

研究论文

甲状腺激素对氧化型低密度脂蛋白诱导的巨噬细胞功能紊乱的影响

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摘要: 甲状腺功能减退症(甲减)患者的动脉粥样硬化(atherosclerosis, AS)及冠心病风险指数显著增加, 但机制不清楚。巨噬细胞功能紊乱是促进AS斑块形成及发展的重要环节, 本研究旨在探讨甲状腺激素对巨噬细胞功能的直接影响, 为甲减相关AS的机制研究提供新思路。用氧化型低密度脂蛋白(oxidized low-density lipoprotein, oxLDL)诱导小鼠单核巨噬细胞系RAW264.7, 建立体外巨噬泡沫细胞模型, 并观察不同浓度甲状腺素(thyroxine, T4)对巨噬泡沫细胞功能的改善效应。分别采用MTT法、划痕实验、 β -半乳糖苷酶染色实验检测巨噬细胞增殖、迁移功能及细胞衰老情况; ELISA法检测巨噬细胞分泌肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α)、单核细胞趋化蛋白-1 (monocyte chemoattractant protein-1, MCP-1)及白细胞介素-1 β (interleukin-1 β , IL-1 β)情况; Western blot检测参与巨噬细胞增殖、迁移等过程的粘着斑激酶(focal adhesion kinase, FAK)的磷酸化水平。结果显示: oxLDL可显著抑制巨噬细胞增殖和迁移、促进其衰老及分泌 TNF- α 、MCP-1和IL-1 β , 而T4可浓度依赖性逆转oxLDL对巨噬细胞上述功能的影响; oxLDL可使巨噬细胞磷酸化FAK蛋白表达上调, 而T4可浓度依赖性降低FAK蛋白磷酸化水平。上述结果提示, T4可呈浓度依赖性地调控巨噬细胞增殖、迁移、衰老及炎症因子分泌等功能。

关键词: 甲状腺激素; 氧化型低密度脂蛋白; 巨噬细胞; 甲状腺功能减退症; 动脉粥样硬化

中图分类号: R363; R581.2; R541.4

Effects of thyroid hormone on macrophage dysfunction induced by oxidized low-density lipoprotein

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Abstract: It has been recognized that patients with hypothyroidism have higher risks of atherosclerosis and coronary heart disease, however, the mechanisms are largely unknown. Considering that macrophage dysfunction plays an important role in the formation and development of atherosclerosis plaques, this study aimed to investigate the direct effects of thyroid hormone on macrophage functions and to provide new insight for the mechanism of hypothyroid atherosclerosis. RAW264.7 cells (mouse leukaemic monocyte macrophage cell line) were incubated with oxidized low-density lipoprotein (oxLDL) to establish macrophage foam cells model *in vitro*, and the protective effects of different concentration of thyroxine (T4) on the macrophage foam cells function were explored. The proliferation, migration and cell aging of macrophages were detected by MTT method, scratch test and β -galactosidase staining respectively. The ELISA method was used to detect the secretion of tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-

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1), and interleukin-1 β (IL-1 β). Western blot analysis was applied to measure the phosphorylation of focal adhesion kinase (FAK), which was required for the process of proliferation and migration of macrophages. The results showed that oxLDL significantly inhibited the macrophage proliferation and migration, induced cell senescence, and promoted the secretion of TNF- α , MCP-1, and IL-1 β ; while T4 reversed those effects of oxLDL on macrophage in a concentration-dependent manner. Moreover, oxLDL increased the phosphorylation of FAK in macrophage, while T4 concentration-dependently reversed the effect. These results suggest that T4 modulates macrophage proliferation, migration, senescence, and secretion of inflammation factors in a concentration-dependent way.

Key words: thyroid hormone; oxidized low-density lipoprotein; macrophage; hypothyroidism; atherosclerosis

甲状腺功能减退症(甲减)是动脉粥样硬化(atherosclerosis, AS)和冠心病的危险因素之一^[1],其血清学表现为促甲状腺激素升高、游离甲状腺素降低,目前临床上治疗甲减的有效方法是补充左旋甲状腺素。甲减引起或加重AS的机制研究主要集中在甲状腺激素对血脂、内皮细胞及血管平滑肌细胞功能的影响等方面^[2]。巨噬细胞在AS斑块的发生、发展过程中起重要作用^[3,4]。在早期脂质浸润中,巨噬细胞可通过摄取氧化型低密度脂蛋白(oxidized low-density lipoprotein, oxLDL)形成巨噬泡沫细胞,这是AS早期最基本的特征之一。但是,甲状腺激素是否对巨噬细胞功能有直接作用并不清楚。细胞增殖、迁移、衰老及分泌因子是巨噬细胞的基本且重要的功能。因此,本研究拟通过检测甲状腺素(thyroxine, T4)对巨噬细胞以上功能的影响进一步探究甲减致AS的机制。

1 材料与方 法

1.1 主要材料 小鼠单核巨噬细胞系RAW264.7细胞系购于中国医学科学院基础医学研究所。OxLDL购于广州奕源生物科技有限公司。T4、四甲基偶氮唑蓝[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, MTT]购于Sigma-Aldrich公司。细胞衰老 β -半乳糖苷酶(β -galactosidase, β -Gal)染色试剂盒购于杭州达文生物有限公司。肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α)试剂盒、单核细胞趋化蛋白-1(monocyte chemoattractant protein-1, MCP-1)试剂盒及白细胞介素-1 β (interleukin-1 β , IL-1 β)试剂盒购于Elabscience。粘着斑激酶(focal adhesion kinase, FAK)和磷酸化粘着斑激酶(phospho-focal adhesion kinase, P-FAK)抗体购于Abcam。胎牛血清(fetal bovine serum, FBS)、DMEM培养基均购自Hyclone。

1.2 细胞培养及实验分组 RAW264.7细胞系用含10% FBS的DMEM培养基培养。采用100 μ g/mL

的oxLDL诱导RAW264.7建立巨噬泡沫细胞模型^[5]。实验分为空白对照组(control check, CK)、巨噬泡沫细胞对照组(oxLDL)、oxLDL+T4低浓度组(25 nmol/L)、oxLDL+T4中浓度组(50 nmol/L)以及oxLDL+T4高浓度组(100 nmol/L)。其中,后3组为oxLDL与T4同时加入共作用于细胞。各实验每组设置3个复孔。

1.3 MTT法检测细胞增殖 待细胞长至汇合期后,调整细胞浓度为 3×10^4 个细胞/mL,接种于96孔培养板,于37 $^{\circ}$ C、5% CO₂培养箱内培养24 h后按实验分组加入不同浓度的药物,并分别于药物作用24、48、72 h后在每孔加入15 μ L的MTT,于37 $^{\circ}$ C、5% CO₂培养箱内避光孵育3~4 h。吸净孔内的液体,加入200 μ L二甲基亚砜,室温于摇床震荡10 min。酶标仪492 nm波长检测同一时间点吸光(optical density, OD)值,并计算不同处理对巨噬细胞的抑制率。抑制率=(1-实验组OD值/CK组OD值) \times 100%。

1.4 细胞划痕实验 在6孔板中加入约含 3×10^5 个细胞/孔的细胞悬液,第二天用200 μ L移液枪枪头沿培养板底部划痕。按实验分组处理细胞,于37 $^{\circ}$ C、5% CO₂培养箱培养24 h后于倒置显微镜下观察划痕中细胞迁移情况。

1.5 β -Gal染色检测细胞衰老 将细胞接种至48孔板中,接种密度为 3×10^4 个细胞/mL。按照上述实验分组处理细胞,分别培养24、48、72 h后吸出培养液,PBS洗涤2次,加入300 μ L β -Gal染色固定液,室温固定15 min。吸除细胞固定液,PBS洗涤3次,每次3 min。每孔加入300 μ L染色工作液,37 $^{\circ}$ C孵育过夜。倒置显微镜下观察拍照。

1.6 ELISA分析 按照Elabscience公司提供的ELISA试剂盒说明检测各组细胞上清中TNF- α 、MCP-1及IL-1 β 的相对含量。

1.7 Western blot分析 药物处理48 h后提取细胞总蛋白,采用BCA法定量。各组取30 μ g蛋白上样,经十二烷基硫酸钠-聚丙烯酰胺凝胶电泳后,

电转至 PVDF 膜并封闭。加入一抗 4 °C 摇床过夜，再用辣根过氧化物酶标记的二抗室温孵育 1 h，加入化学发光液上机检测。使用 ImageLab 软件进行灰度分析，并以 β -actin 条带标准化。

1.8 统计学方法 采用 SPSS 20.0 统计软件进行实验数据分析。计量资料以 $\text{mean} \pm \text{SD}$ 表示。多组间均数比较采用单因素方差分析，两两比较采用 Tukey 法。 $P < 0.05$ 时认为差异具有统计学意义。

2 结果

2.1 T4呈浓度依赖性促进巨噬泡沫细胞增殖

为了探索 T4 对巨噬细胞增殖及活性的影响，我们设置了不同浓度 T4 组 (25、50、100 nmol/L) 及三个时间点 (24、48、72 h) 进行 MTT 实验。MTT 结果显示：与 CK 组相比，oxLDL 作用于巨噬细胞 48 h 及 72 h 后显著抑制巨噬细胞增殖，随着给药浓度的增加，T4 可显著缓解 oxLDL 对巨噬细胞增殖的抑制 ($P < 0.01$ ，图 1)。

2.2 T4促进巨噬泡沫细胞迁移

为了观察 T4 对巨噬细胞迁移功能的影响，我们进行了细胞划痕实验，并于 24 h 后观察细胞迁移情况。结果显示，相对于 CK 组，oxLDL 可显著抑制细胞的迁移 ($P < 0.01$)；T4 可浓度依赖性减弱 oxLDL 对细胞迁移的抑制作用 ($P < 0.01$ ，图 2)。

2.3 T4抑制巨噬泡沫细胞衰老

为了检测 T4 是否对巨噬细胞的衰老情况有影响，我们在 24、48、72 h 分别对各组细胞进行了 β -Gal 染色。结果显示：与 CK 组比，oxLDL 随着给药时间的增加促进细胞衰老 ($P < 0.01$)，T4 可浓度依赖性缓解 oxLDL 造成的细胞衰老 ($P < 0.01$ ，图 3)。

2.4 T4抑制巨噬泡沫细胞分泌炎症因子

按实验分组处理细胞 24 h 后，我们取上清用 ELISA 法检测巨噬泡沫细胞分泌炎症因子情况。结果表明，与 CK 组比，oxLDL 可使巨噬细胞分泌 TNF- α 、MCP-1 和 IL-1 β 增加 ($P < 0.01$)；但 T4 可浓度依赖性减少 oxLDL 引起的巨噬细胞炎症因子的分泌 ($P < 0.01$ ，图 4)。

2.5 T4呈浓度依赖性降低巨噬泡沫细胞FAK蛋白磷酸化水平

FAK 是细胞内的一种非受体酪氨酸激酶，可自身磷酸化，参与胞内多条信号通路的传导，与细胞运动、生存等相关。为了检测 T4 是否影响巨噬细胞内 FAK 蛋白的表达及活性，我们进行了 Western blot 实验。结果表明，与 CK 组比较，oxLDL 可使巨噬细胞 FAK 蛋白磷酸化水平显著上调；随着给药浓度的增加，T4 可使 oxLDL 引起的 FAK 蛋白磷酸化水平明显下调，但对 FAK 总蛋白的表达并无影响 (图 5)。

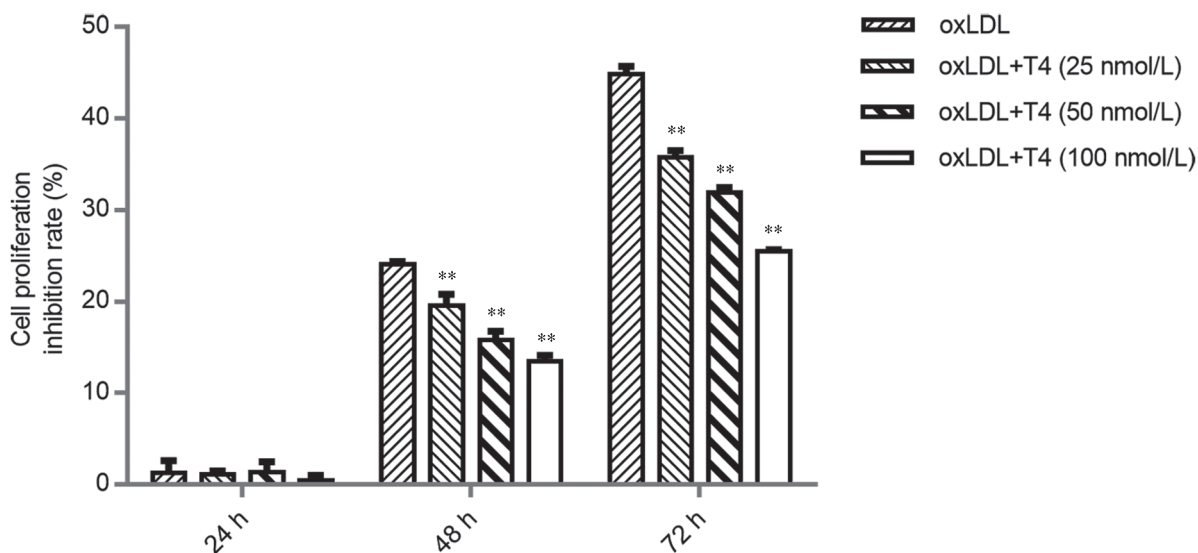


图 1. T4呈浓度依赖性逆转oxLDL对RAW264.7细胞增殖的抑制

Fig. 1. T4 reverses oxLDL-induced RAW264.7 cell proliferation inhibition in a concentration-dependent way. Cells were treated with the indicated concentration of T4 and/or oxLDL (100 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 h respectively. Cell proliferation inhibition rate was determined by MTT assay and expressed relative to control check (CK) group. Mean \pm SD, $n = 3$. ** $P < 0.01$ vs oxLDL group.

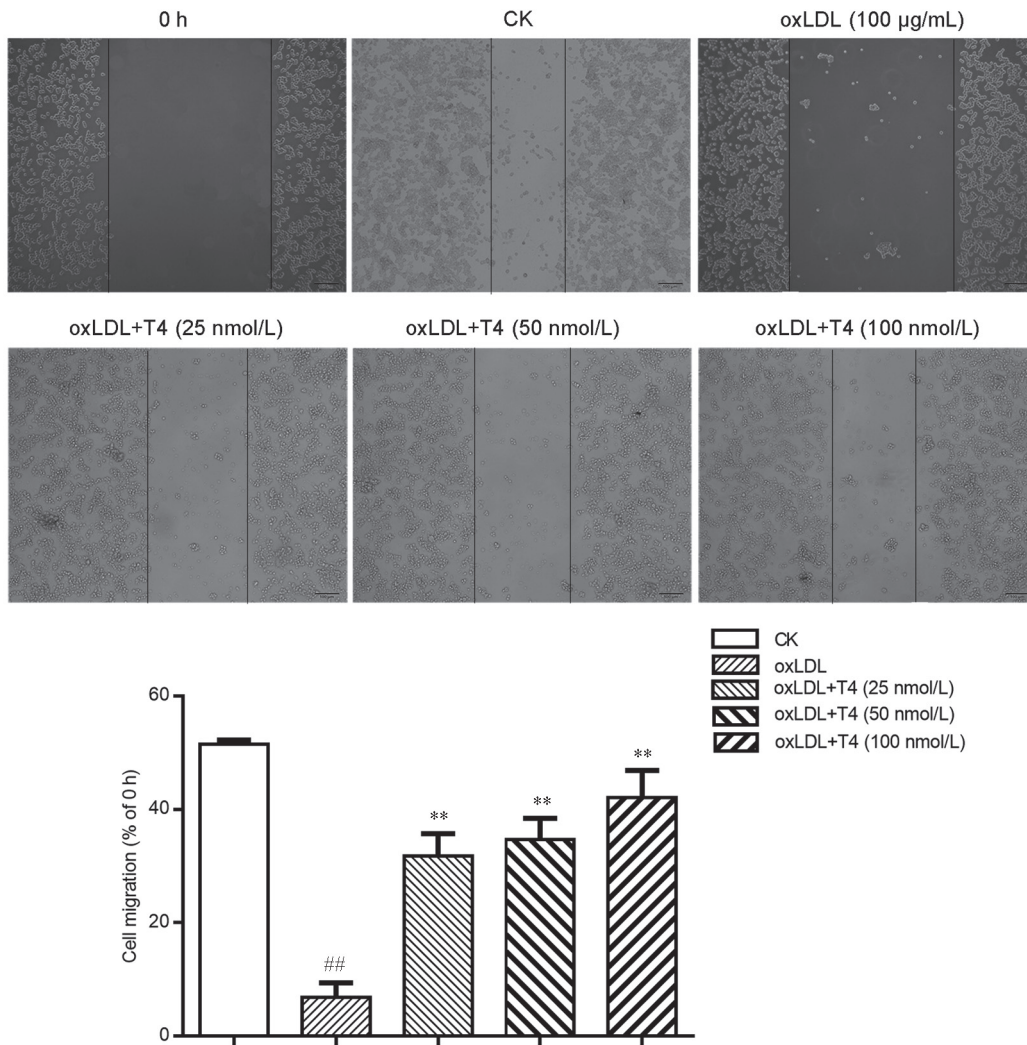


图 2. T4呈浓度依赖性减轻oxLDL对RAW264.7细胞迁移的抑制

Fig. 2. T4 alleviates oxLDL-induced RAW264.7 cell migration inhibition in a concentration-dependent way. Cells were treated with the indicated concentration of T4 and/or oxLDL (100 µg/mL) for 24 h. Cell migration was determined by the wound healing assay. Scale bar, 100 µm. Mean ± SD, $n = 3$. ** $P < 0.01$ vs oxLDL group; ## $P < 0.01$ vs control check (CK) group.

3 讨论

本研究应用 oxLDL 诱导 RAW264.7 细胞系建立了巨噬泡沫细胞模型，并用不同浓度 T4 作用于巨噬泡沫细胞，结果显示 T4 呈浓度依赖性地促进巨噬泡沫细胞增殖及迁移，抑制巨噬泡沫细胞衰老及炎症因子分泌。

鹿特丹研究 (Rotterdam Study) 认为，亚临床甲减与高血压、高脂血症、高血糖等因素一样是缺血性心脏病的独立危险因素^[6]。本研究组前期完成的一项队列研究显示：行经皮冠状动脉介入治疗的冠心病合并甲减患者，其远期主要不良心脑血管事件 (major adverse cardiovascular and cerebral events,

MACCE) 的发生率明显增高，而给予充足的左旋甲状腺素替代治疗可以明显减少 MACCE 发生^[7]。目前有研究表明，甲减时 AS 增加的间接因素是血脂紊乱、舒张期高血压及内皮功能受损；而甲状腺激素具有直接抗 AS 效应，如激活内皮细胞合成一氧化氮促进血管舒张、直接作用于血管平滑肌细胞使其舒张、抑制血管平滑肌细胞增殖及凋亡等^[8,9]。巨噬细胞作为 AS 斑块重要细胞成分之一，在斑块起始、发展、坏死、破裂整个过程中都发挥重要作用。循环中的单核细胞贴附于动脉内膜后，穿透血管内皮分化为巨噬细胞，进而吞噬 oxLDL 等而形成巨噬泡沫细胞。目前，甲状腺激素与巨噬细胞功能的

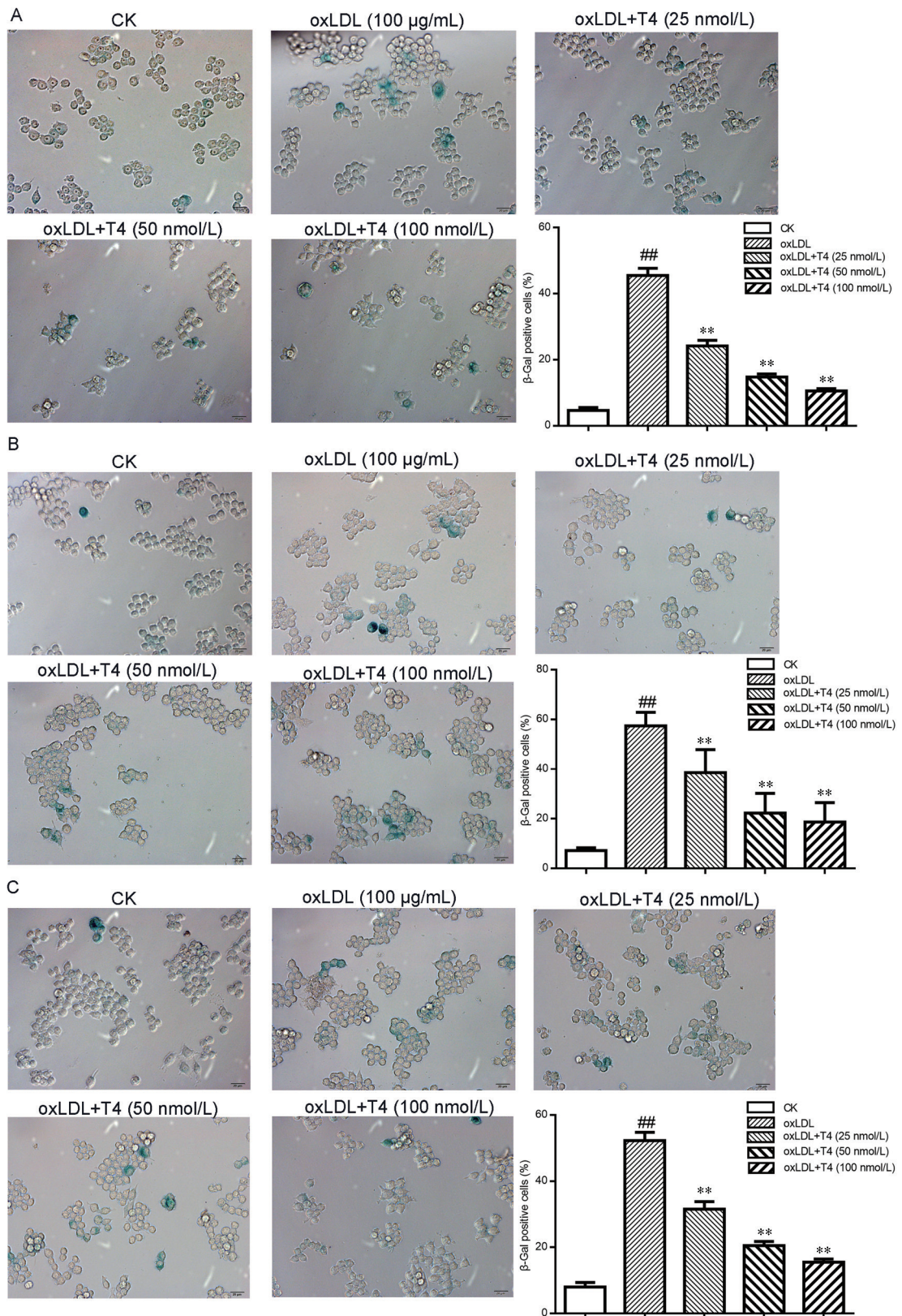


图 3. T4呈浓度依赖性抑制oxLDL诱导的RAW264.7细胞衰老

Fig. 3. T4 inhibits oxLDL-induced RAW264.7 cell senescence in a concentration-dependent way. Cells were treated with the indicated concentration of T4 and/or oxLDL (100 µg/mL) for 24 h (A), 48 h (B), and 72 h (C) respectively. Cell senescence was determined by β-galactosidase staining. Scale bar, 20 µm. Mean ± SD, n = 3. **P < 0.01 vs oxLDL group; ^{##}P < 0.01 vs control check (CK) group.

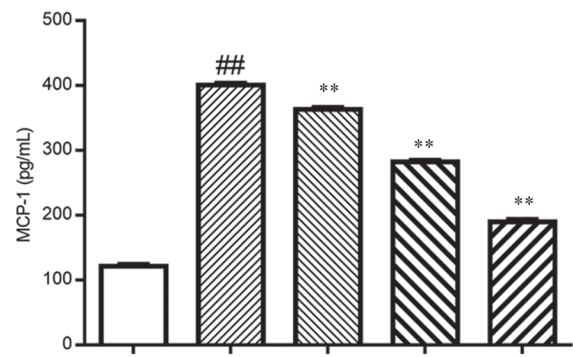
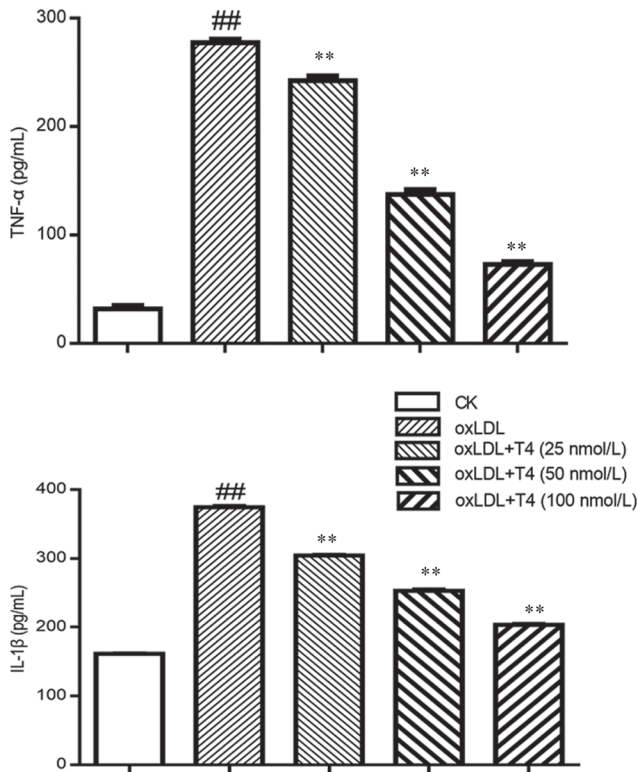


图 4. T4呈浓度依赖性抑制oxLDL诱导的RAW264.7细胞TNF- α 、MCP-1、IL-1 β 分泌
 Fig. 4. T4 inhibits oxLDL-induced secretion of TNF- α , MCP-1, and IL-1 β in RAW264.7 cells. Cells were treated with the indicated concentration of T4 and/or oxLDL (100 μ g/mL) for 24 h. The TNF- α , MCP-1, and IL-1 β levels in cell culture supernatant were determined using ELISA kits. Mean \pm SD, $n = 3$. ** $P < 0.01$ vs oxLDL group; ## $P < 0.01$ vs control check (CK) group.

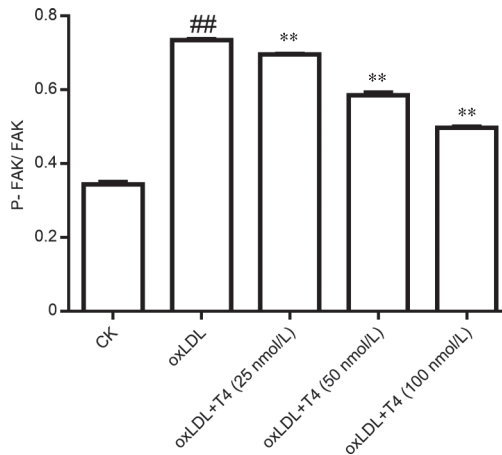
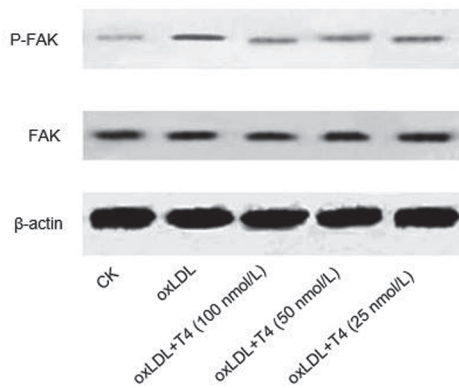


图 5. T4呈浓度依赖性抑制oxLDL诱导的RAW264.7细胞内FAK磷酸化水平
 Fig. 5. T4 inhibits oxLDL-induced phosphorylation of FAK in RAW264.7 cells. Cells were treated with the indicated concentration of T4 and/or oxLDL (100 μ g/mL) for 48 h. The protein levels of FAK and P-FAK were examined by Western blot analysis. Mean \pm SD, $n = 3$. ** $P < 0.01$ vs oxLDL group; ## $P < 0.01$ vs control check (CK) group.

研究存在许多争议。大多数研究表明，生理水平的甲状腺激素能使巨噬细胞的吞噬功能增强^[10-12]；但Rosa等却发现甲减动物的巨噬细胞吞噬功能增强，而甲亢对巨噬细胞吞噬功能无影响^[13]。Perrotta等研究显示，超生理剂量的T3能使小鼠骨髓来源巨噬细胞向炎性M1表型极化并抑制M2极化^[14]。近

几年研究显示，甲状腺激素在细胞内可通过基因组通路和非基因组通路影响巨噬细胞功能，前者通过核受体甲状腺激素受体 α (thyroid hormone receptor, TR α)实现，TR α 可能有抗炎、抗凋亡效应^[9, 15]；甲状腺激素通过非基因组通路可使巨噬细胞诱导型一氧化氮合酶生成增多、吞噬能力增强、细胞因子产

生增多、杀菌能力增强。由此可见, 甲状腺激素对巨噬细胞功能的影响, 受不同实验条件、模型的影响, 结论尚不统一。本研究从体外巨噬泡沫细胞的角度探索 T4 对其功能的直接影响, 并发现 T4 能拮抗 oxLDL 对巨噬细胞功能的影响, 促进巨噬泡沫细胞增殖、迁移, 抑制细胞衰老、分泌炎症因子。

FAK 是整合蛋白介导的信号转导中的重要成员, 有酪氨酸蛋白激酶活性, 可自身磷酸化激活, 酪氨酸 397 (Tyr397) 是一个主要的磷酸化位点。FAK 中 Tyr397 磷酸化位点与 Src 激酶家族的 SH2 结构域结合后, 启动下游信号通路, 调控细胞运动及细胞存活等关键细胞过程^[16]。已有研究表明, FAK 参与调控肿瘤细胞及巨噬细胞等正常细胞的迁移过程^[17-19]。细胞迁移由肌动蛋白聚合驱动的板状伪足伸展、已有的黏着斑瓦解及新的黏着斑形成的过程组成^[20]。因此, 细胞骨架装配与分解间的平衡才能保证细胞移动; 引起 FAK 快速而短暂激活的激动剂会促进迁移, 而引起 FAK 持久激活的激动剂(如 oxLDL)则会抑制细胞迁移^[21]。本研究及前期研究^[21, 22]结果均符合这一点, 即 oxLDL 抑制巨噬泡沫细胞的迁移, 其机制与 oxLDL 引起 FAK 高度磷酸化有关。OxLDL 抑制巨噬细胞迁移是导致 AS 斑块捕获胆固醇负荷泡沫细胞的一个机制。本研究进一步显示, T4 呈浓度依赖性降低巨噬泡沫细胞的 FAK 磷酸化水平并促进细胞迁移, 提示甲状腺激素可能通过促进巨噬泡沫细胞迁移而抑制 AS 斑块进展。然而, T4 是如何降低巨噬泡沫细胞中 FAK 磷酸化水平的, 尚需进一步的研究来揭示。

FAK 与细胞增殖、衰老的研究主要集中在肿瘤细胞, 在巨噬细胞等非肿瘤细胞中研究较少。已有研究表明, FAK 能促进肿瘤细胞增殖、抑制其衰老^[23-25]。而本研究显示, oxLDL 诱导巨噬细胞内 FAK 激活的同时, 抑制细胞增殖、促进细胞衰老。因此, 本研究尚无法说明 FAK 在巨噬泡沫细胞增殖、衰老中的作用。T4 促进巨噬泡沫细胞增殖、抑制其衰老的机制有待进一步实验研究。研究表明, 用 TNF- α 刺激小鼠巨噬细胞后, 可引起 FAK 的表达和活性均增高, 进而使 MCP-1、NF- κ B 等表达上调^[26]。本研究显示, oxLDL 诱导巨噬泡沫细胞分泌 TNF- α 、MCP-1 和 IL-1 β 增加, 我们推测这可能与 FAK 激活有关, T4 可能通过减弱 FAK 磷酸化水平抑制巨噬细胞分泌炎症因子, 这还需进一步实验证实。

本研究在细胞水平初步提示了 T4 对巨噬细胞功能有直接作用, 为甲减致 AS 的机制研究提供了新思路, 进一步的研究应在细胞水平及整体动物水平进行更深入的功能及机制探索。

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