP1途径抑制RAW264.7巨噬细胞自噬

为了阐明ASIC1/RIP1途径激活是否参与胞外酸化诱导的RAW 264.7巨噬细胞自噬流障碍，分别以ASIC1特异性阻断剂PcTx-1和RIP1抑制剂Nec-1干预胞外酸化诱导的RAW264.7细胞24 h，Western blot检测自噬标志物LC3和p62蛋白的表达，以明确抑制ASIC1/RIP1途径后细胞自噬流的变化。结果表明，与pH 7.4+ox-LDL组相比较，胞外酸化组的LC3II/LC3I蛋白比值显著降低

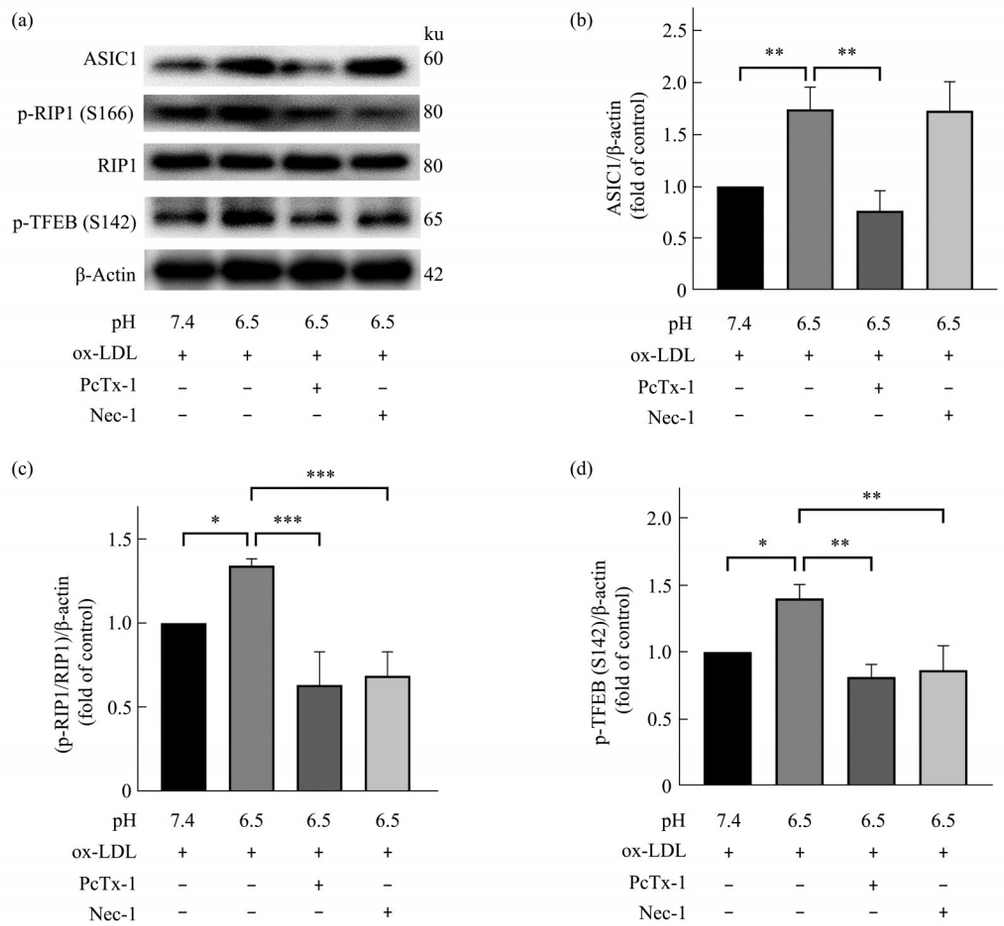

Fig. 4　Extracellular acidification enhances phosphorylation of TFEB *via* activating ASIC1/RIP1 pathway

RAW 264.7 macrophages were cultured in indicated pH medium containing 25 mg/L ox-LDL with or without ASIC1 specific blocker PcTx-1 or RIP1 inhibitor Nec-1 for 24 h. The expressions of ASIC1, total RIP1, p-RIP1 Ser166, total TFEB, p-TFEB Ser142 were detected by Western blot. (a) Representative Western blots of ASIC1, total RIP1, p-RIP1 Ser166, p-TFEB Ser142. (b−d) Quantification of the above proteins by the NIH Image J software. Results are expressed as the *means*±*SEM* (*n*=3) of three independent experiments. Statistical analysis was analyzed by one-way analysis of variance. **P*<0.05, ***P*<0.01, ****P*<0.001.

（图5a，b），p62蛋白的表达显著增加（图5a，c）。与胞外酸化组相比较，PcTx-1组和Nec-1组的LC3II/LC3I比值都明显升高，而p62蛋白的表达则均显著减少。这表明阻断ASIC1/RIP1信号途径可取消胞外酸化对RAW264.7巨噬细胞自噬流的抑制作用。

2.6　胞外酸化通过ASIC1/RIP1途径抑制RAW264.7巨噬细胞脂噬

在明确胞外酸化经ASIC1/RIP1途径抑制自噬的基础上，进一步探讨该信号途径对胞外酸化诱导RAW264.7巨噬细胞脂噬的影响。胞外酸化诱导的RAW264.7巨噬细胞分别以ASIC1阻断剂PcTx-1和RIP1抑制剂Nec-1干预24 h，透射电镜和免疫荧光染色激光共聚焦显微镜观察细胞的脂噬变化。透射电镜观察结果表明（图6a），与pH 7.4组相比较，胞外酸化组巨噬细胞内脂滴数量明显增加，自噬体、溶酶体、自噬溶酶体以及包含脂滴的脂噬泡数量均明显减少，表明胞外酸化抑制RAW264.7巨噬细胞脂噬和促进脂质蓄积。然而，胞外酸化对RAW264.7细胞的上述现象能被PcTx-1和Nec-1所取消。激光共聚焦显微镜下分别观察脂滴（Bodipy，红色）与自噬体（LC3II，绿色）和溶酶体（LAMP1，绿色）共定位的研究结果表明（图6b，c），与pH 7.4组相比，胞外酸化组细胞的脂滴

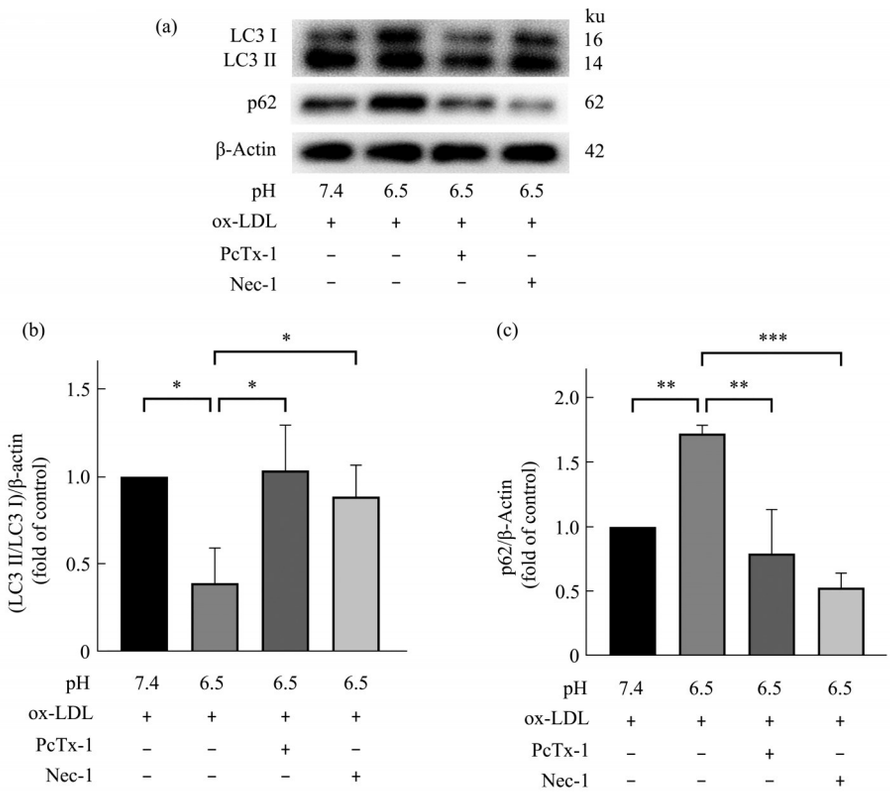

Fig. 5　Inhibiting the activation of ASIC1/RIP1 pathway reverses autophagy deficit induced by extracellular acidification in RAW264.7 macrophages

RAW 264.7 macrophages were cultured in indicated pH medium containing 25 mg/L ox-LDL with or without ASIC1 blocker PcTx-1 or RIP1 inhibitor Nec-1 for 24 h. The protein levels of LC3 and p62 in the cells were evaluated by Western blot. (a) Representative Western blots of LC3I, LC3II, and p62. (b, c) Quantification of LC3I, LC3II, and p62 protein levels by the NIH Image J software. Values were expressed as *means*±*SEM* (*n*=3). Differences were statistically analyzed using one-way analysis of variance. **P*<0.05, ***P*<0.01, ****P*<0.001.

（红色）蓄积增加，自噬体（绿色荧光）和溶酶体的数量（绿色荧光）都则明显减少，脂滴与自噬体、脂滴与溶酶体的共定位（黄色）也显著减少。而与pH 6.5组相比较，PcTx-1和Nec-1干预组组细胞内的脂质蓄积（红色）都明显减少，自噬体和溶酶体与脂滴的共定位（黄色）均显著增加。以上结果表明，胞外酸化通过ASIC1/RIP1途径抑制RAW264.7巨噬细胞的脂噬。

2.7　抑制ASIC1/RIP1途径促进RAW24.7巨噬细胞的胆固醇流出和减少细胞内的脂质蓄积

脂噬是将富含胆固醇酯的脂滴降解为游离胆固醇后经ABCA1转运至细胞外的生物学过程。为了进一步验证ASIC1/RIP1途径介导的脂噬障碍促成脂质蓄积，胞外酸化诱导的RAW264.7巨噬细胞分别使用ASIC1阻断剂PcTx-1和RIP1抑制剂Nec-1干预24 h，胆固醇荧光试剂盒检测巨噬细胞中ABCA1介导的胆固醇流出，油红O染色检测细胞内的脂质蓄积。胆固醇流出检测结果（图7a）表明，与pH 7.4对照组相比较，胞外酸化组RAW264.7细胞的胆固醇流出显著减少，而与pH 6.5处理组相比，PcTx-1组和Nec-1组细胞的胆固醇流出均显著增加。此外，油红O染色结果（图7b，c）表明，与pH 7.4组相比，胞外酸化组巨噬细胞内脂质蓄积（红色区域）明显增加。而与胞外酸化组相比较，ASIC1抑制剂组和RIP1抑制剂组细胞内的脂质蓄积均显著减少。上述结果表明，胞外酸化通过激活ASIC1/RIP1途径抑制脂噬，减少RAW264.7巨噬细胞ABCA1介导的胆固醇流出，从而促进细胞内的脂质蓄积。

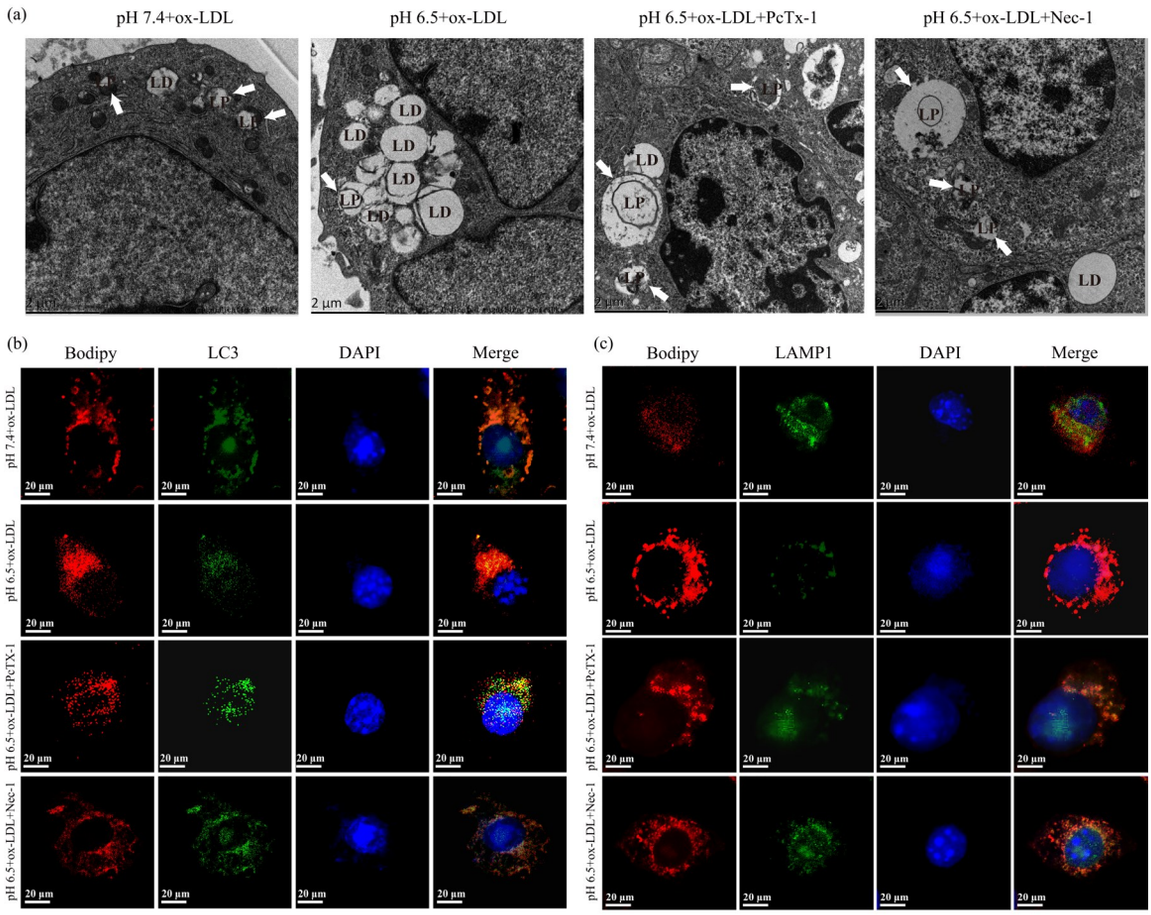

Fig. 6　Extracellular acidification inhibits lipophagy *via* activating ASIC1/RIP1 pathway in RAW 264.7 macrophages

RAW 264.7 macrophages were cultured in indicated pH medium containing 25 mg/L ox-LDL with or without ASIC1 specific blocker PcTx-1 or RIP1 inhibitor Nec-1 for 24 h. (a) The ultrastructure changes in the cells were observed by TEM. LD: lipid droplet; LP: lipophagy. (b, c) Representative images of cellular fluorescent co-location of Bodipy (indicated droplet) with LC3II (indicated autophagosome), and LAMP1 (indicated lysosome) were evaluated by confocal microscopy, respectively.

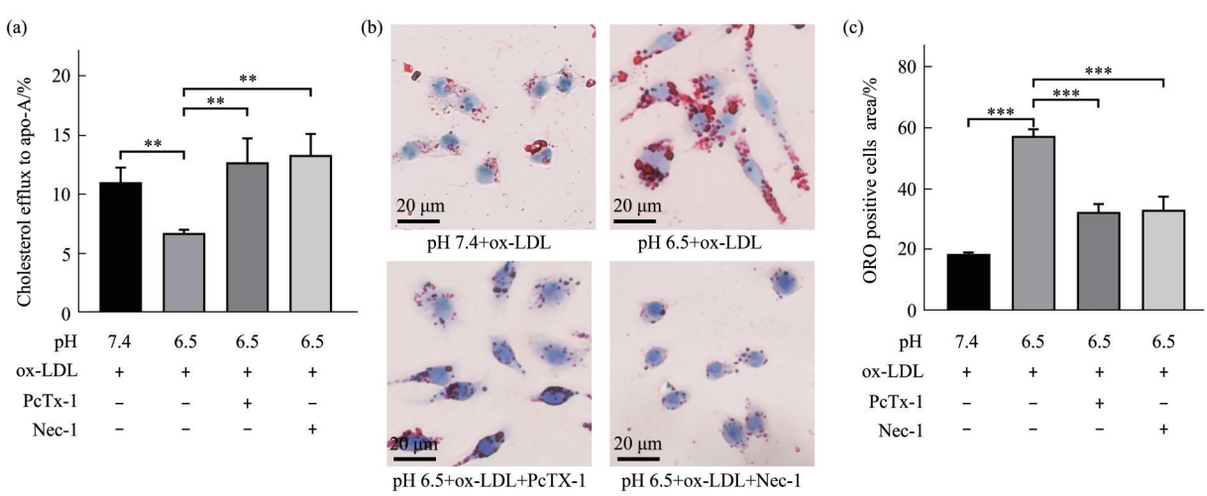

Fig. 7　Extracellular acidification inhibits ABCA1−mediated cholesterol efflux *via* activating ASIC1/RIP1 pathway

(a) RAW 264.7 cells were cultured in different pH medium containing NBD-cholesterol (20 mg/L) for 24 h to loading cholesterol. The cells were then incubated with the indicated pH medium containing 20 mg/L ApoA-1 with or without ASIC1 blocker PcTx-1 or RIP1 inhibitor Nec-1 for 24 h. Then cholesterol efflux was analyzed by NBD-cholesterol kits. (b) Lipid deposition was detected by oil red O (ORO) staining. (c) ORO staining positive areas were quantified. Results are expressed as the *means*±*SEM* of three independent experiments. Statistical analysis was analyzed by one-way analysis of variance. ***P*<0.01, ****P*<0.001.

3 讨 论

AS病变处常出现微环境酸化和脂噬障碍，但该酸化微环境对巨噬细胞的脂噬影响及其机制目前尚不清楚。本课题探讨胞外酸化是否通过激活ASIC1/RIP1途径抑制巨噬细胞脂噬。研究发现：a. 胞外酸化促进RAW264.7巨噬细胞的脂质蓄积和ASIC1膜转位，诱导RIP1和TFEB磷酸化，抑制巨噬细胞的脂噬；b. 抑制巨噬细胞ASIC1激活显著降低胞外酸化诱导RIP1和TFEB的磷酸化水平，且促进脂噬；c. RIP1抑制剂显著降低胞外酸化诱导的RAW264.7巨噬细胞TFEB磷酸化水平，促进脂噬和减少细胞内的脂质蓄积。研究结果表明，胞外酸化通过激活巨噬细胞ASIC1/RIP1途径促进TFEB的Ser166位点磷酸化，从而抑制巨噬细胞脂噬。

本研究首先构建胞外酸化诱导的巨噬细胞源性泡沫细胞模型。以RAW264.7巨噬细胞为研究对象，以pH 6.5酸性培养液模拟AS病变血管内膜液中巨噬细胞所处的酸化环境，明确胞外酸化对巨噬细胞脂质蓄积的影响。为了防止pH大幅度波动对实验结果的影响，采用生物缓冲剂MES配制酸性培养基以维持细胞外pH值的相对稳定。研究发现，胞外酸化显著促进RAW264.7巨噬细胞内的脂质蓄积和泡沫细胞形成。这与前期研究结果相一致[10]。此外，新近研究结果证实，胞外酸化促进人单核细胞源性巨噬细胞（THP-1）内的脂质蓄积[19]，抑制细胞外酸化减轻巨噬细胞源性泡沫细胞形成[18]。尽管本研究结果和文献资料证实，细胞外酸化加速巨噬细胞的脂质蓄积，但巨噬细胞感知胞外酸化信号进而促脂质蓄积的机制尚不清楚。这是接下来探讨的主要科学问题。

ASIC1蛋白作为感受胞外升高H^+的重要膜受体，但在正常pH环境，其主要位于细胞质中[20]。当胞外发生酸化时，该通道蛋白迅速从胞质向细胞膜转位，继而与H^+结合以转导胞外酸化信号，参与神经元损伤等多种生理与病理生理进程[21]。本研究结果表明，ASIC1表达于RAW264.7巨噬细胞，pH 6.5的胞外酸化环境不仅促进ASIC1蛋白表达和膜转位，而且显著增加细胞内的脂质蓄积。有趣的是，本研究结果（图2a）显示细胞膜蛋白Na^+/K^+ ATPase的表达比总蛋白质高，该现象与文献［22-23］报道相一致，但目前对这一异常现象的机制尚不清楚。值得注意的是，ASIC1的特异性阻断剂PcTx-1抑制该受体激活后，胞外酸化诱导的RAW264.7巨噬细胞的脂质蓄积显著减少。这些实验结果表明，激活ASIC1是胞外酸化促进RAW264.7巨噬细胞脂质蓄积的重要机制。

越来越多的证据表明，ASIC1通过调控自噬参与各种组织酸化性疾病的病理生理过程。在肝癌、脑胶质瘤等实体肿瘤中，肿瘤微环境酸化通过ASIC1激活自噬促进肿瘤细胞的增殖和迁移[24-25]。在类风湿性关节炎病理进程中，关节滑膜酸化通过激活ASIC1/AMPK/FoxO3a途径诱导关节软骨细胞自噬[26]。然而，胞外酸化对巨噬细胞自噬的影响目前尚不清楚。本研究从自噬新视角探讨了ASIC1在胞外酸化诱导RAW264.7巨噬细胞脂质蓄积中的作用及其机制。研究结果表明，胞外酸化显著增加p-RIP1 Ser166和p-TFEB Ser142蛋白的水平，降低LC3II/LC3I的比值和升高p62蛋白的表达。重要的是，胞外酸化对巨噬细胞的上述作用能被ASIC1的阻断剂PcTx-1所取消。鉴于LC3II/LC3I比值升高和p62蛋白表达降低反映自噬被激活，反之则被抑制[27]。上述研究结果表明，胞外酸化通过激活ASIC1促进RIP1和TFEB磷酸化抑制RAW264.7巨噬细胞自噬。然而，本研究结果与文献报道胞外酸化经ASIC1促进肿瘤和关节软骨细胞自噬不一致，其原因可能是胞外酸化对不同类型细胞的自噬信号途径的作用不同。

脂噬是一种清除富含胆固醇酯脂滴的选择性自噬，在防止细胞内脂质异常蓄积中起着重要作用[16]。当脂噬受到抑制时，因细胞内的脂滴降解能力降低而导致大量脂质蓄积形成泡沫细胞，进而促进AS的发生[28]。研究发现，细胞脂噬受RIP1/TFEB信号途径调控[29-30]。在该信号途径中，RIP1是自噬及溶酶体活性的重要负性调节因子，RIP1抑制自噬的机制与其Ser166位点磷酸化后升高p-Ser142 TFEB水平有关[30]。TFEB为核转录因子，其通过调控自噬关键基因LC3和LAMP1的表达来参与自噬过程调节[31]。磷酸化激活的RIP1募集下游信号分子TFEB，并促使后者142号位点的丝氨酸残基磷酸化（p-Ser142 TFEB）[32]。当其142位点的丝氨酸磷酸化时，TFEB从胞质向细胞核的移位被抑制，下调LC3和LAMP1的表达，进而抑制脂噬[14]。文献资料证实，胞外酸化促使神经元ASIC1羧基端靶向募集RIP1，升高p-RIP1 Ser166水平[12]。那么，胞外酸化是否通过ASIC1/RIP1/TFEB途径抑制RAW264.7巨噬细胞脂噬呢？

本文在已明确胞外酸化通过ASIC1促进RIP1和TFEB磷酸化基础上，进一步探讨了该信号途径对胞外酸化诱导RAW264.7巨噬细胞脂噬的影响及作用机制。透射电镜结果表明，与正常对照组相比较，胞外酸化组细胞内的脂滴大量蓄积，包含脂滴的脂噬体的数量显著减少。免疫荧光染色、激光共聚焦显微镜结果表明，胞外酸化组细胞内脂滴与自噬体和溶酶体共定位区域都明显减少，而细胞内的脂质蓄积明显增加。此外，与对照组相比较，胞外酸化组细胞的LC3II蛋白水平显著降低和p62蛋白的表达明显增加，ABCA1介导的胆固醇流出显著减少。以上结果证实，胞外酸化抑制RAW264.7巨噬细胞的脂噬。然而，胞外酸化对巨噬细胞的脂噬抑制作用能分别被ASIC1的特异性阻断剂PcTx-1和RIP1的抑制剂Nec-1所抵消。这表明胞外酸化通过ASIC1/RIP1途径抑制TFEB介导的巨噬细胞脂噬。其机制为阻断ASIC1/RIP1信号途径后，胞外酸化诱导的巨噬细胞RIP1Ser166位点磷酸化被抑制，继而降低TFEB Ser142磷酸化水平，促进TFEB的核转位，上调脂噬相关基因LC3和LAMP1的表达，从而逆转胞外酸化对RAW264.7巨噬细胞脂噬的抑制。

4 结　论

本研究探讨了ASIC1/RIP1途径在胞外酸化诱导巨噬细胞脂质蓄积中的作用及其机制。发现胞外酸化通过激活ASIC1和RIP1升高p-Ser142 TFEB水平，继而下调自噬相关基因LC3和溶酶体相关基因LAMP1的表达，抑制巨噬细胞脂噬，从而促进脂质蓄积和泡沫细胞形成。本研究从激活ASIC1/RIP1途径调控脂噬的新视角揭示了酸化微环境促巨噬细胞脂质蓄积的作用机理，将为以ASIC1新靶点的AS性疾病防治提供研究基础。

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Extracellular Acidification Impairs Macrophage Lipophagy Through ASIC1/RIP1 Pathway*

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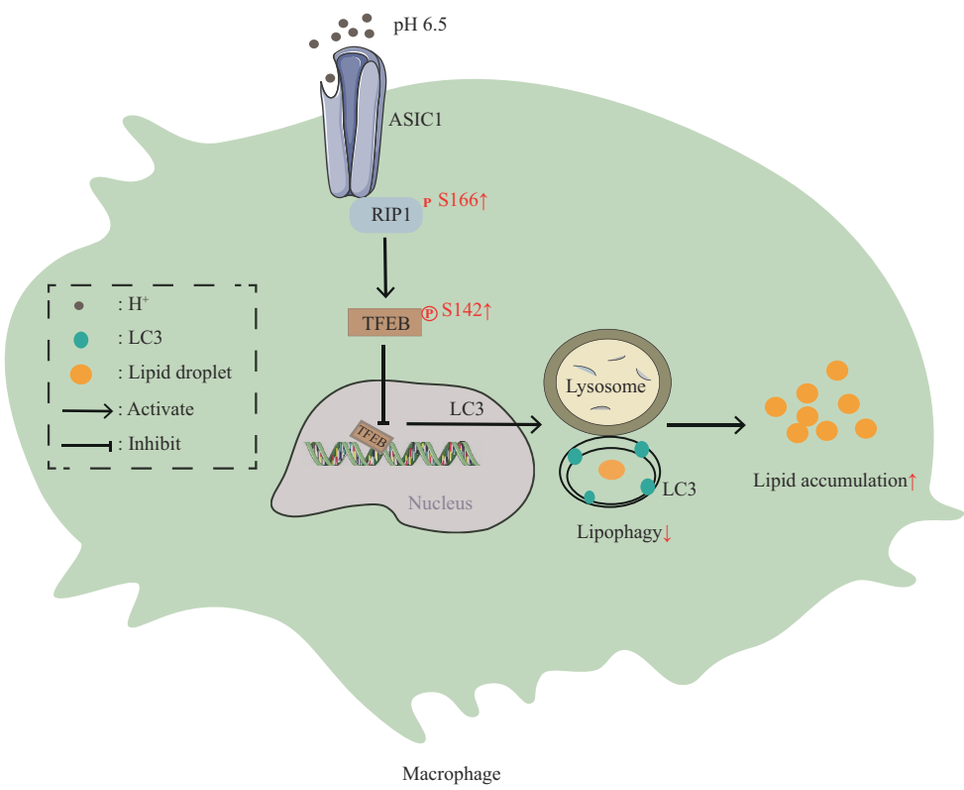
Graphical abstract



Abstract **Objective** Our recent study has demonstrated that extracellular acidification promotes lipid accumulation in macrophages *via* the activation of acid sensing ion channel 1 (ASIC1), but the underlying mechanism remains unclear. This study aims to explore the effect of extracellular acidification on macrophage

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lipophagy and the underlying mechanism. **Methods** RAW264.7 macrophages were incubated with 25 mg/L ox-LDL in a pH 6.5 culture medium for 24 h to build macrophage-derived foam cell models induced by extracellular acidification. Then, RAW264.7 macrophages were cultured in the acidic medium of pH 6.5 with or without PcTx-1 (ASIC1 specific blocker, 10 μg/L) or Nec-1 (RIP1 specific inhibitor, 20 μmol/L) for 24 h, intracellular lipid accumulation was observed by oil red O staining. The expressions of total ASIC1, plasma membrane ASIC1, RIP1, p-RIP1 Ser166, TFEB, p-TFEB Ser142, LC3 and p62 were measured by Western blot. The co-localization of lipids (indicated by Bodipy) with LC3II (autophagosomes) and LAMP1 (lysosomes) was analyzed by a confocal laser scanning microscopy, respectively. Morphological changes of lipophagy in the cells were observed by using transmission electron microscopy. ABCA1-mediated cholesterol efflux was determined by cholesterol fluorescence kits. **Results** Compared with pH 7.4+ox-LDL group, the intracellular lipid accumulation in the pH 6.5+ox-LDL group was significantly increased. Meanwhile, the expressions of plasma membrane ASIC1, p-RIP1 Ser166, p-TFEB Ser142, and p62 proteins were elevated significantly, while LC3II protein level and LC3II/LC3I ratio were decreased. Accordingly, compared with pH 7.4+ox-LDL group, the macrophage lipophagy of the pH 6.5+ox-LDL group was inhibited as indicated by the decreased localization of lipid droplets with LC3 and LAMP1, a decrease in the number of lipophagosomes as well as an increase in lipid droplets. Furthermore, ATP binding cassette transporter A1 (ABCA1) -dependent cholesterol efflux from the macrophages of pH 6.5+ox-LDL group reduced dramatically. However, these above effects of extracellular acidification on RAW264.7 macrophages were abolished by PcTx-1 and Nec-1, respectively. **Conclusion** These findings suggest extracellular acidification promotes the phosphorylation of TFEB at Ser142 *via* activating ASIC1/RIP1 pathway, thereby impeding lipophagy in RAW 264.7 macrophages, and that ASIC1 may be a new potential target for preventing aberrant lipid accumulation diseases including atherosclerosis.

Key words ASIC1, extracellular acidification, atherosclerosis, lipophagy, RIP1, TFEB
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