白藜芦醇抑制巨噬细胞细胞外基质金属蛋白酶诱导物的表达

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摘要: 目的 探讨白藜芦醇对细胞外基质金属蛋白酶诱导物(EMMPRIN)表达的影响。方法 将人类单核细胞系 THP-1和 MCF-7细胞共培养,测定上清液中 MMP-9活性。用 PMA诱导 THP-1为巨噬细胞,加入白藜芦醇,观察 EMMPRIN表达和 MMP-9活性变化。细胞共转染实验测定白藜芦醇对 PPARγ的激动作用。用 PPARγ的拮抗剂 GW9662预处理细胞后,测定白藜芦醇对 EMMPRIN表达的影响。结果 PMA诱导使单核细胞 EMMPRIN表达明显增强,与 EMMPRIN高表达的 MCF-7细胞共培养显著增加单核细胞表达 MMP-9。白藜芦醇显著抑制 EMMPRIN和 MMP-9生成。白藜芦醇明显激动 PPARγ, GW9662大幅减弱白藜芦醇对 EMMPRIN和 MMP-9的作用。结论 单核细胞向巨噬细胞分化过程中表达明显增强的 EMMPRIN可能是促进 MMPs表达的主要因子。白藜芦醇通过激动 PPARγ抑制 EMMPRIN的表达,可能是其抑制巨噬细胞 MMPs产生的机制。

关键词: 细胞外基质金属蛋白酶诱导物; 过氧化物酶体增殖剂激活受体 γ; 基质金属蛋白酶; 白藜芦醇; 单核/巨噬细胞

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Resveratrol inhibits expression of EMMPRIN from macrophages

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Abstract: Aim To investigate the effect of resveratrol on EMMPRIN expression of macrophages. **Methods** Human monocytic cell line THP-1 cells were co-cultured with EMMPRIN-highly-expressed MCF-7 cells; MMP-9 production was assayed by zymography. THP-1 cells were induced by PMA, expression of EMMPRIN was assayed by Western blotting. Cells were treated with resveratrol or PPARγ agonist — pioglitazone during differentiation, EMMPRIN expression and MMP-9 activity were assayed. U937 cells were co-transfected with PPARγ expression and luciferase-coding reporter vector, then cultured with pioglitazone or resveratrol, the activating capability of resveratrol on PPARγ was evaluated by measuring the luciferase activity. THP-1 cells were pretreated with PPARγ antagonist — GW9662 before pioglitazone or resveratrol treatment, then assayed for EMMPRIN expression and MMP-9 production. **Results** EMMPRIN expression was greatly increased during the differentiation from monocytes to macrophages; co-culturing with MCF-7 cells significantly increased MMP-9 production by monocytes. Both resveratrol and pioglitazone markedly inhibited EMMPRIN expression during monocytes differentiation. Resveratrol significantly activated PPARγ and GW9662 greatly decreased the effect of resveratrol on EMMPRIN and MMP-9. **Conclusion** EMMPRIN expression is greatly up-regulated from monocytes to macrophages, which may play a role in inducing MMPs production by monocytes/macrophages. Resveratrol can significantly inhibit EMMPRIN expression via activating PPARγ, which may be the

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underlying mechanism of its inhibitory effect on MMPs production by monocytes/macrophages.

Key words: EMMPRIN; PPARγ; MMPs; resveratrol; monocytes/macrophages

冠状动脉粥样硬化性心脏病是严重危害人类健康的疾病,冠脉内粥样斑块的破裂导致具有高致凝性的脂核成分与血液接触,引起的血栓迅速堵塞冠状动脉,造成急性心肌梗死,是冠心病致死致残的主要原因。研究表明,循环中的单核细胞迁移、浸润粥样斑块并分化为巨噬细胞,表达分泌大量基质金属蛋白酶(matrix metalloproteinases, MMPs),后者降解包绕在粥样斑块表面的纤维帽胶原成分,削弱其厚度和强度,是诱导斑块破裂的主要因素[1,2]。因而,抑制粥样斑块内 MMPs的产生具有重要的临床价值。

近期研究发现,细胞外基质金属蛋白酶诱导物(extracellular matrix metalloproteinase inducer, EMMPRIN)可能与 MMP表达调节密切相关,本文对单核/巨噬细胞 EMMPRIN的表达进行了研究,并且发现天然药物白藜芦醇可以通过激活细胞内过氧化物酶体增殖剂激活受体γ(peroxisome proliferator-activated receptor γ, PPARγ)显著抑制单核/巨噬细胞 EMMPRIN和 MMP-9的表达,从而为白藜芦醇的抗冠心病作用提供了新的依据。

材料和方法

材料 人类乳腺癌细胞系 MCF-7和 U937细胞系由上海市健康科学中心卢珞教授赠与。人类单核细胞系 THP-1购自中国科学院上海细胞研究所;弗波醇(PMA)、二甲基亚砜(DMSO)、明胶和 GW9662均购自 Sigma公司;吡格列酮和植物提取白藜芦醇均购自 Merck公司;青霉素、链霉素、DMEM、RPMI 1640培养基和胎牛血清均购自澳大利亚 PAA laboratories GmBH公司;兔抗人 EMMPRIN抗体购自 Zymed公司;辣根过氧化物酶标记驴抗兔二抗购自 Amersham公司; 4 mm电转染杯购自 BIO-RAD公司;电转染采用 Gene Pulser X cell转染仪(BIO-RAD公司);人 PPARγ全长表达质粒 PSG5-hPPARγ和 PPARγ报告质粒 pACO-TKpGL3均由法国巴斯德研究所 Dr. Celine Haby友情提供;内参质粒为 pSV-β-Galactosidase Control Vector(Promega公司); β-gal活性测定采用 β-Galactosidase Enzyme Assay System kit(Promega公司)。

细胞培养 THP-1和 U937细胞使用 RPMI 1640培养基,MCF-7细胞使用 DMEM培养基,细胞在 37 ℃, 5% CO_2环境中培养。培养基中加入 10%胎牛血清、青霉素(100 u·mL^{-1})、链霉素(100 μg·mL^{-1})和 HEPES(10 mmol·L^{-1})。

细胞共培养 MCF-7和 THP-1细胞分别在无血清培养基中培养 2 d,留取培养上清液,以 1∶3的比例将 MCF-7和 THP-1细胞在 RPMI 1640培养基中共培养,分别在 12, 24, 48和 72 h时留取细胞培养上清液。

Western blotting分析 细胞经 PBS冲洗后,使用 4 ℃预冷的 RIPA裂解液提取蛋白,2×上样缓冲液煮沸变性 5 min。在 11% SDS-PAGE胶中每梳孔加入总蛋白 30 μg进行垂直电泳(150 V, 90 min), 4 ℃ 100 V转膜,5%牛奶-TBST室温封闭 1 h,5%牛奶-TBST稀释 EMMPRIN一抗(1∶1 000) 4 ℃轻摇过夜,二抗(1∶6 000)室温孵育 1 h后化学发光显色剂(PIECE)显色,感光、洗片。

白藜芦醇激动 PPARγ能力测定 U937细胞培养到指数生长期,取 2×10^7个细胞,PBS洗涤,电转染液(含 0.1 mmol·L^{-1} DTT, 10 mmol·L^{-1}葡萄糖, 10 mmol·L^{-1} HEPES, 100 u·mL^{-1}青霉素和 100 μg·mL^{-1}链霉素的 RPMI 1640培养基) 400 μL重悬细胞。加入 PPARγ表达质粒 5 μg,报告质粒 10 μg,内参质粒(pSV-β-Galactosidase Control Vector, Promega) 5 μg,混匀。另取细胞,仅加入报告质粒和内参质粒作为对照。细胞悬液室温静置 5 min后电转染(电压 250 V,电容 950 μF,电阻无限大)。室温静置 10 min,移入 6孔板中,加入适量的完全培养基,37 ℃, 5% CO_2条件下孵育 1 h后加入各种药物。继续培养 24 h。使用 Luciferase Assay System kit(Promega)裂解细胞,制备虫荧光素酶分析液并使用荧光定量仪(Lumat LB9507, Berthold Technologies)测定各组细胞荧光值。同时测定各组细胞中的 β-gal活性用以校正转染效率。

酶谱法(gelatin zymography)测定 MMP-9的活性 细胞培养上清液经含 0.1%明胶的 SDS凝胶行非变性垂直电泳(125 V, 90 min),凝胶置于复性缓冲液 2.5%(*v*/*v*) Triton X-100中室温振荡孵育 30 min。换用孵育液(50 mmol·L^{-1} Tris-base Tris-HCl, 0.2 mol·L^{-1} NaCl, 5 mmol·L^{-1} $CaCl_2$, 0.02% Brij 35)室温温和振荡 30 min,更换新鲜孵育液 37 ℃孵育过夜,使用 0.5%(*w*/*v*)考马斯亮蓝 R-250

染色 20 ~ 30 min,脱色液[甲醇-醋酸-水(50∶10∶40)]脱色。

统计学处理　相关数据使用 SPSS 11.0软件进行 t检验,$P < 0.05$为有统计学差异。

结果

1　单核细胞分化为巨噬细胞过程中 EMMPRIN表达明显增强

为了测定单核细胞向巨噬细胞分化过程中EMMPRIN的表达变化,用 PMA诱导分化人类单核细胞系 THP-1细胞,如图 1,细胞向巨噬细胞分化后EMMPRIN表达明显增强。

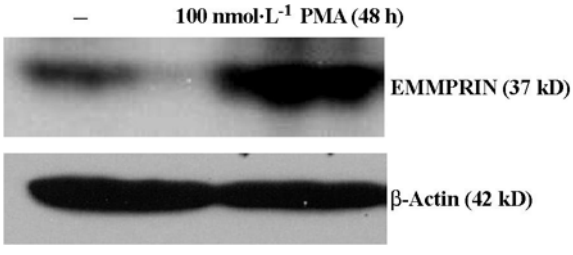

Figure 1　EMMPRIN expression was greatly induced during cell differentiation from monocytes to macrophages. THP-1 cells were cultured in presence or absence of PMA (100 nmol·L^{-1}) for 48 h. EMMPRIN expression in two groups was compared by Western blotting

2　MCF-7细胞和单核细胞共培养对单核细胞 MMP-9产生的影响

为了进一步明确 EMMPRIN诱导单核/巨噬细胞产生 MMP的作用,将 THP-1细胞和高表达EMMPRIN的肿瘤细胞 MCF-7共培养,然后使用酶谱法测定细胞上清液 MMP-9的活性,如图 2。单独培养的 MCF-7细胞上清液中几乎不含 MMP-9,而 THP-1细胞上清液中仅测得少量 MMP-9。MCF-7细胞和 THP-1细胞共培养后,随时间增加,上清液中MMP-9含量明显增强,提示 EMMPRIN很可能是单核/巨噬细胞表达分泌 MMP的强诱导剂。

3　白藜芦醇对分化单核细胞 EMMPRIN和 MMP-9表达的影响

为了明确白藜芦醇对单核/巨噬细胞 EMMPRIN表达的作用,在 PMA诱导 THP-1细胞的同时加入白藜芦醇,细胞分化没有受到影响,而分化细胞EMMPRIN的表达随白藜芦醇浓度增加而显著抑制(图 3A)。此外,白藜芦醇同样显著抑制了单核细胞向巨噬细胞分化中 MMP-9的生成(图 3B)。

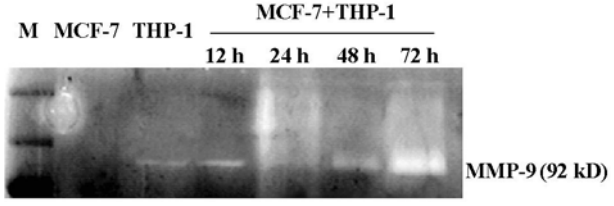

Figure 2　Co-cultured with MCF-7 cells greatly increased MMP-9 production by THP-1 cells. MCF-7 and THP-1 cells were either cultured alone or co-cultured in non-serum medium for 72 h. Medium from co-cultured cells at 12, 24, 48 and 72 h as well as medium from singly cultured cells were assayed by zymography for MMP-9 activity. M: Marker

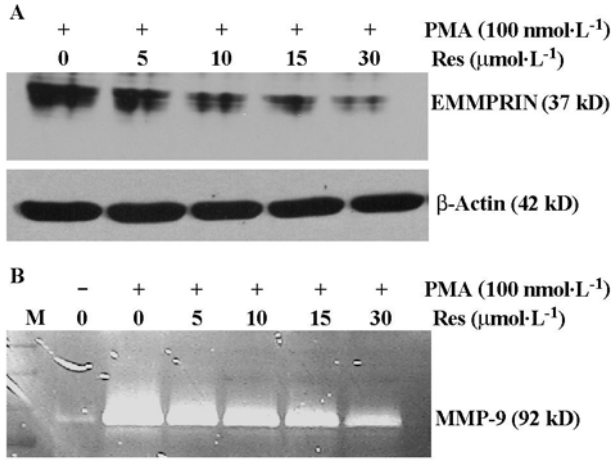

Figure 3　EMMPRIN and MMP-9 expressions were both significantly inhibited by resveratrol (Res). THP-1 cells were cultured in presence or absence of PMA, plus increasing concentrations of resveratrol for 48 h. EMMPRIN expression was assayed by Western blotting (A). MMP-9 gelatinolytic activity was assayed by zymography (B). M: Marker

4　吡格列酮对分化单核细胞中 EMMPRIN表达的影响

为了测定激活的 PPARγ对单核/巨噬细胞EMMPRIN表达的作用,在 PMA诱导 THP-1细胞同时加入 PPARγ的激动剂吡格列酮,发现吡格列酮剂量依赖性地显著抑制了 EMMPRIN的表达,而使用 PPARγ抑制剂 GW9662预处理细胞 1 h后,吡格列酮的抑制作用明显降低(图 4)。

5　白藜芦醇对 PPARγ的作用

为了明确白藜芦醇是否对 PPARγ具有激动作用,在 U937细胞中同时转染 PPARγ的表达质粒和编码虫荧光素酶的激活 PPARγ报告质粒。报告质粒的启动子上含有 3个 PPARγ结合序列,其表达虫

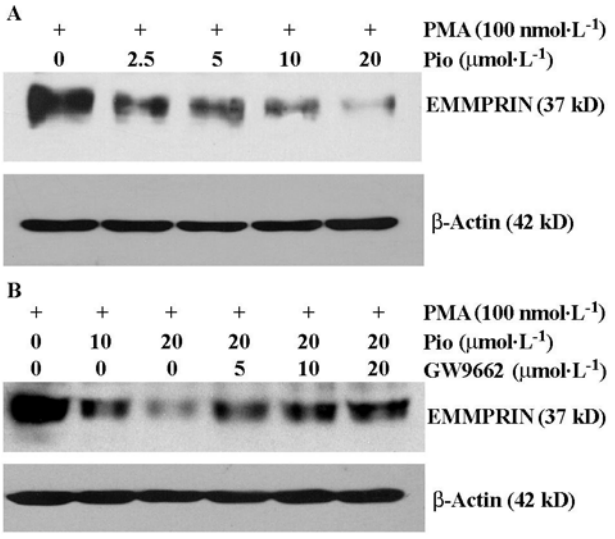

Figure 4　Pioglitazone (Pio) greatly inhibited expression of EMMPRIN of PMA induced THP-1 cells. THP-1 cells were induced by PMA (100 nmol· L^{-1}) for 48 h, in the presence of different concentrations of pioglitazone, EMMPRIN expression was assayed by Western blotting (A). PMA induced THP-1 cells were pretreated with increasing concentrations of GW9662 for 1 h before culturing with pioglitazone for 48 h (B). EMMPRIN expression was assayed by Western blotting

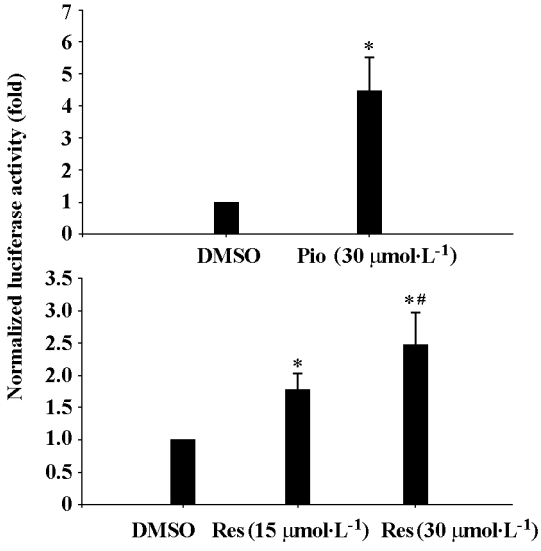

Figure 5　Resveratrol (Res) significantly activated PPARγ. U937 cells were co-transfected with PPARγ expression and reporter vector. Transfected cells were cultured with DMSO, 30 mol· L^{-1} pioglitazone (Pio), 15 or 30 μmol· L^{-1} resveratrol for 24 h and then lysated for reporter luciferase activity assay. Data from control group, which was treated with DMSO, were set as 1 and data from other groups were expressed as fold inductions to the control group. Means of four independent experiments were shown and assayed by *t*-test. * $P < 0.05$ *vs* DMSO treated control group; $^{\#}$ $P < 0.05$ *vs* resveratrol (15 μmol· L^{-1}) treated group

荧光素酶的程度由 PPARγ控制,因而,转染细胞中虫荧光素酶的含量代表了细胞中 PPARγ的激活程度。如图 5,加入 DMSO的转染细胞仅表达少量的虫荧光素酶;吡格列酮(30 μmol· L^{-1})则显著激动了 PPARγ,使加入虫荧光素酶底物的细胞裂解液荧光强度与 DMSO组相比增加 4.48倍($P < 0.001$),15和 30 μmol· L^{-1}白藜芦醇分别使荧光强度提高 1.78倍($P = 0.033$)和 2.47倍($P = 0.01$)且与剂量相关(15与 30 μmol· L^{-1}比较 $P = 0.04$);而在单独转染 PPARγ报告质粒的细胞中,吡格列酮、白藜芦醇和 DMSO都未能使荧光增强。

6　PPARγ抑制剂对白藜芦醇抑制 EMMPRIN作用的影响

为了进一步证明白藜芦醇通过激动 PPARγ发挥抑制 EMMPRIN的作用,用 GW9662预处理 THP-1细胞 1 h后加入白藜芦醇和 PMA(图 6)。白藜芦醇抑制分化细胞 EMMPRIN表达的作用被显著减弱。与之对应的是,白藜芦醇对分化细胞 MMP-9生成的抑制作用也明显下降(图 7)。

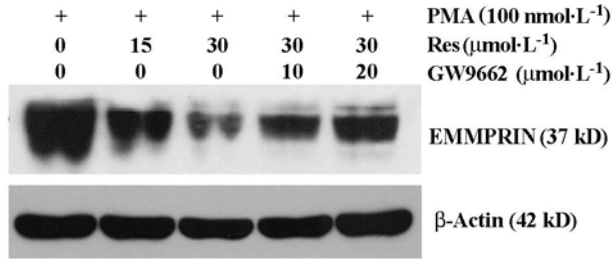

Figure 6　PPARγ antagonist decreased inhibitory effect of resveratrol (Res) on EMMPRIN expression. PMA induced THP-1 cells were pretreated with 10 or 20 μmol· L^{-1} GW9662 for 1 h, then cultured with resveratrol for 48 h. EMMPRIN expression was assayed by Western blotting

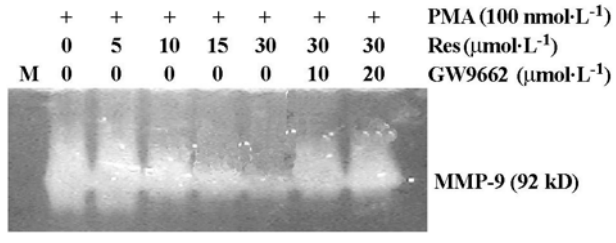

Figure 7　PPARγ antagonist decreased inhibitory effect of resveratrol (Res) on MMP-9 level. PMA induced THP-1 cells were cultured with different concentrations of resveratrol for 48 h, with or without pretreatment by 10 or 20 μmol· L^{-1} GW9662 for 1 h. MMP-9 level in culture medium was assayed by zymography. M: Marker

讨论

多种细胞因子如肿瘤坏死因子、某些白介素等参与了单核/巨噬细胞内 MMPs的转录前和转录后调控[3~5],然而确切的机制依然模糊。肺癌、乳腺癌、皮肤癌等肿瘤细胞及肿瘤组织内的成纤维细胞表面高度表达 EMMPRIN蛋白,通过旁分泌和自分泌的作用,诱导成纤维细胞大量表达 MMPs[6]。使用反义核苷酸抑制 EMMPRIN基因转录后,成纤维细胞 MMPs表达显著减少[7],表明 EMMPRIN是肿瘤组织内上调 MMPs的主要因子。在人类动脉粥样斑块中,EMMPRIN也高度表达且与巨噬细胞分布一致[8,9];培养的人外周血单核细胞中加入炎症因子或氧化低密度脂蛋白后,同时促进了 EMMPRIN和 MMPs(MMP-1和 MMP-9)的表达[9,10]。而在本实验中,单核细胞向巨噬细胞分化过程中 EMMPRIN表达大大增强;与高表达 EMMPRIN的细胞共同培养,显著增强了单核细胞 MMP-9(降解纤维帽作用最强的 MMP之一)的产生。这些结果皆表明:EMMPRIN可能同样参与调控粥样斑块内单核/巨噬细胞表达 MMPs。

流行病学调查发现红葡萄酒可降低冠心病发病率,含有高浓度白藜芦醇被认为是其主要原因。白藜芦醇(resveratrol,3,4,5-三羟基二苯乙烯)是存在于花生、葡萄等植物中的一种多酚类物质。动物实验中,白藜芦醇可以显著减少模型动物粥样斑块的大小[11],缩小冠脉结扎后引起的心梗面积及心功能损害[12],表现出很强的心脏保护作用。分子生物学实验表明,白藜芦醇在巨噬细胞等动脉粥样硬化相关细胞中发挥多种抗氧化和抗炎症作用[13~17],理论上可以通过减少斑块局部氧化脂质生成、炎症细胞的浸润以及炎性细胞因子的产生而防止血管的进一步损害。最近发现,白藜芦醇可以显著抑制小鼠巨噬细胞 MMP-9的产生[18],提示其可能具有稳定粥样斑块的作用。在本实验中,白藜芦醇同样抑制人类单核细胞分化的巨噬细胞产生 MMP-9,与之对应的是,巨噬细胞 EMMPRIN蛋白的表达也显著降低。MMP-9和 EMMPRIN的同向变化不但进一步证明 EMMPRIN是促进巨噬细胞表达 MMPs的重要因子,而且首次提示白藜芦醇抑制巨噬细胞 MMP产生的机制与其减少 EMMPRIN表达有关。

令作者感兴趣的是,许多白藜芦醇的药理学效应和 PPARγ介导的作用非常相似。PPARγ是一种配体激动的细胞核内转录因子,被配体激活后与靶基因启动子上特异性序列结合,从而调控基因的转录。PPARγ在心血管系统内有重要的抗炎、抗氧化作用,其配体激动剂噻唑烷二酮类(TZD)药物能显著抑制巨噬细胞表达包括 MMPs在内的多种炎性细胞因子[19~21]。因而,作者推测白藜芦醇对 EMMPRIN的抑制作用可能与激动 PPARγ相关,实验中发现,和白藜芦醇一样,TZD类药物吡格列酮可以明显抑制巨噬细胞 EMMPRIN的表达,而 PPARγ的拮抗剂 GW9662则阻止了这一作用。细胞共转染激动实验表明,白藜芦醇能够明显激动 PPARγ,其激动能力在本研究中相当于吡格列酮的 60%。最后,GW9662预处理细胞后,白藜芦醇对巨噬细胞 EMMPRIN和 MMP-9表达的抑制作用又一次同时被显著减弱,这些结果表明:PPARγ对 EMMPRIN的基因起到转录抑制作用,而白藜芦醇则通过激动单核/巨噬细胞内的 PPARγ来抑制 EMMPRIN表达,进而减少 MMP的产生,可能是其防治冠心病的药理机制之一。

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