Induction of heme oxygenase 1 and inhibition of TNF-α-induced ICAM-1 expression by andrographolide in EA.hy926 cells

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中文摘要

穿心蓮內酯(Andrographolide)是穿心蓮中含量最多的二萜類，穿心蓮是東南亞國家廣為流傳的傳統中草藥，常用於治療感染、發炎、感冒、發燒和腹瀉。血基質氧化酶 Heme oxygenase (HO)-1 是一個壓力反應的基因，也是抗氧化酵素的一種。過去文獻指出 HO-1 會藉由產生 bilirubin 和減少細胞內游離鐵而抑制內皮細胞黏著分子表現。ICAM-1 是一種發炎生理指標，會使單核球黏附至內皮細胞上进而滲入細胞。在本研究中，我們探討在 EA.hy926 內皮細胞中，穿心蓮內酯對 TNF-α 誘發 ICAM-1 表現的影響以及可能參與的機制。隨著處理劑量增加(0-7.5 μM)，穿心蓮內酯抑制 TNF-α 所誘發的 ICAM-1 表現及內皮細胞與白血球的黏附作用，具有顯著的抑制效果(\(P<0.05\))。同時，處理穿心蓮內酯誘發 HO-1 表現呈現劑量效應。穿心蓮內酯會增加轉錄因子 Nrf2 的核轉移作用和誘導 antioxidant response element (ARE)-luciferase reporter 活性。利用轉染 HO-1 specific small interfering RNA (siRNA) 壓制細胞中 HO-1 表現，顯著破壞穿心蓮內酯抑制 ICAM-1 的表現。這些結果顯示，穿心蓮內酯藉由增加 Nrf2 細胞核轉移、誘發 ARE-luciferase reporter 活性而刺激 HO-1 表達，進而抑制 TNF-α 所誘發的 ICAM-1 表現。

關鍵詞：穿心蓮內酯‧EA.hy926 cells‧血基質氧化酶‧細胞間黏著分子‧腫瘤壞死因子
Andrographolide is the most abundant diterpene lactone in Andrographis paniculata, which is widely used as a traditional medicine in Southeast Asia. Heme oxygenase 1 (HO-1) is an antioxidant enzyme encoded by a stress-responsive gene. HO-1 has been reported to inhibit the expression of adhesion molecules in vascular endothelial cells (EC). Intercellular adhesion molecule (ICAM-1) is an inflammatory biomarker that is involved in the adhesion of monocytes to EC. In this study, we investigated the effect of andrographolide on the expression of ICAM-1 induced by tumor necrosis factor R (TNF-α) in EA.hy926 cells and the possible mechanisms involved. Andrographolide (2.5-7.5 μM) inhibited the TNF-α-induced expression of ICAM-1 in a dose-dependent manner and resulted in a decrease in HL-60 cell adhesion to EA.hy926 cells (p < 0.05). In parallel, andrographolide significantly induced the expression of HO-1 in a concentration-dependent fashion (p < 0.05). Andrographolide increased the rate of nuclear translocation of nuclear factor erythroid 2-related 2 (Nrf2) and induced antioxidant response element-luciferase reporter activity. Transfection with HO-1-specific small interfering RNA knocked down HO-1 expression, and the inhibition of expression of ICAM-1 by andrographolide was significantly reversed. These results suggest that stimulation of Nrf2-dependent HO-1 expression is involved in the suppression of TNF-α-induced ICAM-1 expression exerted by andrographolide.

Key words: Andrographolide; EA.hy926 cells; heme oxygenase 1; ICAM-1; TNF-α
第一部分

前言

根據衛生署統計，心臟疾病與腦血管疾病一直高居國內十大死因的第二、三名，心血管疾病本身就是一種發炎反應，動脈粥狀硬化更是導致心血管疾病的重要病因，黏著分子的表現對於動脈硬化疾病的發展扮演重要角色，因此了解動脈粥狀硬化形成並加以預防是目前預防醫學重要的課題之一。在開發中或已開發中國家使用傳統中草藥的人口比例或市場金額都逐年上升，加上現代人注重健康養生，認為中草藥應用在膳食和疾病療養上會有不錯效果，因此，中草藥在飲食及醫學領域也逐漸受到西方國家的重視。

穿心蓮，拉丁學名為 Andrographis paniculata (Burm. F.) Nees，主要分布於東南亞熱帶地區，而中國大陸與台灣各地也有零星栽培，穿心蓮味苦性寒，具清熱解毒、涼血及消腫止痛之效，過去研究發現，穿心蓮具有廣泛的生理功能，包括抗發炎、抗腹瀉、抗病毒、保護肝臟、抗癌等。因此本研究將探討穿心蓮對於內皮細胞的抗發炎效果及其機制。
文獻回顧

一. 動脈粥狀硬化 (Atherosclerosis)

1. 動脈粥狀硬化的成因

心臟疾病與腦血管疾病一直居國內十大死亡原因的第二、三名，根據衛生署統計，九十八年的心臟與腦血管疾病分別占所有死亡原因的 10.6%及 7.3% (行政院衛生署, 2010)。動脈粥狀硬化是一種慢性發炎疾病，會造成血管內壁脂肪堆積及纖維斑塊形成，是導致心血管疾病的主要原因之一（de Winther et al., 2005）。Ross (1999) 提出動脈粥狀硬化在致病初期血管內壁的內皮細胞有受損現象，造成內皮細胞受損的原因包括: 抽菸、高血壓、高膽固醇飲食、糖尿病等。動脈粥狀硬化的致病機轉十分複雜，主要是因為血漿中低密度脂蛋白 (low-density lipoprotein, LDL) 濃度的增加，高低密度脂蛋白濃度會增加轉變成氧化型低密度脂蛋白 (oxidized-low-density lipoprotein, ox-LDL) 的機會，當血管壁內層長期受到大量的氧化型低密度脂蛋白刺激時，會使內皮細胞的通透性增加，促進前發炎細胞激肽或趨化因子 (chemokines) 的活化與黏著分子表現，進而吸引循環中的白血球包括單核球黏附至內皮細胞，單核球會進一步穿透血管壁，增加對 ox-LDL 的攝入 (uptake) 而分化成巨噬細胞，此階段稱為血管內膜發炎。接著，巨噬細胞會吞噬大量的 ox-LDL，形成泡沫細胞 (foam cell)，進而在血管內膜形成脂肪紋 (fatty streak)，這種富含脂質的脂肪紋除了巨噬細胞也含有膠原蛋白及蛋白多醣，這些脂肪條紋會緩慢的形成纖維斑塊 (fibrous plaque)。纖維斑塊會分泌生長因子及細胞激素使得斑塊增厚，造成斑塊表面纖維化，形成更緊密的粥狀物，而內部堆積更多壞死的細胞與脂質，進而使斑塊往周圍蔓延。動脈粥狀硬化晚期，纖維性的胞外間質被分解導致斑塊不穩定而破裂，形成血栓，增加心肌梗塞或是腦中風發生率（de Winther et al., 2005; Dzau et al., 2002)。
圖 2-1. 動脈粥狀硬化形成過程 (de Winther et al., 2005)

圖 2-2. 血管腔內動脈粥狀硬化進展剖面圖 (Rader and Daugherty, 2008)
2. Cell黏著分子與動脈粥狀硬化的關係：白血球徵募 (leukocyte recruitment)

細胞黏著分子表現與動脈粥狀硬化的初期關係密切。動脈粥狀硬化的早期階段涉及到發炎細胞會從血液循環中徵募 (recruitment) 並遷移至內皮細胞發炎處。此現象是經由一連串的白血球與血管壁內皮細胞黏附作用所調控，主要包括下列步驟 (圖 2-3)：(1) 滾動 (rolling)：當組織受到損害時，會大量表現選擇素 (selectin)，與循環中的白血球產生交互作用，白血球進而在血管壁上滾動，這項作用是由白血球的 L-selectin 和血管內皮細胞上的 P-selectin、E-selectin 所調控；

(2) 黏著 (adhesion)：受傷的組織會釋放趨化因子來活化滾動中的白血球，使它們停止滾動並黏附至內皮細胞上，此階段的血管內皮細胞黏著分子 (ICAM-1、VCAM-1) 會受到細胞激素 (如：TNF-α) 的活化而增加其表現，細胞激素同時也會活化 integrin，活化的 integrin (β1、β2) 則會增強白血球與血管內皮細胞結合的力量；

(3) 遷移 (transmigration)：白血球黏附至內皮細胞後會開始變形，在這個步驟中，參與的黏著分子 PECAM-1 會使白血球的型態產生改變進而穿透血管內皮細胞層，從血管內遷移至血管外的發炎組織處，促使後續動脈粥狀硬化的形成 (Blankenberg et al., 2003；Price and Loscalzo, 1999)。

![](image)

圖 2-3. 白血球黏附作用的連續步驟 (Blankenberg et al., 2003)
細胞黏著分子

在動脈粥狀硬化初期，研究發現細胞黏附的過程中，有許多細胞表面分子參與，表2-1為各種黏著分子參與動脈粥狀硬化初期的過程(Galkina and Ley, 2007)。根據Gene Ontology Consortium定義，細胞黏著分子為表現於細胞表面之分子且能讓細胞黏著到其他細胞或胞外基質的分子，這些細胞黏著分子(cellular adhesion molecules, CAMs)依其不同的結構特性可分為以下三大類(表2-2):分別為選擇素(selectins)、類免疫球蛋白分子(immunoglobulin-like molecules)及整合素(integrins) (Blankenberg et al., 2003; Danese et al., 2005)。

2-1-1 選擇素(selectin)

選擇素是一個含有三個不同醣蛋白(glycoproteins)的結構，其蛋白的N端有一個類似lectin的區域，在血管層內扮演調控細胞和細胞間黏附作用的角色，主要三個成員，分別以發現細胞的位置來命名，例如表現在白血球表面的L-selectin (CD62L)、表現於內皮細胞的E-selectin (CD62E)及儲存在血小板內的P-selectin (CD62P)(Blankenberg et al., 2003)。

L-selectin表現在所有的白血球細胞，文獻指出若淋巴球上的L-selectin產生缺陷，會減少50%淋巴球在主動脈上的移行作用，另外，淋巴球移行到主動脈的粥狀硬化處也受到L-selectin的調控(Galkina et al., 2006)。L-selectin的配體(ligands)是含有碳水化合物的分子，PSGL-1是L-selectin的配體之一，當PSGL-1與L-selectin結合時，會讓循環中的白血球與在內皮細胞上滾動的白血球產生交互作用，造成secondary capture(圖2-4)。因此，研究認為在白血球徵募到動脈血管內皮發炎處可能是透過L-selectin和PSGL-1的交互作用產生secondary capture來達成(Eriksson, 2001)。
E-selectin 只表現在內皮細胞，當內皮細胞受到發炎激素刺激時，例如: tumor necrosis factor (TNF)-α、interleukin (IL)-1 或 platelet factor 4 (PF4)，則會誘發 E-selectin 表現，人類動脈粥狀硬化的內皮細胞處，也會表現 E-selectin (Yu et al., 2005)。

P-selectin主要儲存於血小板內，在生物體中，動脈粥狀硬化的斑塊內可以檢測到P-selectin (Johnson et al., 1994)。為了瞭解P-selectin在白血球徵募至動脈粥狀硬化過程中所扮演的角色，利用頸動脈的體外模式，在 knockdown P-selectin或PSGL-1的 Apoe^{-/-}小鼠模式中發現，單核球滾動和黏附至頸動脈內皮細胞的速度明顯降低。因此推論在單核球的黏附過程中，P-selectin及PSGL-1的相互作用扮演重要的角色(Ramos et al., 1999)。

2-1-2 類免疫球蛋白分子 (Immunoglobulin-like molecules)

Immunoglobulin (Ig) superfamily 細胞黏著分子表現於細胞表面的醣蛋白，包括 ICAM-1, ICAM-2, VCAM-1, platelet–endothelial cell adhesion molecule (PECAM)-1 and the mucosal addressin MAdCAM-1(表 2-2)。
Intercellular adhesion molecules (ICAMs)為此superfamily中的一員，ICAMs含有五個成員，ICAM-1 表達在白血球細胞、纖維細胞、上皮細胞及內皮細胞，具有增加白血球黏附作用，ICAM-2 受到發炎物質的負向調控，表現於白血球、血小板與內皮細胞，ICAM-3 除了表現在內皮細胞及白血球細胞外，也是ICAM分子中唯一表現在嗜中性白血球的黏著分子。另外，ICAM-4 表現於紅血球，ICAM-5 則表現在大腦(Briscoe et al., 1992；Blankenberg et al., 2003)。這些ICAM分子的配體主要包括白血球特異性β2整合素，每個ICAM分子和ligand間的結合，促進白血球的黏附作用，ICAM-1、2、3 可加強白血球牢固的黏附於血管表面(Blankenberg et al., 2003)。

ICAM-1 (CD54)是一個跨膜醣蛋白，含有 505 個胺基酸，因不同程度的醣基化修飾，其分子量介於 80-114 kDa 之間，主要和表現於白血球表面的整合素 LFA-1 結合，ICAM-1 表現於許多少細胞表面，包括上皮細胞、角質細胞、肝臟細胞、淋巴細胞及纖維母細胞，這些細胞平時只會表現少量的 ICAM-1，但是當受到促發炎細胞激素(TNF-α、IL-1、IL-6 及 interferon-γ 等)、氧化壓力、病毒感染等刺激後，會大量增加 ICAM-1 的表現(表 2-3) (Roebuck and Finnegan, 1999)。ICAM-1 對於白血球穿透血管內層的移行作用扮演重要角色，同時也與許多發炎疾病發生有關，除了動脈粥狀硬化外，還包括發炎性腸道疾病(Fiocchi, 1998)及氣喘等疾病(Stanciu and Djukanovic, 1998)。

人類 ICAM-1 基因為位於第十九號染色體上，包括七個 exons 無六個 introns (Voraberger et al., 1991)。ICAM-1 promoter 上有許多特定轉錄因子的結合位，包括 Sp1、NF-κB、AP-1、AP-2、interferon-stimulated response element (ISRE 或稱為 STAT) ，因此 ICAM-1 表現會受到許多轉錄因子的調控 (Roebuck and Finnegan, 1999)。細胞內調控 ICAM-1 基因表現的訊息傳遞路徑包括 MAPK/AP-1 (Karin, 1996)、IKK/NF-κB (Mercurio et al., 1997)、PKC (Sippy et al., 1996)、JAK/STAT (Song et al., 1997; Look et al., 1995)。

以 NF-κB 訊息傳遞路徑為例，TNF-α 和 IL-1β 都會透過活化 NF-κB 來調控
ICAM-1 表現，NF-κB 以 Rel A 的 homodimers 或 heterodimers 形式結合至 NF-κB 的結合區域，啟動 ICAM-1 基因表現(圖 2-5) (Roebuck and Finnegam, 1999)。

Vascular cell adhesion molecule 1 (VCAM-1) 是一個分子量 110 Kda 的蛋白質，主要表現在內皮細胞、巨噬細胞、肌肉細胞及樹突狀細胞。VCAM-1 表現於活化的內皮細胞時，會藉由和白血球表面的整合素α4β1 (very late antigen 4, VLA-4) 或α4β7 結合來促使白血球滲出血管壁，特別是單核球、嗜鹼性球、嗜酸性球以及淋巴球需要有 VCAM-1 來產生黏附作用(Zapolska et al., 2001)。

另外，PECAM-1 也是 Ig family 成員之一，主要表現在白血球、血小板與內皮細胞。PECAM-1 在內皮細胞連接處有密集的表現量，會參與細胞和細胞間的鍵結，與內皮細胞完整性及白血球滲出血管壁有關(Blankenberg et al., 2003)。
表 2-1. 黏著分子參與動脈粥狀硬化的白血球徵募作用（Galkina and Ley, 2007）

<table>
<thead>
<tr>
<th>Migration Step</th>
<th>Adhesion Molecules</th>
<th>Model</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tethering, rolling</td>
<td>L-selectin</td>
<td><em>Sel</em>&lt;sup&gt;T&lt;/sup&gt; vs C57BL/6 and <em>Apoe</em>&lt;sup&gt;T&lt;/sup&gt; lymphocytes (adoptive transfer)</td>
<td>50% reduction in lymphocyte homing</td>
</tr>
<tr>
<td></td>
<td>P-selectin</td>
<td>C57BL/6 and <em>Apoe</em>&lt;sup&gt;T&lt;/sup&gt; mice on western diet</td>
<td>Reduced rolling and attachment with anti-P-selectin or PSGL-1 Abs</td>
</tr>
<tr>
<td></td>
<td>E-selectin</td>
<td><em>Sele</em>&lt;sup&gt;T&lt;/sup&gt; and <em>Apoe</em>&lt;sup&gt;T&lt;/sup&gt; double Knockout(DKO) mice</td>
<td>Slightly reduced lesion area</td>
</tr>
<tr>
<td>Adhesion</td>
<td>VCAM-1</td>
<td>C57BL/6 and <em>Apoe</em>&lt;sup&gt;T&lt;/sup&gt; mice on western diet</td>
<td>Increased rolling velocity with anti-VLA-4 or VCAM-1 Abs</td>
</tr>
<tr>
<td></td>
<td>VLA-4</td>
<td><em>Apoe</em>&lt;sup&gt;T&lt;/sup&gt; mice on western diet</td>
<td>Decrease in neointimal growth and neutrophil and Mψ recruitment with anti-VLA-4 Abs</td>
</tr>
<tr>
<td></td>
<td>ICAM-1</td>
<td><em>Icam1</em>&lt;sup&gt;T&lt;/sup&gt; mice (on western diet)</td>
<td>Slightly decreased lesion size compared to C57BL/6 controls</td>
</tr>
</tbody>
</table>
表2-2. 黏著分子及其配体（Danese et al., 2005）

Selectin, integrin and immunoglobulin superfamily molecules

<table>
<thead>
<tr>
<th>Adhesion molecules</th>
<th>Localisation</th>
<th>Ligated</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Selectin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Selectin</td>
<td>All leucocytes</td>
<td>GlyCAM, MadCAM, P-, E-selectin</td>
<td>Rolling</td>
</tr>
<tr>
<td>E-Selectin</td>
<td>Endothelium</td>
<td>L-Selectin</td>
<td>Rolling</td>
</tr>
<tr>
<td>P-Selectin</td>
<td>Endothelium, platelets</td>
<td>L-Selectin PSLG-1</td>
<td>Rolling</td>
</tr>
<tr>
<td><strong>Integrin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11a/CD18</td>
<td>All leucocytes</td>
<td>ICAM-1, -2</td>
<td>Adhesion</td>
</tr>
<tr>
<td>CD11b/CD18</td>
<td>Neutrophils, monocytes</td>
<td>ICAM-1</td>
<td>Adhesion</td>
</tr>
<tr>
<td>CD11c/CD18</td>
<td>Neutrophils, monocytes</td>
<td>?</td>
<td>Adhesion</td>
</tr>
<tr>
<td>α4β1</td>
<td>Lymphocytes, monocytes</td>
<td>VCAM-1</td>
<td>Adhesion</td>
</tr>
<tr>
<td>α4β7</td>
<td>Lymphocytes, monocytes</td>
<td>VCAM-1 MadCAM-1</td>
<td>Adhesion</td>
</tr>
<tr>
<td><strong>Immunoglobulin superfamily</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Endothelium, monocytes</td>
<td>CD11a/CD18</td>
<td>Adhesion</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>Endothelium</td>
<td>CD11a/CD18</td>
<td>Adhesion</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Endothelium</td>
<td>α4β1</td>
<td>Adhesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α4β7</td>
<td>Adhesion</td>
</tr>
<tr>
<td>MadCAM-1</td>
<td>Endothelium</td>
<td>α4β7</td>
<td>Adhesion</td>
</tr>
<tr>
<td><strong>Chemokine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractalkine</td>
<td>Endothelium</td>
<td>α4β7</td>
<td>Adhesion</td>
</tr>
</tbody>
</table>

![Medicall MEDICAL UNIVERSITY](image)
表 2-3. 不同类型的细胞可受到不同刺激而引发ICAM-1的表

(Reobuck and Finnegan, 1999)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cell</td>
<td>TNF-α, IL-1, IL-6, thrombin, X-ray, PDTC, IFN-γ, endothelin-1, substance P, estradiol, shear stress, UV, TPA, LPS, measles virus oxidized LDL, H₂O₂, metal ions</td>
</tr>
<tr>
<td>Epithelial cell</td>
<td>TNF-α, IL-1, LPS, TPA, histamine, EBV, CMV, RSV, parainfluenza virus, rhinovirus</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>TNF-α, IL-1, IL-4, IFN-γ, retinoic acid, mycoplasma, PGE₂</td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>TNF-α, histamine</td>
</tr>
<tr>
<td>Hepatocyte</td>
<td>TNF-α, IL-1, IFN-γ, IL-6</td>
</tr>
<tr>
<td>Leukocyte</td>
<td>TNF-α, IL-1, IFN-γ, IL-3, GM-CSF, TPA</td>
</tr>
<tr>
<td>Smooth muscle cell</td>
<td>TNF-α, PDGF</td>
</tr>
</tbody>
</table>

圖 2-5. 細胞激素調控ICAM-1的訊息傳遞路徑(Roebuck and Finnegan, 1999)
2-1-3 整合素 (integrins)

整合素家族含有 24 個次家族，為 αβ 異質二聚體 (heterodimer)，由 18 種 α 次單元與 8 種 β 次單元所組成，藉由不同的組合配對可得到 24 種的 integrin (表 2-4)(van der Flier and Sonnenberg, 2001)，其主要功能為形成細胞間或胞外基質黏著的橋梁，調控細胞與細胞、細胞與胞外基質及細胞與病原體的接觸(Galkina and Ley, 2007)。LFA-1 (αLβ2) (lymphocyte function-related antigen) 是細胞表面抗原，會表現在所有的白血球，也稱為CD11a/CD18， 屬於整合素的β2次家族; VLA-4 (α4β1) 整合素是α4的次家族成員，主要表現在單核球及淋巴球，另外，VLA-4 也會與免疫球蛋白家族的VCAM-1 及淋巴球細胞相互作用並且黏附至內皮細胞發炎處(Danese et al., 2005)。
### Table 2-4. Integrins and their Ligands (van der Flier and Sonnenberg, 2001)

<table>
<thead>
<tr>
<th>Integrin</th>
<th>ECM</th>
<th>Soluble</th>
<th>Cell-cell</th>
<th>Pathogens/toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁β₁</td>
<td>Co; Ln</td>
<td>MMP-1</td>
<td></td>
<td>Echovirus 1,8; Rotavirus; leech (rLAP)</td>
</tr>
<tr>
<td>α₂β₁</td>
<td>Co; Ln; Chad</td>
<td></td>
<td></td>
<td><em>Yersinia</em> spp. (invasin)</td>
</tr>
<tr>
<td>α₅β₃</td>
<td>Fn; Fn; Tsp-1 (Co; Fn)</td>
<td>(α₅β₃; α₅β₃)</td>
<td>VCAM-1; (α₅)</td>
<td>Rotavirus</td>
</tr>
<tr>
<td>β₅</td>
<td>Fn</td>
<td>VCAM-1; MAcAM; (α₅)</td>
<td></td>
<td><em>Yersinia</em> spp. (invasin); rotavirus</td>
</tr>
<tr>
<td>α₅β₄</td>
<td>Fn</td>
<td>tTG; endostatin</td>
<td>ADAM-15,17; L1</td>
<td><em>Yersinia</em> spp.; <em>B. burgdorferi</em>; <em>Shigella</em> spp. (ipa); <em>B. pertussis</em> (fimD); foot-and-mouth disease virus</td>
</tr>
<tr>
<td>α₆β₁</td>
<td>Ln</td>
<td>Fisp12-mCTGF; Cyr61</td>
<td>ADAM-2,9</td>
<td>Papilloma virus; <em>Yersinia</em> spp. (invasin)</td>
</tr>
<tr>
<td>β₄</td>
<td>Ln</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₇β₁</td>
<td>Ln</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₇β₁</td>
<td>Fn; Ln; Nu</td>
<td>TGFβ1-LAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₉β₁</td>
<td>Tn; Op; Co; Ln</td>
<td>pp-β1; tTG; FXIII; angiostatin</td>
<td>VCAM-1</td>
<td></td>
</tr>
<tr>
<td>α₁₀β₁</td>
<td>Co</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁₁β₁</td>
<td>Co</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁β₁</td>
<td>Fn; Vn</td>
<td>TGFβ1-LAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β₃</td>
<td>Vn; Fn; vWF; Op; Tn; Bsp; Tsp-1</td>
<td>Fg; Cyr61; Fisp12-mCTGF; MMP2; endostatin; angiostatin; tissustatin</td>
<td>ADAM-15, 23; CD31</td>
<td>Parechovirus 1</td>
</tr>
<tr>
<td>α₂β₁</td>
<td>Vn; Bsp</td>
<td>TGFβ1-LAP; Cyr61; endostatin</td>
<td></td>
<td>Snake venoms (disintegrins); adeno virus; rotavirus; foot-and-mouth disease virus; coxsackievirus A9; parechovirus 1; hantaviruses; HIV (tat protein)</td>
</tr>
<tr>
<td>α₂β₁</td>
<td>Fn; Tn</td>
<td>TGFβ1-LAP</td>
<td></td>
<td>HIV (tat protein)</td>
</tr>
<tr>
<td>α₂β₁</td>
<td>Co; Ln; Fn</td>
<td>TGFβ1-LAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₂β₁</td>
<td>Vn; Fn; vWF</td>
<td>Fg; Cyr61; Fisp12-mCTGF; prothrombin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₄β₁</td>
<td>Fg; iC3b; FX</td>
<td>ICAM-1-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₄β₁</td>
<td>Fg; iC3b</td>
<td>ICAM-1; VCAM-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₅β₁</td>
<td>Fg; iC3b</td>
<td>ICAM-1; VCAM-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₅β₁</td>
<td>Fg; iC3b</td>
<td>ICAM-3; VCAM-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₅β₁</td>
<td>Fg; iC3b</td>
<td>E-cadherin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. 腫瘤壞死因子 (tumor necrosis factor-α，TNF-α)

免疫系統是生物體的防禦系統之一，生物體藉由辨別並殺死病原體及腫瘤細胞的過程，來保護宿主免於疾病的傷害。發炎現象是血管組織在傷害刺激下(例如，病原體、受損細胞或刺激物)所產生的一種生物反應(Calder and Kew, 2002)。前發炎細胞激素(pro-inflammatory cytokines)例如：TNF-α、IL-1α、IL-1β、IFN-γ 及 gram-negative bacterial endotoxins (lipopolysaccharide, LPS)等都已被證實可以刺激內皮細胞並增加白血球的黏附作用(表 2-5) (Meager, 1999)。TNF-α 屬於 TNF/TNFR cytokine superfamily 的成員，會引發局部發炎反應，活化的巨噬細胞會分泌 TNF-α，產生活性氧物質(ROS)，另外，TNF-α 也會透過活化轉錄因子 NF-κB 刺激內皮細胞，增加 ICAM-1、VCAM-1、MCP-1 等發炎基因的表現，並促使白血球黏附於內皮細胞上，因此 TNF-α 在動脈粥狀硬化過程中也扮演重要角色 (Meager, 1999)。

### 表 2-5. 細胞激素誘發細胞黏著分子(CAM)的表現 (Meager, 1999)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CAM Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α, TNF-β, IL-1α, IL-1β</td>
<td>E-selectin, P-selectin, L-selectin ligand, ICAM-1, VCAM-1, MadCAM-1 strongly up-regulated.</td>
</tr>
<tr>
<td>IL-4, IL-13</td>
<td>Selective up-regulation of VCAM-1 (synergize with TNF-α); late expression of E-selectin stimulated by TNF-α or IL-1 inhibited.</td>
</tr>
<tr>
<td>IL-10</td>
<td>Potential inhibitor of TNF-α induced ICAM-1 expression.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Weak inducer of ICAM-1 expression, but strong synergy with either TNF or IL-1. Can maintain E-selectin expression levels.</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>E-selectin expression inhibited (additive inhibitory effect with IL-4); up-regulation of PECAM-1.</td>
</tr>
</tbody>
</table>
二. Heme Oxygenase-1 (HO-1)

1. HO-1 介紹

文獻指出血基質氧化酶-1 (HO-1) 對心血管疾病包括動脈粥狀硬化有重要的影響 (Immenschuh and Schroder, 2006)。HO-1為一種具有保護作用的蛋白質，許多研究證實它在內皮細胞、上皮細胞、平滑肌細胞中扮演抗發炎、抗凋亡、抗增生的作用 (Brouard et al., 2000；Lee et al., 1996；Peyton et al., 2002；Tobiasch et al., 2001)。HO-1是血基質代謝的速率限制酵素 (Maines, 2005; Maines and Gibbs, 2005)，它將血基質代謝成膽紅素(biliverdin)、一氧化碳 (CO)和遊離鐵 (free iron)（圖2-6）。HO-1的表現受到氧化壓力的調控，可藉由增加膽紅素和促進CO產生來達到抗發炎和保護細胞的作用 (Ryter et al., 2006)。

圖2-6. 血基質分解路徑 (Farombi and Surh, 2006)
哺乳動物的血基質氧化酶(HO)有三種異構物，分別命名為HO-1、HO-2和HO-3 (Maines, 1988; McCoubrey et al., 1997)。HO-1又稱為熱休克蛋白32 (heat-shock protein 32, Hsp 32)，人類HO-1是由288個胺基酸所組成，分子量為32.8 kDa，其中約80%的胺基酸序列和大鼠HO-1相同(Yoshida et al., 1988)。人類HO-2分子量為36 kDa，由316個胺基酸所組成(Ishikawa et al., 1995)。HO-3分子量為33 kDa，研究指出HO-3對血基質的催化作用不強，且無法受誘發而大量表現(Farombi and Surh., 2006)。相反地，HO-1容易被血基質或其他非血基質物質所誘發，例如：植化物(phytochemicals)，以蘿蔔硫素(sulforaphane)為例，sulforaphane為含硫配醣體(glucosinolate)之水解物，廣存於十字花科蔬菜，如青花菜、甘藍菜及花椰菜等。根據文獻指出，在人類血管內皮細胞(human vascular endothelial cell)，sulforaphane會透過誘發HO-1來抑制lipopolysaccharide (LPS)誘發的發炎損傷(Shan et al., 2010)。另外，ultraviolet (UV)、過氧化氫、重金屬以及一氧化氮也會誘發HO-1 (Motterlini et al., 2000)。

2. HO-1 的生理角色

透過各種刺激，被誘發的HO-1扮演保護的角色。文獻指出，在誘導肝損傷的實驗中，大鼠經過缺血再灌注的損傷模式，HO-1的mRNA及蛋白質表現增加，這和心臟、腎臟及肝臟的器官功能改善具有相關性(Masini et al., 2003)。Pannen等人(1998)證實HO-1在大鼠出血性休克和復甦的模式中具有保護肝臟的作用。近期研究發現，誘發HO-1表現可以保護人類肝臟缺氧所帶來的傷害，同時誘發HO-1表現也被認為可以修復發炎性損傷(Tuzuner et al., 2004)。HO-1在調控肝損傷的角色也同樣被證實，研究發現向上調控HO-1表現可以挽救小鼠肝損傷所造成的細胞凋亡(Sass et al., 2003)。另外，HO-1過度表現也會改善肝臟損傷造成的細胞凋亡及組織發炎反應併發症狀(McCarter et al., 2004)，因此調控HO-1表現可以做為肝臟疾病發炎的治療方式。雖然HO-1對於肝臟保護作用的機制還未完全被釐清，但可
以確定的是和其代謝產物biliverdin/ bilirubin、CO和ferritin有關。

3. HO-1 代謝產物對細胞的保護作用

3-1. Biliverdin and bilirubin

Biliverdin、bilirubin和CO是heme的代謝產物，在過去常被認為是沒有用或有毒的產物，而今它們被認為是重要的內生性抗氧化劑(圖2-6)。在體內及體外試驗中均已證實biliverdin和bilirubin具有抗氧化的活性(Llesuy and Tomaro, 1994)。最近研究顯示，biliverdin對大鼠肝臟局部缺血再灌流所造成的傷害具有保護作用(Fondevila et al., 2004)；10 nM bilirubin也被發現可以回復子宮頸癌細胞(HeLa cells)在200 μM過氧化氫誘導下造成的傷害(Baranano et al., 2002)。HO-1和bilirubin的抗氧化活性在大鼠肝臟暴露於UVA模式中也獲得證實(Ossola and Tomaro, 1998)。另外，在大鼠AH 136B肝癌細胞中，HO-1會透過增加bilirubin濃度、降低細胞內促氧化劑濃度，而達到抗氧化效果(Tanaka et al., 2003; Fang et al., 2004)。

3-2 Carbon monoxide (CO)

一氧化碳(CO)是血基質代謝的副產物之一，在組織損傷時會透過誘導HO-1表現而代謝生成較多的CO，幫助減緩發炎反應。一氧化碳具有抗發炎的保護角色最初是在急性肺損傷的模式中被發現，另外在小鼠心臟移植的模式中也被證實(Sato et al., 2001)。文獻指出，一氧化碳在急性和慢性發炎反應中，具有保護的角色(Moore et al., 2005)，一氧化碳可以抑制前發炎反應並提高巨噬細胞的抗發炎作用(Yachie et al., 1999)。在RAW 264.7鼠科動物的巨噬細胞中，CO被證實會減弱受lipopolysaccharide (LPS)所誘發的免疫反應，並且可調控大鼠肝臟中誘發型一氧化氮合成酵素(inducible nitric oxide synthase) (iNOS)表現和一氧化氮(nitric
oxide) (NO)產生(Sarady et al., 2004) (Sawle et al., 2005)。CO在體內和體外試驗中也被證實會影響其它細胞的增生作用，例如，CO在體內試驗中會抑制平滑肌細胞的增生(Otterbein et al., 2003)。大鼠預處理250 ppm的CO1小時，可以明顯降低球囊損傷後的內膜增生作用(Gerard et al., 1993)。

3-2-1 參與一氧化碳(CO)作用的訊息傳遞路徑
有研究發現 guanylyl cyclase-cyclic (c)GMP 或 p38 mitogen-activated protein kinase (MAPK)在 CO 的抗增生及抗凋亡作用中扮演重要角色。舉例來說，在纖維母細胞和 BTC3 細胞中，藉由誘導 cGMP 的產生來達到 CO 的抗細胞凋亡作用；相反地，在內皮細胞中 CO 則需透過活化 p38 MAPK，才具有抗細胞凋亡的作用(Brouard et al., 2000)。文獻指出，CO 抗平滑肌細胞增生作用和抗血小板凝集作用皆需要 cGMP 和 p38 的參與(Petrache et al., 2000)。CO 會經由活化 p38 和產生 cGMP 的 guanylyl cyclase 來抑制巨噬細胞 IL-10 和 TNF-α 的生成(Yachie et al., 1999)。在一些含有血基質的蛋白質中，有可能透過 CO 來調控訊息傳遞，例如：血紅素(hemoglobin)、肌紅蛋白(myoglobin)、鳥苷酸環化酶(guanylyl cyclase)、環氧合酶(cyclooxygenase)、cytochrome P450 oxidase、inducible nitric oxide synthase (iNOS)等(Otterbein et al., 2003)。CO 會透過與血基質內部構造中的鐵結合，改變這些蛋白質的構形而調控其生理活性，例如，當 CO 與 iNOS 結合時，iNOS 的活性會降低。CO 也會和含有其他金屬離子的蛋白質例如：Zinc(鋅)、Copper(銅)、Manganese(錳)產生結合作用(Otterbein et al., 2003)。

3-3 Fe^{2+} and ferritin
血基質經HO-1催化產生游離鐵，一般來說，Fe^{2+}在低濃度時會被羟自由基(hydroxyl radical, OH)催化而對細胞產生毒性，但有研究顯示，當HO-1被誘發時，鐵蛋白(ferritin)濃度和Fe^{2+}-sequestering protein量會被提升；而當HO-1活性被抑制時，則會減少(Eisenstein et al., 1991)。雖然誘發HO-1而產生ferritin對細胞保護作
用這部分仍有爭議，但最近有些文獻對ferritin和HO-1間的關係皆持認同看法，例如：過氧化氫會對人類內皮細胞(ECV 304)造成毒性，但它也會誘發HO-1和ferritin合成(Grosser et al., 2004)；Gonzales等人(2005)研究證實在大鼠肝臟中鈷氯化物(cobalt chloride)誘發HO-1表現後會增加鐵蛋白和含鐵儲鐵蛋白的濃度；另外，也有文獻證實在非酒精性脂肪肝病症中，HO-1增加和鐵蛋白的濃度有顯著相關性(Malaguanera et al., 2005)。

4. 調控HO-1基因表現的訊息傳遞路徑

胞內調控HO-1基因表現的訊息傳遞路徑如圖2-7所示，例如mitogen activated protein kinases (MAPKs)、phosphatidylinositol 3-kinase PI3K/Akt pathway及protein kinase C(PKC)皆被認為與調控HO-1基因表現有關。MAPKs pathways包含extracellular signal-regulated kinase 1/2 (ERK1/2)、c-Jun-N-terminal kinase (JNK)和p38，它們會透過磷酸化下游轉錄因子而影響標的基因表現。亞砷酸鈉在肝癌細胞株中會透過ERK1/2和p38訊息傳遞路徑來誘發HO-1基因轉錄(Elbirt et al., 1998)。最近研究證實當angiotensin II誘發HO-1表現及增加CO釋放時，PKC參與angiotensin II (Ang II)調控HO-1基因表現(Li et al., 2004)。此外與HO-1表現有關的訊息傳遞路徑還包括protein kinase A (PKA)，舉例來說，賀爾蒙和外在刺激會增加胞內cAMP濃度進而活化PKA，Immenschuh等人(1998)研究發現，大鼠初代肝細胞給予PKA活化劑，例如Bt2cAMP和glucagon會誘發HO-1表現，並呈現劑量關係。另外，活性氧分子被證實會透過PI3K/Akt訊息傳遞路徑來誘發大鼠腎上腺嗜铬細胞(pheochromocytoma 12 cell ) HO-1表現(Salinas et al., 2003)。另外也有研究指出，在人類乳腺癌細胞(MCF-7)中，15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2)會透過PI3K/Akt訊息傳遞路徑來誘發HO-1基因表現(Kim et al., 2004)。在人類神經母細胞(SH-SY5Y)實驗中證實，HO-1基因表現是透過PI3K調控nuclear factor E2-related factor-2 (Nrf2)來達成(Nakaso et al., 2003)。
5. 參與 HO-1 表現的轉錄因子

目前文獻已證實 activator protein (AP)-1、NF-κB、Nrf2 等轉錄因子會參與調控 HO-1 基因表現 (Farombi and Surh, 2006)。

* Activator protein (AP)-1

AP-1 是一個由 fos、Jun 或 ATF 家族所組成的 homodimer 或 heterodimer，藉由和基因啓動區結合啓動基因轉錄作用。透過活化 AP-1 可提升 HO-1 表現，細胞激素、生長因子、氧化壓力及細菌和病毒感染等外來刺激會活化 AP-1 進而刺激基因表現，AP-1 同時也參與細胞分化、增生和凋亡過程 (Karin et al., 1997)。有研究證實在外來刺激下，例如：血基質、亞砷酸鈉、氯化鈷和 cobalt protoporphyrin (CoPP)，它們誘發 HO-1 表現是透過 AP-1 轉錄因子 (Shan et al., 2004) (Lu et al., 2000) (Shan et al., 2004)。

* Nuclear factor E2-related factor-2 (Nrf2)
Nrf2是一個分子量66-kDa的蛋白質，屬於basic region-leucine zipper (bZIP)家族的轉錄因子之一，對活化帶有antioxidant response element (ARE)的基因表現扮演調控者的角色(Mann et al., 2007) (圖2-8)。

ARE位於許多phase II解毒酵素(phase II detoxifying enzymes)與抗氧化酵素的promoter中，例如: HO-1、NQO1、glutathione S-transferase (GST)。Nrf2有六個domains，從Neh 1到Neh 6 (Nrf2-ECH homology)，在C端終點，Nrf2有一段basic leucine zipper structure；在N端終點，Nrf2有一個高活化區的domain，Neh 2，Keap-1和Nrf2結合時，是結合至Nrf2上的Neh 2 domain (Itoh et al., 2004) (圖2-9)。在C端位置上的basic leucine zipper(bZip)，bZip區域中的basic region主要負責與DNA結合，而leucine zipper則是會與其它的bZip轉錄因子結合形成dimer。Nrf2調控基因轉錄時必須先和其它的bZip蛋白質包括Jun (c-Jun、Jun-D、Jun-B)及small Maf (MafG、MafK、MafF)等，形成heterodimerization (Kaspar et al., 2009)。在無刺激條件下，Nrf2和抑制蛋白Kelch-like ECH-associated protein-1 (Keap-1)結合形成複合體，存在細胞質中，呈不活化狀態，當細胞受到氧化壓力刺激時，會促使Nrf2與Keap-1分離，此時Nrf2轉移至細胞核內並且結合到含有ARE的基因序列上，進而啟動目標基因的轉錄作用，例如: HO-1 (Itoh et al., 2003)。文獻指出Nrf2的核轉錄作用會受到mitogen activated protein kinases (e.g., ERK1/2, JNK, p38 MAPK)、PI3K或protein kinase C的磷酸化作用所調控(Huang et al., 2002)。以人類主動脈內皮細胞(human aortic endothelial cells, HAECs)為實驗模式，當Nrf2在細胞內的蛋白質及mRNA表現增加，可抑制促發炎細胞激素TNF-α誘發p38 MAPK的磷酸化、單核球黏附作用與發炎基因，例如VCAM-1及MCP-1的表現，進而達到預防發炎疾病發生的作用(Chen et al., 2006)。

Keap-1是一個含有624個胺基酸的多勝肽，具有五個domains: (1) the N-terminal region (NTR); (2) the BTB domain; (3) the intervening region (IVR); (4) the double glycine repeat (DGR) or Kelch domain; and (5) the C-terminal region (CTR) (Itoh et al., 2004)。Nrf2的Neh 2 domain會跟Keap-1上的DGR domain結合，當Keap-1
與Nrf2結合時會抑制Nrf2的活化，將Nrf2隔離在細胞質內(Zipper and Mulcahy., 2002)。Keap-1是Nrf2的抑制蛋白，在無刺激環境下，Keap-1會與Nrf2結合並增加Nrf2的蛋白質降解作用(McMahon et al., 2003)。有文獻指出Keap-1含有好幾個具有高反應性的cysteine residues，在活化ARE基因序列的過程中，會包含一個具有高反應性的cysteine residues之胞內氧化還原接受器(redox receptor)，此氧化還原接受器可能是Keap-1(Dhakshinamoorthy et al., 2001)。有研究發現，在Keap-1上有四個硫氫基(sulfhydryl)會受到細胞內氧化壓力的影響，當硫氫基受到氧化壓力修飾後，造成Nrf2與Keap1分開，進而促使Nrf2結合至ARE基因序列(Dinkova-Kostova et al., 2002)。因此，當細胞受到氧化壓力刺激後，調控活化ARE基因序列的過程中，Keap1及Nrf2兩者同時扮演重要角色。

※ Nuclear factor-κB (NF-κB)

除了Nrf2和AP-1，NF-κB也被證實會向上調控HO-1基因表現。在刺激物如血紅素(hemin)、鎘(cadmium)及LPS刺激下，會活化NF-κB而向上調控HO-1基因表現(Liu et al., 2004; Chen et al., 2004; Wijayanti et al., 2004)。人類肝癌母細胞株(human hepatoblastoma-derived HepG2 cells)過度表現NF-κB，使得HO-1 mRNA表現也增加(Lavrovsky et al., 2000)。
圖2-8. Nrf2/ARE的活化路徑 (Mann et al., 2007)

圖2-9. Nrf2的調控網路 (Itoh et al., 2004)
三．穿心蓮

1．簡介

穿心蓮，拉丁學名為 *Andrographis paniculata* (Burm. f.) Nees，屬於唇形目 (Lamiales)、爵床科 (Acatheaceae)、穿心蓮屬 (*Andrographis*)，為一年生草本植物，別名一見喜、苦膽草、印度草、斬蛇劍、金香草、欖核蓮等，主要分布於東南亞熱帶地區如印度、斯里蘭卡、巴基斯坦、爪哇、緬甸、泰國、越南、印尼等地，非洲及南美洲也有其蹤跡，中國大陸與台灣各地也有零星栽培。植株直立、多枝，花期 9 月~10 月，採收時視生長旺盛欲開花前，割取地上部分或全株拔起，洗淨，曬乾，可內服 (煎湯) 及外用 (煎汁塗抹或研末調敷)。穿心蓮味苦性寒，具清熱解毒、涼血及消腫止痛之效，在傳統治療上常用於感冒發熱、咽喉腫痛、口舌生瘡、上呼吸道感染、氣管炎及毒蛇咬傷等。穿心蓮的葉子及根部在傳統藥理上已被使用數百年，在亞洲和歐洲民間更廣泛用來作為疾病治療或預防保健 (表 2-6) (Jarukamjorn and Nemoto, 2008)。穿心蓮的葉子和莖部可萃取出具有活性的植物化學成份 (phytochemicals)，研究指出穿心蓮的活性成分中含有豐富的 diterpenoids and 2′-oxygenated flavonoids，包括 andrographolide, neoandrographolide, 14-deoxy-11, 12-didehydroandrographolide, 14-deoxyandrographolide, isoandrographolide, 及 14-deoxyandrographolide, homoandrographolide, andrographan, andrographosterin, and stigmasterol 等 (Pholphana et al., 2004)。在這些 diterpenoids 中，穿心蓮內酯 (andrographolide) 是最重要的活性成分，其結構式為

\[
(3-[2-\{decahydro-6-hydroxy-5-(hydroxymethyl)-5,8α-dimethyl-2-methylene-1-napthalenyl/ethylidene\}dihydro-4-hydroxy-2(3H)-furanone)；
\]
分子式 C_{20}H_{30}O_{5}；分子量為 350.5，它存在植株的各部分，在全株乾燥的穿心蓮中，其莖內穿心蓮內酯約含 0.8~1.2%，而葉子內的含量最多 (>2%)。根據研究發現，穿心蓮具有廣
泛的生理功效，包括抗發炎 (Chiou et al., 2000；Sheeja et al., 2006)、抗腹瀉 (antidiarrhoeal) (Gupta et al., 1990)、抗病毒 (Wiart et al., 2005)、保護肝臟 (Trivedi and Rawal, 2001)、抗癌 (Kumar et al., 2004；Li et al., 2007；Zhou et al., 2006) 等。

表 2-6. 穿心蓮的傳統醫療作用（Jarukamjorn and Nemoto, 2008）

<table>
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<th>Traditional Uses</th>
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<tbody>
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<td>Maha-tikta</td>
<td>Helminth infection</td>
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<td>Bhunimba</td>
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<td></td>
<td>Peptic ulcer</td>
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<td></td>
<td>Skin infections (topical use)</td>
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<td>Snake-bites (topical use)</td>
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圖 2-10. 穿心蓮及穿心蓮內酯結構 (Jarukamjorn and Nemoto, 2008；Singha et al., 2007)
2. 生理功效

2-1 抗發炎作用

許多文獻指出穿心蓮及穿心蓮內酯皆具有抗發炎的功效，過去研究發現穿心蓮萃取物可用來預防或治療上呼吸道感染(Poolsup et al., 2004)，Shen 等人(2002)提出，穿心蓮內酯的抗發炎作用機制可能是藉由預防 ROS 的產生，以N-formyl-methionyl-leucyl-phenylalanine (fMLP)誘導人類嗜中性白血球為模式，給予穿心蓮內酯 (0.1~10 μM)，可以透過調控 protein kinase C (PKC) 路徑減少 ROS 的產生，進而降低 Mac-1 表現而抑制白血球的 adhesion 和 transmigration。也有研究指出，穿心蓮內酯可以直接影響 NF-κB 和 DNA 的結合而減少促發炎激素的表現，例如 cyclooxygenase 2 (Cox-2) 和 nitric-oxide synthase (NOS) (Hidalgo et al., 2005；Iruretagoyena et al., 2005)。穿心蓮內酯在小鼠巨噬細胞株 RAW 264.7 中，會透過抑制 inducible NO synthase (iNOS)表現來減少 LPS 所誘發的 NO，另外，研究也發現以 LPS 刺激小鼠腹腔內巨噬細胞，穿心蓮內酯會透過抑制 ERK1/2 的活化而減少 tumor necrosis factor-α (TNF-α) 及 interleukin-12 (IL-12)產生(Qin et al., 2006)。此外，Bao 等人(2009)證實，在 BALB/c 小鼠以 ovalbumin (OVA)誘發 Th2 細胞過敏導致氣喘的模式中，給與穿心蓮內酯可以抑制 nuclear factor-κB (NF-κB)的活化，而達到抗發炎的作用。另外，Chao 等人(2009)在最近的研究也發現，以 LPS 刺激 Raw264.7 巨噬細胞株的模式中，穿心蓮的乙酸乙酯萃出物可以透過抑制 NF-κB 的活化而減少 TNF-α、IL-6、MIP-2、NO 的產生；穿心蓮的乙酸乙酯萃出物也會抑制實驗小鼠的初代巨噬細胞產生 TNF-α、IL-12p40、MIP-2 及 NO 進而抑制發炎作用。

2-2 抗癌作用

在現代醫學中，大多數的抗癌藥物透過直接抑制癌細胞增生，或是誘導癌細胞
胞凋亡壞死、以及造成細胞週期停滯來達到抗癌的作用，穿心蓮被證實也可透過類似的機制，直接或間接調控癌細胞，而達到抗癌的功效(Vojdani and Erde, 2006)。Kumar等人(2004)研究發現，穿心蓮的甲醇萃取物可以顯著抑制人類結腸癌細胞(HT-29 cell)的增生，在人類急性骨髓白血病癌細胞(human acute myeloid leukemic HL-60 cells)中，穿心蓮乙醇萃出物及穿心蓮內酯會誘導細胞週期停止，並且透過調控促細胞凋亡(pro-apoptotic)分子的表現來影響癌細胞進行細胞凹亡反應(Cheung et al., 2005)。另外，穿心蓮也可以抑制肺癌細胞，研究發現穿心蓮內酯會藉由抑制 PI3K/Akt/AP-1 訊息傳遞路徑來降低 matrix metalloproteinase-7 (MMP-7) 表現，進而減少非小細胞肺癌細胞(A549)的侵襲性(Lee et al., 2010)。血管內皮生長因子(vascular endothelial growth factor, VEGF)是血管內皮細胞生長和存活重要的因素之一，它會調控血管內皮細胞增生、血管新生和血管通透性，VEGF會被腫瘤細胞大量利用以利其存活，有研究證實穿心蓮內酯可以抑制 VEGF 和 nitric oxide (NO)產生，並提高細胞中抗血管新生因子表現，如 IL-2 和組織金屬蛋白酵素抑制劑(tissue inhibitor of metalloproteinase ,TIMP-1) (Sheeja et al., 2007)。這些研究皆顯示穿心蓮內酯具有抑制腫瘤生長的潛力。

2-3 肝臟保護作用

過去文獻指出穿心蓮具有保護肝臟的功效，研究顯示在小鼠肝臟中，穿心蓮可以預防 hexachlorocyclohexane (BHC)刺激下所造成的γ-glutamyl transpeptidase (γ-轉胺基胜肽酶)、glutathione-S-transferase (GST)及脂質過氧化的增加，顯示穿心蓮具有潛在的抗氧化和保肝作用(Trivedi and Rawal, 2001)。在動物實驗中給予穿心蓮內酯，對半乳醣胺(galactosamine)誘發的肝損傷具有保護的功能(Handa and Sharma, 1990)。另外，小鼠在以四氯化碳(carbon tetrachloride)誘發肝損傷模式下，穿心蓮內酯會藉由降低malondialdehyde (MDA)、glutamate pyruvate transaminase (GPT)及alkaline phosphatase (ALP)並維持血漿中高濃度的glutathione (GSH)而達到保護肝臟的功效(Kapil et al., 1993)。
3. 使用風險

穿心蓮在傳統醫學上常用於治療感冒，因此評估穿心蓮萃取物是否具有毒性值得注意。急性口服毒性數據測試可用來評估人類在使用藥物上的風險。Chandrasekaran 等人(2009)以 8-12 週的大鼠來做急性口服毒性試驗，研究發現每公斤體重的大鼠給予穿心蓮萃出物 5000 mg，研究結束大鼠仍存活，並在 14 天的實驗期間，並沒有顯示出任何的不良症狀，也沒有任何不正常的體重增加情形。但是一些文獻指出，每天給予雄性小白鼠乾燥的穿心蓮葉磨成的粉末 20 mg，持續 60 天會停止精子的產生(Akbarsha et al., 1990)。廣州中醫藥大學徐等人(2005)，以小鼠來做急性毒性試驗，每公斤體重管灌穿心蓮軟膠囊內容物 15 ml(每粒膠囊含有 2.1 g 的穿心蓮生藥)，一天兩次，一日總給藥量為 110.25 g/kg，並連續觀察 14 天，結果發現給藥後的小鼠無躁動不安、呼吸困難、突眼等急性毒性表現，給藥後連續觀察 14 天並無小鼠死亡，且藥後兩周內小鼠的正常生長週期並未顯著受影響，此急性毒性試驗發現，小鼠一日最大耐受量為 110.25 g/kg，相當於成人臨床推薦用量的 525 倍，顯示穿心蓮在臨床上的應用是安全的。此外，在中草藥被廣泛應用的現代社會，中草藥與藥物間的交互作用也是一個嚴重的問題，以動物模式利用 HPLC/UV 來分析穿心蓮萃出物的成分，實驗發現預先給予穿心蓮內酯可以增加血液內茶鹼(theophylline)的清除，若長期使用穿心蓮則會提高茶鹼在血液內的濃度，研究中指出穿心蓮萃出物和其中的草藥成分會和 CYP1A2 產生交互作用，因此在使用上應多加留意(Chien et al., 2010)。
研究目的

動脈粥狀硬化是一種慢性發炎疾病，會造成血管內壁脂肪堆積及纖維斑塊形成，是導致心血管疾病的主要原因之一。許多文獻指出穿心蓮及穿心蓮內酯皆具有抗發炎的藥理作用，而穿心蓮內酯更是穿心蓮中的重要活性成分，因此本實驗將利用內皮細胞株 EA. hy926 以腫瘤壞死因子 TNF-α 誘發其發炎反應，之後探討穿心蓮內酯對於內皮細胞發炎反應的影響及其作用機制。
文獻參考


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Induction of Heme Oxygenase 1 and Inhibition of Tumor Necrosis Factor α-Induced Intercellular Adhesion Molecule Expression by Andrographolide in EA.hy926 Cells

INTRODUCTION

Current epidemiologic predictions suggest that cardiovascular disease (CVD) is reaching pandemic proportions (1), and CVD is the leading cause of death worldwide, accounting for ~16.7 million deaths each year (2). This number is predicted to reach approximately 25 million by 2020, if current trends continue (3). The reasons for the increased global incidence of CVD include the aging of the world’s population and lifestyles in lower- and middle-income countries becoming more akin to those of wealthier nations (1).

CVD is partially characterized by chronic inflammation and an increased level of expression of inflammatory biomarkers, such as intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and E-selectin, on the surface of the activated endothelial cells (EC) and in the blood circulation (4). Increased circulating levels of these adhesion molecules are considered to be predictive of CVD risk because they indicate a proinflammatory state in the vasculature (4). In vivo inflammatory processes are mediated by the involvement of proinflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), IL-12, and interferon γ (INF-γ). EC play a key role in coordinating leukocyte trafficking to specific tissues that
regulate their activation (5). When confronted by an inflammatory stimulus, e.g.,
bacterial lipopolysaccharide (LPS) (6), TNF-α (7), or IL-1β (8), EC become activated as
a result of modification of their phenotype. This is accompanied primarily by the
upregulation of a series of proinflammatory genes, e.g., E-selectin, P-selectin, VCAM-1,
and ICAM-1 (6-8). The expression of these genes is regulated primarily by the
activation of transcription factor NF-κB (9). The proper production of proinflammatory
cytokines aids in innate immune responses; however, overproduction causes undesirable
side effects such as tissue injury, septic shock, and even death (10). To avoid the
consequences of uncontrolled production, the expression of proinflammatory genes
must be strictly regulated (11). One of the mechanisms by which inflammation is
counteracted is the expression of the anti-inflammatory genes of activated EC. Because
of their dual ability to inhibit the expression of proinflammatory genes associated with
EC activation and to protect EC from undergoing apoptosis, Bach and colleagues (11)
referred to these genes as protective genes. Soares et al. (12) have hypothesized that the
stress-responsive gene encoding heme oxygenase 1 (HO-1) acts in such a manner.

HO-1 is an inducible enzyme that catalyzes the rate-limiting step in the oxidative
degradation of free heme into Fe²⁺, carbon monoxide, and biliverdin, which is
subsequently catabolized into bilirubin by biliverdin reductase (13). Induction of the
gene encoding HO-1 is primarily regulated at the transcriptional level, and transcription
factor Nrf2 plays a critical role in the inducibility of the gene (14). Under unstimulated
conditions, Nrf2 is sequestered in the cytoplasm by binding to Klech-like
ECh-associated protein 1 (Keap1) (15). This complex is disrupted by several
electrophilic antioxidants, and Nrf2 is freed and translocated to the nucleus where it
binds to antioxidant response element (ARE) sequences in the HO-1 gene promoter (16,
17). HO-1 can also be induced by its substrate, free heme, and by a diversity of
proinflammatory stimuli, which suggests that HO-1 is involved in heme degradation as
well as in resolution of inflammation (18).

Andrographis paniculata (Burm. f) Nees, a Chinese herb, is a member of the Acanthaceae family. It is widely cultivated in Southeast Asia and is widely used as a traditional medicine in India, China, Thailand, and Scandinavia (19). Andrographolide (Figure 1) is the most abundant diterpene lactone in the leaves and stems of A. paniculata and has high biological activity and therapeutic potential (20). Andrographolide has been studied for its anti-inflammatory (21-23), chemopreventive (24), antiangiogenic (25), antiproliferative (26), and antiapoptotic (27) activities. Because of the significant role of inflammation in CVD development, the cardiovascular benefits of andrographolide as the result of its anti-inflammatory activity cannot be ignored.

Therefore, on the basis of the well-known anti-inflammatory activity of andrographolide, this study was designed to explore the effect of andrographolide on the TNF-α-induced expression of ICAM-1 in EA hy.926 cells and the mechanism by which andrographolide acts to influence ICAM-1 expression.

MATERIALS AND METHODS

Materials. Monocytic HL-60 cells were obtained from Bioresources Collection and Research Center (BCRC, Taiwan). Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640, 25% trypsin-EDTA, penicillin-streptomycin, RPMI-1640 without phenol red, and OPTI-MEM were from GIBCO/BRL (Grand Island, NY). Fetal bovine serum (FBS) was from HyClone (Logan, UT). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium
bicarbonate, sodium phosphate dibasic heptahydrate, TNF-R, and all other chemicals were from Sigma (St. Louis, MO). TRIzol reagent was from Invitrogen (Carlsbad, CA). Andrographolide and antibody against HO-1 were from Calbiochem (Darmstadt, Germany). The antibody raised against ICAM-1 was from Cell Signaling Technology (Boston, MA). The antibody raised against Nrf2 was from Santa Cruz Biotechnology (Santa Cruz, CA). The luciferase assay kit was from Promega (Madison, WI). Bis-(carboxyethyl) carboxyfluorescein acetoxyethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR).

**Cell Cultures.** The human endothelial cell line EA.hy926 was cultured in DMEM supplemented with 3.7 g/L NaHCO₃, 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO₂ humidified incubator. The HL-60 cells were cultured in T-75 tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100000 units/L penicillin, and 100 mg/L streptomycin.

**Cell Viability Assay.** Cell viability was assessed by the MTT assay. The MTT assay measures the ability of viable cells to reduce a yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan by mitochondrial succinate dehydrogenase. EA.hy926 cells were grown to 70-80% confluence and were then treated with different concentrations of andrographolide (0-20 μM) for 16 h followed by incubation with TNF-α (1 ng/mL) for an additional 6 h. Finally, the DMEM medium was removed, and the cells were washed with PBS. The cells were then incubated with MTT (0.5 mg/mL) in DMEM medium at 37 °C for an additional 3 h. The medium was removed, and 2-propanol was added to dissolve the formazan. After centrifugation at 20000g for 5 min, the supernatant of each sample was transferred to 96-well plates, and the absorbance was read at 570 nm in an ELISA reader. The absorbance in cultures treated with 0.1% DMSO was regarded as 100% cell viability.
Monocyte Adhesion Assay. EA.hy926 cells in 12-well plates were allowed to grow to 80% confluence and were then pretreated with andrographolide for 16 h followed by incubation with 1 ng/mL TNF-R for an additional 6 h. The human monocytic HL-60 cells cultured in RPMI 1640 medium with 10% FBS were labeled with 1 μM 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM). At the end of the andrographolide and TNF-α treatment, a total of 1×10⁶ BCECF-AM-labeled HL-60 cells were added to each well, and the cells were co-incubated with EA.hy926 cells at 37 °C for 30 min. The wells were washed and filled with cell culture medium, and the plates were sealed, inverted, and centrifuged at 100g for 5 min to remove nonadherent HL-60 cells. Bound HL-60 cells were lysed in a 1% SDS solution, and the fluorescence intensity was determined in a fluoroscan ELISA plate reader (FLX800, Bio-Tek, Winooski, VT) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. A control study showed that fluorescence is a linear function of HL-60 cell density in the range of 3000-80000 cells/well. The results are reported on the basis of the standard curve obtained.

Western Blotting Analysis. After each experiment, cells were washed twice with cold PBS and were harvested in 150 μL of lysis buffer [10 mM Tris-HCl (pH 8), 0.1% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/L leupeptin, 1 mg/L aprotinin, and 2 mM dithiothreitol]. Cell homogenates were centrifuged at 14000g for 20 min at 4 °C. The resulting supernatant was used as a cellular protein for Western blot analysis. The total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Pierce Biotechnology Inc., Rockford, IL). Equal amounts of cellular proteins were electrophoresed in an sodium dodecyl sulfate-polyacrylamide gel, and proteins were then transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites on themembranes were blocked with
5% nonfat milk in 15mM Tris/150mM NaCl buffer (pH 7.4) at room temperature for 2 h. Membranes were probed with rabbit anti-human ICAM-1, HO-1, and β-actin antibodies. The membranes were then probed with the secondary antibody labeled with horseradish peroxidase. The bands were visualized by using an enhanced chemiluminescence kit (PerkinElmer Life Science, Boston, MA) and were quantitated with an ImageGauge (Fuji Film).

**RNA Isolation and RT-PCR.** Total RNA of EA.hy926 cells was extracted by using Trizol reagent. We used 4 µg of total RNA for the synthesis of first-strand cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega) in a final volume of 20 µL containing 250 ng of oligo-dT and 40 units of RNase inhibitor. PCR was conducted in a thermocycler in a reaction volume of 50 µL containing 20 µL of cDNA, BioTaq PCR buffer, 50 µmol of each deoxyribonucleotide triphosphate, 1.25 mmol/L MgCl$_2$, and 1 unit of BioTaq DNA polymerase (BioLine). Oligonucleotide primers of ICAM-1 (forward, 5’-TGAAGGCCACCCCAGAGGACAAC-3’; reverse, 5’-CCCATTATGACTGCGGCTGCTGCTACC-3’), HO-1 (forward, 5’-CTGAGTTCATGAGGAACTTTCAGAAG-3’; reverse, 5’-TGGTACAGGGAGGCCATCAC-3’), and glyceraldehyde-3-phosphate dehydrogenase (forward, 5’-CCATCACCATCTTCCAGGAG-3’; reverse, 5’-CCATCACCATCTTCCAGGAG-3’; reverse, 5’-CCATCACCATCTTCCAGGAG-3’) were designed on the basis of published sequences (28, 29). Amplification of ICAM-1 and GAPDH was achieved when samples were heated to 95°C for 5 min and then immediately cycled 32 times through a 1 min denaturing step at 94°C, a 1 min annealing step at 56°C, and a 1 min elongation step at 72°C. Amplification of HO-1 and GAPDH was achieved when samples were heated to 95°C for 5 min and then immediately cycled 39 times through
a 1 min denaturing step at 95 °C, a 1 min annealing step at 55 °C, and a 2 min
elongation step at 72 °C. The glyceraldehyde-3-phosphate dehydrogenase cDNA level
was used as the internal standard. PCR products were resolved in a 1% agarose gel and
were scanned by using a Digital Image Analyzer (Alpha Innotech) and quantitated
with an ImageGauge.

Plasmids, Transfection, and Luciferase Assays. A p2xARE/Luc fragment
containing tandem repeats of double-stranded oligonucleotides spanning the Nrf2
binding site, 5’-TGACTCAGCA-3’, as described by Kataoka et al. (30), was
introduced into the pGL3 promoter plasmid (Promega). All subsequent transfection
experiments were performed by using nanofection reagent (PAA,Austria) according to
the manufacturer’s instructions. For luciferase assays, the cell lysate was first mixed
with a luciferase substrate solution (E1500, Promega), and the resulting luciferase
activity was measured by using a microplate luminometer (TROPIX TR-717, Applied
Biosystems). For each experiment, luciferase activity was determined in triplicate and
was normalized with β-galactosidase activity.

RNA Interference by Small Interfering RNA of HO-1. Predesigned small
interfering RNA (siRNA) against human HO-1 and nontargeting control pool siRNA
were purchased from Dharmacon Inc. (Lafayette, CO). EA.hy926 cells were
transfected with HO-1 siRNA SMART pool by using DharmaFECT1 transfection
reagent (Thermo) according to the manufacturer’s instructions. Specific silencing was
confirmed by at least three independent immunoblotting assays with cellular extracts 8
h after transfection.

Nuclear Extract Preparation. After each experiment, cells were washed twice with
cold PBS and were then scraped from the dishes with 1000 μL of PBS. Cell
homogenates were centrifuged at 2000g for 5 min. The supernatant was discarded, and
the cell pellet was allowed to swell on ice for 15 min after the addition of 200 μL of
hypotonic buffer containing 10mM HEPES, 1 mM MgCl₂, 1mM EDTA, 10mM KCl, 0.5mM DTT, 0.5% Nonidet P-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM PMSF. After centrifugation at 7000g for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mM HEPES, 400 mM KCl, 1 mM MgCl₂, 0.25 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2mM PMSF, and 10% glycerol at 4°C for 30min. The samples were then centrifuged at 20000g for 15min. The supernatant containing the nuclear proteins was collected and stored at -80°C until the Western blotting assay.

**Statistical Analysis.** Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference between mean values was determined by one-way analysis of variance followed by Tukey’s test; p values of <0.05 were taken to be statistically significant.

**RESULTS**

**Cell Viability.** The MTT assay was used to test whether the concentration of andrographolide used in the presence of TNF-α caused cell damage. As shown in Figure 2, there were no adverse effects on the growth of EA.hy926 cells up to an andrographolide concentration of 7.5 μM in the presence of 1 ng/mL TNF-α, which was used to induce the expression of ICAM-1. The highest concentration of andrographolide used in this study was 7.5 μM, and thus, the effects of andrographolide observed were not due to its cytotoxicity.

**Effect of Andrographolide on the TNF-α-Induced Expression of ICAM-1 in EA.hy926 Cells.** EA.hy926 cells were pretreated with 7.5 μM andrographolide for the indicated times before being exposed to 1 ng/mL TNF-α for 6 h. The protein
expression of ICAM-1 was significantly suppressed after pretreatment for 4 h compared with that treated with TNF-α alone, and the inhibition was sustained with pretreatment for up to 16 h (Figure 3A).

To determine whether the TNF-α-induced protein and mRNA expression of ICAM-1 were dose-dependently affected by andrographolide, concentrations of andrographolide ranging from 0 to 7.5 μM were studied. As shown in panels B and C of Figure 3, the inhibition of TNF-α-induced protein and mRNA expression of ICAM-1 by andrographolide was dose-dependent. A significant inhibitory effect of andrographolide on ICAM-1 protein expression was observed at concentrations greater than 2.5 μM, and a significant inhibitory effect on ICAM-1 mRNA expression was observed at concentrations greater than 5 μM.

**Andrographolide Inhibits HL-60 Cell Adhesion.** We next determined whether andrographolide pretreatment could inhibit HL-60 cell adhesion. As shown in Figure 4, TNF-α significantly increased the level of HL-60 cell adhesion. However, andrographolide pretreatment inhibited HL-60 cell adhesion in a dosedependent manner, and significant effects were found at 5 and 7.5 μM.

**Effect of Andrographolide on HO-1 Expression of EA.hy926 Cells in the Presence of TNF-α.** The stress-responsive gene encoding HO-1 has been recognized to be a protective gene in EC (12). To determine whether the inhibition of the TNF-α induced expression of ICAM-1 by andrographolide was due to upregulation of HO-1, EA.hy926 cells were pretreated with various concentrations of andrographolide for 16 h before being exposed to TNF-α for an additional 6 h. As shown in panels A and B of Figure 5, TNF-α did not affect the protein or mRNA expression of HO-1. However, pretreatment with andrographolide for 16 h significantly enhanced both protein and
mRNA expression of HO-1 in a concentration-dependent manner.

**Andrographolide Increases the Rate of Nuclear Translocation of Nrf2 and Induces ARE-Luciferase Reporter Activity.** Nrf2 is a major transcription factor that regulates ARE-driven gene expression (31). We further determined whether Nrf2 is activated by andrographolide. As shown in Figure 6A, cells treated with 7.5 μM andrographolide had a higher level of Nrf2 accumulation in the nuclear fraction as early as 1 h, and this accumulation was sustained until 6 h. We used cells transfected with luciferase reporter constructs harboring the ARE to determine the specificity of andrographolide for this sequence. As shown in Figure 6B, andrographolide increased ARE-luciferase activity in a dosedependent manner, and a significant effect was found at 5 and 7.5 μM.

**HO-1 siRNA Alleviates Andrographolide Inhibition of ICAM-1 Expression in the Presence of TNF-α.** The role of HO-1 in the inhibition of ICAM-1 expression by andrographolide in the presence of TNF-α was examined by using an siRNA SMARTpool system to create an HO-1 knockdown model. EA.hy926 cells were transfected with an siHO-1 construct for 8 h, followed by treatment with 7.5 μM andrographolide for 16 h and TNF-α for an additional 6 h. Control cells were transfected with a nontargeting siRNA construct (NC). The efficiency of the siRNA SMARTpool system in knocking down HO-1 was assayed by Western blot and RT-PCR assay (Figure 7A). HO-1 siRNA alleviated the andrographolide inhibition of protein (Figure 7B) and the mRNA expression (Figure 7C) of ICAM-1 in the presence of TNF-α. These results imply the importance of HO-1 for the inhibition of TNF-α-induced ICAM-1 expression by andrographolide.
DISCUSSION

In this study, we have demonstrated that andrographolide, an active phytocompound of A. paniculata, effectively suppressed ICAM-1 expression in vascular EC exposed to TNF-α and that this protection is likely associated with Nrf2-dependent HO-1 induction.

A. paniculata is widely used in Asia to treat infection, inflammation, cold, fever, and diarrhea and as an antidote to snakebite (32). The anti-inflammatory activities of A. paniculata and of andrographolide, the most abundant diterpene lactone in A. paniculata, have been extensively studied. The concentrations of andrographolide used (2.5-7.5 μM) in this study are approximately equivalent to those achieved in subjects ingesting A. paniculata (33). The highest concentration of andrographolide used was 7.5 μM because the MTT results showed that concentrations greater than this caused cell damage in the presence of 1 ng/mL TNF-α (Figure 2). These results were consistent with those of a previous study (34). According to the cell viability and anti-inflammatory results, andrographolide exhibited a narrow therapeutic window. It would be prudent to use A. paniculata and its bioactive compound, andrographolide.

Inhibition of the abnormal induction of adhesion molecules is believed to be one of the mechanisms attributed to the CVD protection of a diversity of phytochemicals, e.g., diallyl disulfide (DADS), diallyl trisulfide (DATS), apigenin, luteolin, and cinnamaldehyde (35-37). The suppression of ox-LDL-induced E-selectin and VCAM-1 expression by DADS and DATS and, thus, monocyte adhesion to EC is likely dependent on the PI3K/ PKB or PKA/CREB signaling pathway in an adhesionmoleculespecific manner (35). The reactive oxygen species scavenging capabilities of apigenin and luteolin proceed dose-dependently in the presence of ox-LDL (36). The inhibitory effects of apigenin and luteolin on ICAM-1 and E-selctin
expression are, at least partially, attributed to their antioxidant activity and modulation of the PI3K/Akt signaling pathway. In TNF-α-treated EA.hy926 cells, pretreatment with cinnamaldehyde inhibits the expression of ICAM-1 and VCAM-1 and results in the suppression of the adherence of monocytes to EC (37).

The essential role of ICAM-1 in mediating the adhesion of WEHI 274.1 cells to ox-LDL-stimulated mouse aortic endothelium was demonstrated in a previous study (38). In the study presented here, inhibition of ICAM-1 expression by andrographolide resulted in the suppression of adhesion of HL-60 cells to TNF-α-stimulated EA.hy926 cells (Figure 4). The suppressive pattern was dose-dependent, and a significant effect was observed at 5 and 7.5 μM. These results suggest that inhibition of ICAM-1 expression by andrographolide contributes to the suppression of the adhesion of monocytes to inflammatory EC.

The importance of TNF-α in the expression of adhesion molecules has been well recognized, and the role of TNF-α in CVD has been demonstrated in vivo. Mice in which the TNF-α gene is disrupted develop significantly fewer atherosclerotic lesions in the proximal aorta than do their normal counterparts (39). The role of NF-κB in the TNF-α-induced expression of cell adhesion molecules has been convincingly demonstrated (40). NF-κB is a transcription factor that resides in the cytoplasm under unstimulated circumstances in an inactive form via its association with I-κB. After stimulation with pro-inflammatory agents, I-κB undergoes proteolytic degradation and frees NF-κB. Freed NF-κB translocates from the cytoplasm to the nucleus where it activates target gene transcription (41).

The transcriptional activation of genes encoding various antioxidant enzymes and phase II detoxifying enzymes such as HO-1 is regulated by the ARE (42). Andrographolide increased the rate of nuclear translocation of Nrf2 and induced ARE-luciferase activity (Figure 6A,B), which suggests that andrographolide may
induce the expression of ARE-regulated genes. The Keap1/Nrf2/ARE signaling pathway is thought to play an important role in protecting cells from endogenous and exogenous stresses (43). In this study, andrographolide induced the expression of HO-1 through the Nrf2/ARE signaling pathway.

Although both in vivo and in vitro studies have demonstrated the potent anti-inflammatory activity of A. paniculata and andrographolide (44, 45), the mechanism by which andrographolide acts to prevent adhesion molecule expression is not clear. HO-1 is an inducible enzyme that catalyzes the rate-limiting step in the oxidative degradation of free heme into Fe^{2+}, carbon monoxide, and biliverdin and is regarded as an important cellular antioxidant enzyme. Because of its antioxidant characteristics, HO-1 has been reported to inhibit the expression of adhesion molecules in EC via the generation of bilirubin and the decrease in the level of intracellular free Fe^{2+} (12). Additionally, Lee et al. (46) indicated that the inhibition of TNF-α-induced adhesion molecule expression by overexpression of HO-1 is associated with the reduced level of formation of a TNFR1-c-Src-p47^{phox} complex and subsequent inhibition of NF-κB activation. Furthermore, inhibition of RelA phosphorylation at Ser276 by HO-1 is considered to be involved in suppression of ICAM-1 expression associated with EC activation (47). We did not measure the direct effect of siHO-1 on NF-κB activation in this study. Brunt et al. (48) found that overexpression of HO-1 reduced NF-κB promoter activity and NF-κB DNA binding activity in response to H_2O_2 in HL-1 cardiomyocytes. Their results might suggest the possibility that andrographolide inhibits TNFα-induced ICAM-1 expression is via suppression of NF-κB activation by HO-1 overexpression in our study. The blockage of the degradation of the inhibitory protein inhibitor κB (I-κB) and the induction of the expression of Nrf2-mediated HO-1 have been shown to make up the working
mechanism by which cinnamaldehyde abolishes TNF-α-induced ICAM-1 and VCAM-1 transcription. Recently, HO-1 gene transcription was noted to be induced by one kind of diterpene carnosol via activation of the Nrf2/ARE pathway in PC12 cells (49). Andrographolide is a phytocompound with a diterpene structure. On the basis of these findings, the possibility that the inhibition of ICAM-1 expression by andrographolide is via its induction of HO-1 transcription cannot be excluded. In this study, our results revealed that andrographolide dose-dependently inhibited TNF-α-induced protein expression of ICAM-1 in EA.hy926 cells (Figure 3B). In contrast with the suppression of ICAM-1 expression, however, HO-1 was dose-dependently upregulated by andrographolide (Figure 5A,B). The importance of HO-1 in the inhibition of ICAM-1 expression was further confirmed by using HO-1 siRNA. In cells transfected with siHO-1, the inhibition of TNF-α-induced ICAM-1 expression by andrographolide was abolished (Figure 7B,C). The PI3K/Akt, MAPK, and PKCδ pathways have been reported to be involved in HO-1 expression induced by a variety of phytochemicals (50-52). We demonstrated in this study that andrographolide induces HO-1 expression. However, signaling pathways involved in nuclear translocation of Nrf2 were not determined, and they are under investigation.

The findings of this study are schematically presented in Figure 8. Stimulation of HO-1 by andrographolide is involved in the inhibition of expression of ICAM-1 and the subsequent adhesion of monocytes to EA.hy926 cells. Induction of expression of HO-1 by andrographolide occurs through the Nrf2/ARE signaling pathway. The anti-inflammatory activity of andrographolide confers A. paniculata with CVD-protective potential.
Fig. 1. Chemical structure of andrographolide.

![Chemical structure of andrographolide](image)

Fig. 2. Effect of andrographolide (AP) on the cell viability of EA.hy926 cells in the presence of TNF-α. Cells were pretreated with 0 to 20 μM andrographolide for 16 h followed by incubation with 1 ng/ml TNF-α for another 6 h. Cell viability was measured by using the MTT assay. Values are the mean ± S.D. of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$).
Fig. 3. Andrographolide (AP) inhibits TNF-α-induced ICAM-1 expression in EA.hy926 cells. (A) Cells were pretreated with 7.5 μM andrographolide for various time periods. (B) Cells were pretreated with 0 to 7.5 μM andrographolide for 16 h followed by incubation with 1 ng/ml TNF-α for another 6 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (C) Total RNA was isolated from cells and was subjected to RT-PCR with specific ICAM-1 and GAPDH primers as described in Materials and Methods. Values are the mean ± S.D. of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$). One representative immunoblot out of three independent experiments is shown.
**Fig. 4.** Effect of andrographolide (AP) on TNF-α-induced HL-60 cell adhesion. Cells were pretreated with andrographolide for 16 h before being challenged with 1 ng/ml TNF-α for another 6 h. Values are the mean ± S.D. of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$).
Fig. 5. Andrographolide (AP) induces HO-1 expression in EA.hy926 cell. Cells were pretreated with andrographolide for 16 h followed by incubation with TNF-α for another 6 h. (A) Aliquots of total protein (20 μg) were used for Western blot analysis. (B) Total RNA was isolated from cells and was subjected to RT-PCR with specific HO-1 and GAPDH primers as described in Materials and Methods. Values are the mean ± S.D. of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$). One representative immunoblot out of three independent experiments is shown.
Fig. 6. Effect of andrographolide (AP) on Nrf2 nuclear translocation and ARE-luciferase reporter activity. (A) Nuclear extracts from cells were prepared after treatment with 7.5 μM andrographolide for the indicated time periods. Immunoblots of nuclear extracts from treated cells were then probed with Nrf2-specific antibody. (B) Cells were transfected with the ARE-luciferase construct (ARE) and after 12 h were maintained in low serum medium for a further 1 h and were then stimulated with 0 to 7.5 μM andrographolide for an additional 24 h. The cells were then lysed and analyzed for luciferase activity. Induction is shown as an increase in the normalized luciferase activity in the treated cells relative to the control. Values not sharing the same letter are significantly different ($p < 0.05$). One representative immunoblot out of three independent experiments is shown.
Fig. 7. Effect of HO-1 siRNA on the inhibition of ICAM-1 expression by andrographolide (AP). An HO-1 siRNA system was used to silence HO-1 mRNA in cells and to create an siRNA knock-down EA.hy926 cell model. (A) Western blot and Northern blot analyses of HO-1 expression in EA.hy926 cells transfected with HO-1 siRNA. (B) Aliquots of total protein (20 μg) were used for Western blot analysis. (C) Total RNA was isolated from cells and was subjected to RT-PCR with specific ICAM-1 and GAPDH primers. Values are the mean ± S.D. of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$). One representative immunoblot out of three independent experiments is shown.
Fig. 8. Model showing pathways that mediate the inhibition of expression of ICAM-1 and HL-60 cell adhesion to EA.hy926 cells by andrographolide under inflammatory conditions. Andrographolide causes the dissociation of Nrf2 from Keap1 and its nuclear translocation. Nrf2 then binds to the ARE, which leads to an induction of HO-1 expression and inhibits ICAM-1 expression and subsequent HL-60 cell adhesion.


[13] Ryter SW, Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant


[27] Zhao F, He EQ, Wang L, Liu K. Anti-tumor activities of a diterpene from Andrographis


RNA 干擾術 (RNA interference by small interfering RNA of HO-1)

原理: 藉由雙股 RNA 片段送入細胞後，經 Dicer 作用分解成小片段 RNA (small interfering RNA, siRNAs)，此雙股的 siRNA 會與 RNA-induced silencing complex (Risc) 產生交互作用形成單股 siRNA，並與目標基因的 mRNA 互補結合。而此互補結合的 RNA 片段會被切下並降解，使得目標基因無法進行轉譯作用，抑制該基因的表現。

材料: 人類 HO-1 siRNA 與 non-targeting control-pool siRNA 購自 Dharmacon Inc. (Lafayette, Colorado)，人類 HO-1 siRNA 序列如下：(1) AUGCUAGUCUUGAUGAAG，(2) ACACUCAGCUUUCUGGGG，(3) CAGUGCUAGGCGCUUUA，(4) AGAUGAGCGCAACAAGGA。

實驗方法: EA.hy926 細胞培養至八分滿時進行 siRNA 轉染，取三支試管，於試管 1 中加入 OPTI medium 100 μl 及 2 μM non-targeting control-pool siRNA 100 μl，試管 2 中加入 OPTI medium 100 μl 與 2 μM 人類 HO-1 siRNA 100 μl，試管 3 則依 3 μl: 197 μl 的比例取 DharmaFECT1 transfection reagent 與 OPTI medium 混合，室溫下反應 5 分鐘後由試管 3 取出 200 μl，分別加入試管 1 與試管 2 中混合，並以 1 ml autopipette 以吐氣的方式 mix 均勻，於室溫反應 20 分鐘。接著以回溫的 PBS 清洗細胞兩次後分別將試管 1 與試管 2 的 mixture 各自加到培養皿中，並補足至 2 ml OPTI medium，使欲轉染的 siRNA 最後濃度為 0.1 μM。轉染 8 小時後換成含有 10% FBS 的 DMEM medium 並預處理穿心蓮內酯 16 小時後再與 TNF-α 共同培養 6 小時，收取細胞蛋白質以西方墨點法分析蛋白質表現。