中國醫藥大學營養學系碩士班

碩 士 論 文

二十二碳六烯酸(DHA)誘發血基質氧化酶-1 與抑制腫瘤壞死因子-α 誘發的細胞間黏附分子-1 表現關係之探討

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

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中華民國一百年七月

July, 2011

中國醫藥大學營養學系碩士班

論文口試委員審定書

論文題目：

中文：二十二碳六烯酸(DHA)誘發血基質氧化酶-1與抑制腫瘤壞死因子-α誘發的細胞間黏附分子-1表現關係之探討

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

本論文係 魏瑜伶 於中國醫藥大學營養學系碩士班完成之碩士論文，經考試委員審查及考試合格，特此證明。

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中華民國 一百 年 七 月 六 日

中國醫藥大學營養學系

論文指導教授推薦書

營養學系碩士班：魏瑜伶君所提之論文

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中華民國 100 年 7 月 6 日

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縮寫表

Act D	actinomycin D
AHA	American Heart Association
ALA	alpha-linolenic acid
AP-1	activator protein-1
ARE	antioxidant response element
ATF4	activating transcription factor 4
BR	bilirubin
BV	biliverdin
C/EBP	CCAT/enhancer binding protein
CHX	cycloheximide
CKⅡ	casein kinase Ⅱ
CNC-bZIP	cap'n'Collar-basic leucine zipper
CO	carbon monoxide
DHA	docosahexaenoic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
EpRE	electrophile response element
HLH	helix-loop-helix domain
HO	heme oxygenase
Hsp32	heat shock protein 32
ICAM	intercellular adhesion molecule
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor-1
IgSF	immunoglobulin superfamily
IKK	IκB kinase
IL-1	interleukin-1
IRE	interferon-stimulated response element
IκB	inhibitor κB
Keap1	Klech-like ECH-associated protein 1
LA	linoleic acid
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LZ	leucine zipper
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotoactic protein-1

MCSF	monocyte-colony-stimulating factor
MMP-9	matrix metalloproteinase-9
NFAT	nuclear factor of activated T cells
NF-E2	nuclear factor-erythroid 2
NF-κB	nuclear factor-kappa B
NIK	NF-κB-inducing kinase
NLS	nuclear localization signal
NOS	nitric oxide synthase
PARP	poly (ADP-ribose) polymerase
PDGF	platelet derived growth factor
PECAM	platelet endothelial cellular adhesion molecule
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PPAR	peroxisome proliferators activated receptor
PUFA	polyunsaturated fatty acid
RHD	Rel homology domain
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
sGC	soluble guanylate cyclase
STAT	Janus kinases (JAK)-signal transducers and activators of transcription
TGF-β	transforming growth factor-beta
TNFR1	tumor necrosis factor receptor 1
TNF-α	tumor necrosis factor-alpha
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells

中文摘要

許多研究顯示，發炎反應和血管內皮細胞功能異常是動脈粥樣硬化的重要起始關鍵。細胞間黏附分子 1 (ICAM-1)是一種發炎生理指標，與單核球黏附至內皮細胞上的作用有關。文獻指出，飲食中攝取富含 n-3 多元不飽和脂肪酸的魚油，例如二十二碳六烯酸 (DHA)，可以有效地減少罹患心血管疾病的風險。因此本實驗將以腫瘤壞死因子-α (TNF-α)誘發內皮細胞株 EA.hy926 產生發炎反應，來探討 DHA 對於 TNF-α 所誘發 ICAM-1 表現之影響以及可能參與的機制。結果發現，DHA (50, 100 μM)除了會抑制 TNF-α 所誘發的 ICAM-1 蛋白質、mRNA 表現、報導基因活性；亦能進一步地降低 IKK 磷酸化、IκB 磷酸化和降解、p65 核轉移，以及 NF-κB 與 DNA 的結合能力。另一方面，處理 DHA 會顯著增加血基質氧化酶 1 (HO-1)和轉錄因子 Nrf2 的蛋白質表現，並誘導 Nrf2 轉移進入細胞核內，向上調節 antioxidant response element (ARE)報導基因活性。同時，我們也發現 DHA 調節 HO-1 的表現主要是在轉錄階段。另外，利用 siRNA 干擾技術抑制 HO-1 的表現，會部分逆轉 DHA 對於 ICAM-1 的抑制作用。綜合以上結果得知，在 EA.hy926 細胞中，DHA 會藉由抑制 NF-κB 訊息傳遞路徑及增加 Nrf2-dependent HO-1 之表現來降低 TNF-α 所誘發的 ICAM-1 表現；本研究證實 DHA 具有預防心血管等發炎性疾病之潛力。

關鍵字：二十二碳六烯酸 (DHA)、細胞間黏附分子 1 (ICAM-1)、腫瘤壞死因子-α (TNF-α)、血基質氧化酶 1 (HO-1)、NF-κB、Nrf2、發炎反應

英文摘要

Several studies indicate that inflammation and endothelial cell dysfunction are important initiating events in atherosclerosis. Intercellular adhesion molecule 1 (ICAM-1), an inflammatory biomarker, plays a pivotal role in cardiovascular disease (CVD) progression. Dietary intake of fish oil rich in n-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), has been associated with reduced CVD risk. In this study, we investigated the effect of DHA on the tumor necrosis factor-alpha (TNF-α)-induced ICAM-1 expression in EA.hy926 cells and the possible mechanisms involved. The results showed that DHA (50 and 100 μM) inhibited TNF-α-induced ICAM-1 protein, mRNA expression, and promoter activity. In addition, TNF-α-stimulated IKK phosphorylation, IκB phosphorylation and degradation, p65 nuclear translocation, and NF-κB and DNA binding activity were attenuated by pretreatment with DHA. Furthermore, DHA significantly increased the protein expression of heme oxygenase 1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2), induced Nrf2 translocation to the nucleus, and up-regulated antioxidant response element (ARE)-luciferase reporter activity. HO-1 expression is primarily regulated by DHA at the transcriptional level. Transfection with HO-1 siRNA knocked down HO-1 expression and partially reversed the DHA-mediated inhibition of ICAM-1 expression. In conclusion, these results suggested that DHA inhibits TNF-α-induced ICAM-1 expression is through attenuation of NF-κB signaling pathway and stimulation of Nrf2-dependent HO-1 expression in EA.hy926 cells. The anti-inflammatory effects of DHA may implicate its CVD-protective potential.

Key words: DHA, HO-1, ICAM-1, inflammation, NF-κB, Nrf2, TNF-α

第一部份

第一章 前言

營養過剩、肥胖、高血脂、高膽固醇、高血壓等問題是目前已開發國家中多數人口所面臨的營養相關問題，台灣十大死因當中，心臟病、腦中風等心血管疾病就占了很高的比例，心血管疾病一直是全球性的重大公共衛生議題，其死亡率逐年攀高，對於健康的影響確實不容忽視。許多研究顯示，動脈粥狀硬化(atherosclerosis)是一種慢性發炎反應(Glass & Witztum, 2001)，而且在動脈硬化初期，內皮細胞的發炎反應扮演了極重要的角色。所以，深入瞭解動脈硬化的成因及其調控機轉，有助於預防及治療動脈硬化。

同時，大量的醫學證據發現，採取積極的預防措施可降低罹患心血管疾病之風險。西元 1971 年，丹麥兩位科學家班(Bang)和戴柏格(Dyerberg)，在著名醫學雜誌 Lancet 針對 n-3 不飽和脂肪酸發表驚人結果：他們觀察到，住在北極冰原格陵蘭島上的愛斯基摩人，在缺乏綠色蔬菜和水果的環境中，罹患心血管疾病的比例卻比一般人低很多，主要的原因是他們所攝取的食物大部份是深海魚類，如鮭魚、鯖魚等，飲食中富含大量的魚油，因此很少罹患心肌梗塞及腦栓塞等疾病(Bang et al., 1971)。魚油(n-3 多元不飽和脂肪酸)作為輔助防治心血管疾病的工具已有多年，且有許多研究報導顯示，補充魚油對於降低心血管疾病之發生率有正面的功效(Wang et al., 2006; Casós et al., 2008)。因此本研究將以內皮細胞所表現的黏附分子為指標，探討 DHA 對其影響及相關之分子機制，期望對魚油的預防保健功效能有更深入地了解。

第二章 文獻回顧

一、動脈粥狀硬化(atherosclerosis)

1. 動脈粥狀硬化之成因

動脈粥狀硬化的形成過程為一複雜且漸進式的致病過程，就病理特徵大致可分為幾個階段：(1)血管內層增厚(intimal thickening)；(2)脂肪紋(fatty streak)；(3)中度病灶(intermediate lesion)；(4)纖維斑塊(fibrous plaque)；(5)複雜性病灶(complicated lesion) (Hegele, 1996)。其主要的致病機轉學說是由Ross提出的「內皮細胞受傷後之反應假說」(The response to injury) (Ross, 1993)，造成內皮細胞受損的原因很多，包括血液中LDL的增加及被修飾、自由基(free radicals)、高血壓、糖尿病、泡疹病毒(herpesviruses)感染、衣原體感染(*Chlamydia pneumoniae*)等，皆會造成內皮細胞功能失調(dysfunction)。在動脈硬化初期，受到傷害的內皮細胞功能受損，進而產生代償性反應，改變了細胞的恆定狀態(homeostasis)，及改變內皮層的通透性。內皮層通透性增加，使得一些大分子物質容易通透，例如低密度脂蛋白膽固醇(low-density lipoprotein, LDL)，並在血管壁上堆積及被氧化成ox-LDL (Steinberg et al., 1989)。而且當內皮細胞受到刺激時，會導致許多趨化物質及生長因子(growth factor)被釋出，包括MCP-1 (monocyte chemotactic protein-1)、MCSF (monocyte-colony-stimulating factor)、TGF-β (transforming growth factor-beta)，會促使血液循環中的單核球附著於血管壁，並移行至內皮下空間。單核球會受MCSF影響而分化成巨噬細胞(Qiao et al., 1997)，並吞噬過多的ox-LDL，使得大量的膽固醇、脂質堆積在細胞內而形成泡沫細胞(foam cell)，並分泌大量的前發炎細胞激素(proinflammatory cytokines)，例如：IL-1 (interleukin-1)、TNF-α (tumor necrosis factor-alpha)、IFN-γ (interferon-gamma)，而這些細胞激素會協同生長因子，例如：VEGF (vascular endothelial growth factor)、

PDGF (platelet derive growth factor-1)、IGF-1 (insulin-like growth factor-1)刺激平滑肌細胞的增生，也會向上調節黏附分子之表現，促使單核球的黏附、移行及分化，影響內皮細胞及平滑肌細胞，而使得病灶更加惡化(Kim et al., 2001)。

2. 細胞黏附分子與動脈粥狀硬化之關係

細胞黏附分子(cellular adhesion molecules)是表現於細胞表面，調控細胞與細胞之間或細胞與細胞外基質(extracellular matrix)間的黏附作用。在動脈硬化的病理過程中，最明顯的特徵為受傷的內皮細胞表面會大量增加白血球及黏附分子，接著會因為內皮層的通透性增加，使得白血球容易穿透血管壁，稱為白血球徵募作用(recruitment)，而這些過程在發炎反應中是非常重要的，其中細胞表面的黏附分子則扮演著重要的角色。

白血球黏附到內皮細胞上，再轉移進入內皮下空間，主要包括了四個階段(圖2.1)：(1)首先，白血球會黏附到內皮細胞上(Capture/Tethering)；(2)接著被選擇素(selectins)活化後會沿著血管壁滾動(rolling)；(3)並透過細胞激素(chemokines)的作用，改變整合素(integrins)的構型，促使 integrins 與 CAMs 進行交互作用，穩固地黏附(firm adhesion)在內皮細胞上；(4)白血球開始變形，穿透內皮層(transmigration)至內皮下空間，主要是由 integrins 與 PECAM 來調控，最後促使動脈粥狀硬化的形成(Carlos & Harlan, 1994; Blankenberg et al., 2003; Lawson & Wolf, 2009)。

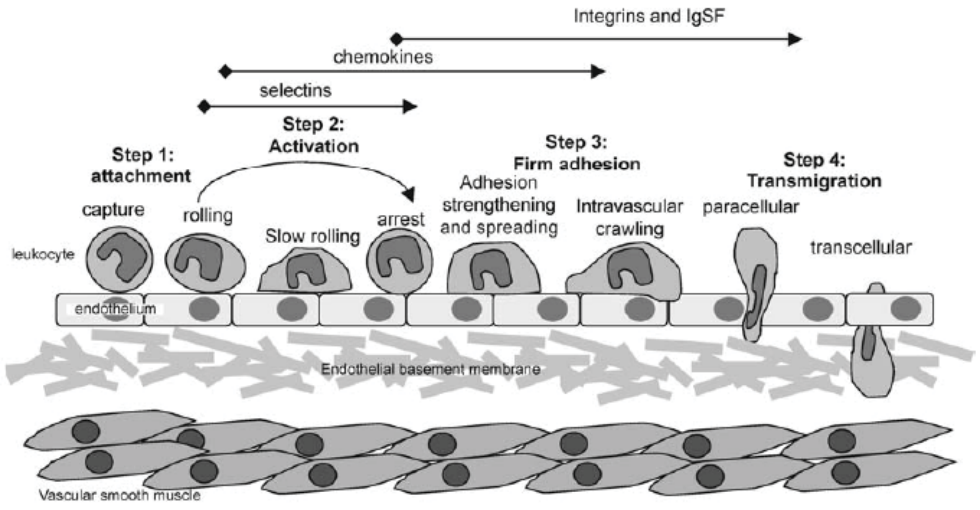


圖 2.1 白血球徵募作用(Lawson & Wolf, 2009)

這一連串的過程分別需要不同的黏附分子參與，其廣泛表現及分佈於各種細胞，主要可分為三類：selectins、IgSF 和 integrins (表 2.1) (Blankenberg et al., 2003)。類免疫球蛋白分子(Immunoglobulin-like molecules or Immunoglobulin superfamily, IgSF)是細胞膜上的醣蛋白接受器(glycoprotein receptor)，由於細胞外的 Ig domain 不同，而有不同的 isoform，例如 ICAM、VCAM、PECAM。

表 2.1 參與動脈粥狀硬化之黏附分子(Blankenberg et al., 2003)

Description of adhesion molecules involved in atherosclerosis, their main ligands, their functions, and their tissue distribution

Adhesion molecules	Other names	Ligands	Functions	Tissue distribution			Soluble form
				Endoth	Leuko	Platelets	
Selectins/ligands							
P-selectin	CD62P, GMP140	PSGL-1, Lewis X, CD24	Rolling/tethering	+		+	+
E-selectin	CD62E, ELAM1	ESL-1, Lewis X, PSGL-1, L-set	Rolling/tethering	+			+
L-selectin	CD62L	Lewis X, CD34, PSGL-1, GlyCAM	Rolling/tethering		+		+
E-selectin ligand 1	ESL-1	E-selectin	Rolling/tethering		+		
P-selectin ligand 1	CD162, PSGL-1	P-, L-, E-selectin	Rolling/tethering		+		+
Immunoglobulins							
ICAM-1	CD54	αLβ2, αMβ2, αXβ2	Firm adhesion	+	+		+
ICAM-2	CD102	αLβ2, αMβ2	Firm adhesion	+	+	+	+
ICAM-3	CD50	αLβ2, αDβ2, DC-SIGN	Firm adhesion	+	+		+
VCAM-1	CD106	α4β1, α4β7, αDβ2	Firm adhesion	+			+
PECAM-1	CD31	PECAM-1, αVβ3	Endothelial integrity, leukocyte extravasation	+	+	+	+
Integrins							
Integrin α2/β1	CD49b/CD29, VLA2	Collagen, laminin	Platelet receptor			+	
Integrin α4/β1	CD49d/CD29, VLA4	VCAM-1, FN	Firm adhesion	+			
Integrin αL/β2	CD11a/CD18, LFA1	ICAMs	Firm adhesion		+		
Integrin αM/β2	CD11b/CD18, Mac1	ICAMs,iC3b, FX, FG	Firm adhesion		+		
Integrin αX/β2	CD11c/CD18	ICAM-1, FG, iC3b, CD23	Firm adhesion		+		
Integrin αD/β2	CD11d/CD18	ICAM-3, VCAM-1	Firm adhesion		+		
Integrin α2B/α3	GPIIb/IIIa	vWF, FN, FG, VN, thrombospondin	Platelet receptor			+	
Integrin αV/β3	VNR, CD51/CD61	PECAM-1, VN, FN, FG, vWF	Proliferation, migration	+		+	
Integrin αV/β5		VN	Proliferation, migration	+			

The last column refers to the demonstrated existence of a soluble isoform. FG, fibrinogen; FN, fibronectin; FX, Factor X; VN, vitronectin; vWF, von Willebrand factor.

細胞間黏附分子(Intercellular Adhesion Molecules, ICAMs)共有五種亞型(subtypes)，其中最常見的是 ICAM-1 (又稱 CD-54)，在內皮細胞、上皮細胞、纖維母細胞、平滑肌細胞、白血球等都有表現；平時維持在很低的含量，當受到前發炎細胞激素(TNF-α、IL-1、IFN-γ)、LPS (lipopolysaccharide)、ox-LDL、氧化壓力(oxidative stress)、病毒感染等刺激時，會大量表現此黏附分子(表 2.2) (Roebuck & Finnegan, 1999)，透過與白血球上之 integrins 產生交互作用，使白血球緊密黏附(firm adhesion)於內皮細胞上(Blankenberg et al., 2003)。利用抗體阻斷 ICAM-1 作用或藉由 ICAM-1 knockdown 模式證實：ICAM-1 在白血球黏附與穿透內皮層的移行過程中確實扮演著重要的角色(Reiss & Engelhardt, 1999; Lehmann et al., 2003)。人類 ICAM-1 基因的啟動子(promoter)序列已被選殖(clone)出來，許多轉錄因子(transcription factor)會參與調控 ICAM-1 表現，包括 AP-1 (activator protein-1) (Son et al., 2006)、Sp1 (Berendji-Grun et al., 2001)與 NF-κB (nuclear

factor-κB) (Zhou et al., 2007; Lian et al., 2010)、STAT (Janus kinases (JAK)-signal transducers and activators of transcription) (Audette et al., 2001)。TNF-α、IL-1 主要是透過活化 NF-κB 來調控 ICAM-1，例如：在以 TNF-α 誘發人類肺上皮細胞(human pulmonary epithelial cells)表現 ICAM-1 的模式中，證實了 NF-κB 的重要性(Oh et al., 2010)；而 IFN-γ 則是透過 STAT 傳遞路徑來調控 ICAM-1 (圖 2.2)。ICAM-1 除了會影響白血球的移行作用及 T 細胞的活化作用；同時，ICAM-1 也與許多發炎疾病有關，例如氣喘(asthma)、動脈粥狀硬化(atherosclerosis)、急性呼吸窘迫症候群(acute respiratory distress syndrome)、缺血性缺氧再灌流損傷(ischemia reperfusion injury)、自體免疫疾病(autoimmune disease)等(Roebuck & Finnegan, 1999)。

表 2.2 誘發 ICAM-1 表現的不同刺激(Roebuck & Finnegan, 1999)

Table 2.2 Cell Type-Specific Induction of ICAM-1 Expression

Cell type	Stimuli
Endothelial cell	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, H_2O_2, metal ions
Epithelial cell	TNF-α, IL-1, LPS, TPA, histamine, EBV, CMV, RSV, parainfluenza virus, rhinovirus
Fibroblast	TNF-α, IL-1, IL-4, IFN-γ, retinoic acid, mycoplasma, PGE_2
Keratinocyte	TNF-α, histamine
Hepatocyte	TNF-α, IL-1, IFN-γ, IL-6
Leukocyte	TNF-α, IL-1, IFN-γ, IL-3, GM-CSF, TPA
Smooth muscle cell	TNF-α, PDGF

Abbreviations: TNF-α, tumor necrosis factor α; IL, interleukin; IFN-γ, interferon-γ; GM-CSF, granulocyte-monocyte colony-stimulating factor; LPS, lipopolysaccharide; PMA, phorbol 12-myristate-13-acetate; RSV, respiratory syncytial virus; CMV, cytomegalovirus; UV, ultraviolet light A; LDL, low-density lipoprotein; PDGF, platelet-derived growth factor; EBV, Epstein-Barr virus; PGE, prostaglandin E; PDTC, pyrrolidine dithiocarbamate.

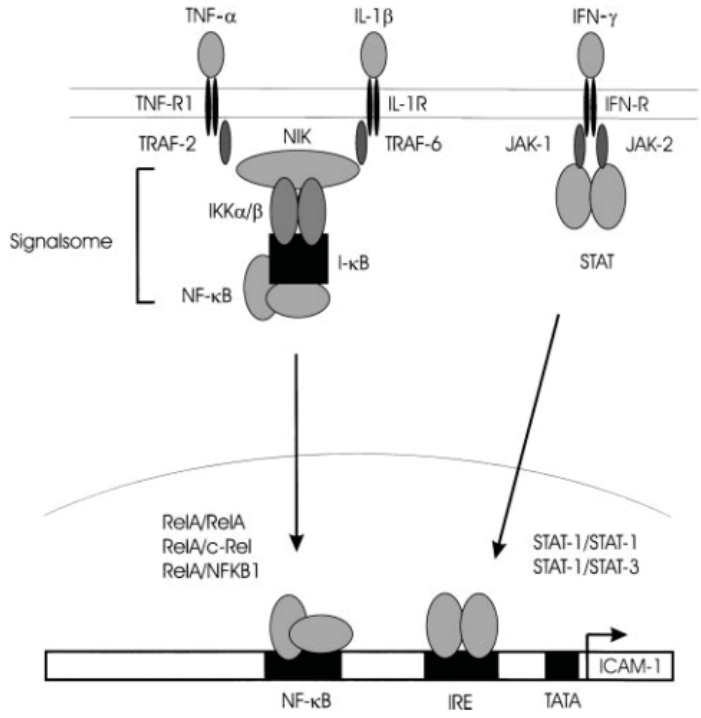


圖 2.2 TNF-α、IL-1β、IFN-γ 調控 ICAM-1 的分子傳遞路徑(Roebuck & Finnegan, 1999)

3. 腫瘤壞死因子與動脈粥狀硬化之關係

腫瘤壞死因子(Tumor Necrosis Factor-alpha, TNF-α)在各種生理及病理過程中扮演著重要的角色，包括細胞增生、分化、凋亡及發炎反應(Gaur & Aggarwal, 2003; Liu, 2005)。TNF-α 藉由和細胞表面的受器結合而產生生物活性，依據分子量的不同可將受器分為 TNFR1 (tumor necrosis factor receptor 1) (55 kDa)及 TNFR2 (75 kDa)。TNFR1 在所有種類的細胞膜上都有表現，也是 TNF-α 主要的受器；而 TNFR2 則表現在免疫細胞及內皮細胞(Gaur & Aggarwal, 2003)。TNF-α 是一種已知的前發炎細胞激素，依細胞種類不同及生理狀態之差異而有獨特的生物功能，通常在動脈粥狀硬化損傷處可發現它的蹤跡(Sana et al., 2005)。TNF-α 會刺激內皮細胞表現黏附分子(如 ICAM-1、VCAM-1、E-selectin)，及促進白血球黏附作用(Zhou et al., 2007; Oh et al., 2010)，而此誘發作用是決定於轉錄因子 NF-κB 的活化。在某些情況下，TNF-α 會造成血管受到傷害、內皮細胞失去功能，因此常被用來作為誘發細胞產生發炎反應的模式。

4. 轉錄因子 NF-κB 訊息傳遞路徑

4-1. NF-κB

NF-κB (Nuclear factor-κB)是與發炎及免疫相關的轉錄因子，屬於 Rel 家族的一種蛋白質。目前已知 NF-κB 家族由五個成員所組成：p65 (RelA)、c-Rel、RelB、p50/p105 (NF-κB_1)、p52/p100 (NF-κB_2)，以同質複合體(homodimer)或異質複合體(heterodimer)的方式存在(Jost & Ruland, 2007)：大部分 NF-κB 複合體在細胞質與 IκB 連結時無法進入細胞核內，例如 p50/p65 是典型的 NF-κB 複合體；有些 NF-κB 複合體，例如 p52/RelB，不與 IκB 連結，便能直接進入細胞核；而 p105 與 p100 在 C 端有 Ankyrin domain (ANK)，平時只能留在細胞質內，當水解成 p50 與 p52 時，才具有進入細胞核的能力(圖 2.3、圖 2.4) (Perkins, 2000)。這些蛋白質都具有一段相似的胺基酸序列，稱之為 Rel homology domain (RHD)，這段序列含有 dimerization domain、nuclear localization signal (NLS)、DNA-binding domain，分別可使 NF-κB 單體進行雙體化(dimerization)；當 IκB 與 NLS 結合時會抑制 NF-κB 的活性，無法進行核轉位(nuclear translocation)；與目標基因(target genes)上游的啟動子(稱之為 κB site)結合，調控目標基因的表現(May & Ghosh, 1998; Papa et al., 2006)。

NF-κB 常見的活化形式為 p50/p65 異質複合體。正常情況下，NF-κB 是不活化且留在細胞質中，因為 NF-κB 的抑制蛋白 inhibitor κB (IκB)，會蓋住促使 NF-κB 進入細胞核中的胺基酸序列(NLS)，使 NF-κB 滯留在細胞質中。當細胞受到發炎介質刺激時(表 2.3)，IκB 會受到上游蛋白 IκB kinase (IKK)磷酸化，接著 IκB 會被泛素化(ubiquitinated)並進入蛋白酶體(proteasome)中分解，進而造成 NF-κB 的活化，使其進入細胞核內和 DNA 結合，啟動發炎基因的表現，例如細胞激素、黏附分子等(圖 2.5、表 2.4) (Barnes et al., 1997; Ghosh & Karin, 2002; Hayden et al., 2006; Sun & Karin, 2008; Rahman & McFadden, 2011)。研究指出，在人類肺泡上

皮細胞(human alveolar epithelial cell)中，TNF-α 會經由活化蛋白激酶 C (protein kinase C, PKC)，活化 c-Src，活化的 c-Src 會經由磷酸化 IKK 而活化 IKK；另一方面，IKK 也會經由 NF-κB-inducing kinase (NIK)這條傳遞路徑受到活化(Huang et al., 2003)。

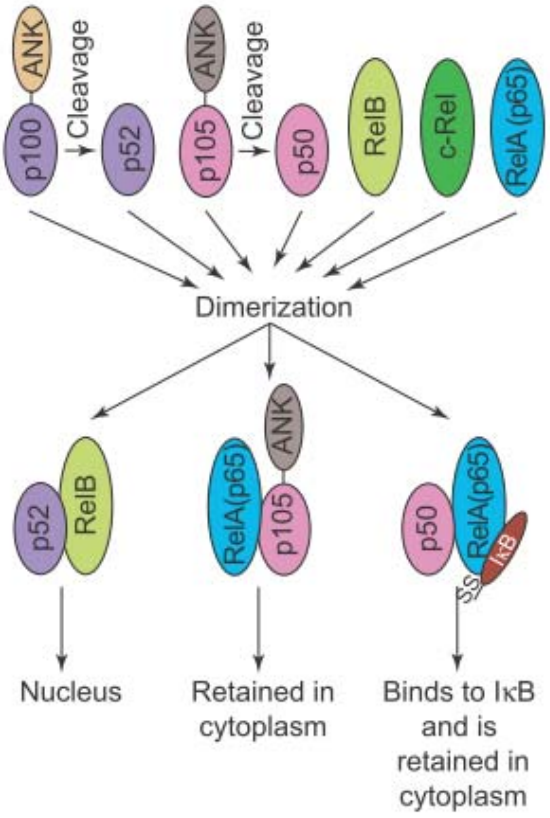


圖 2.3 NF-κB 組成型式(Perkins, 2000)

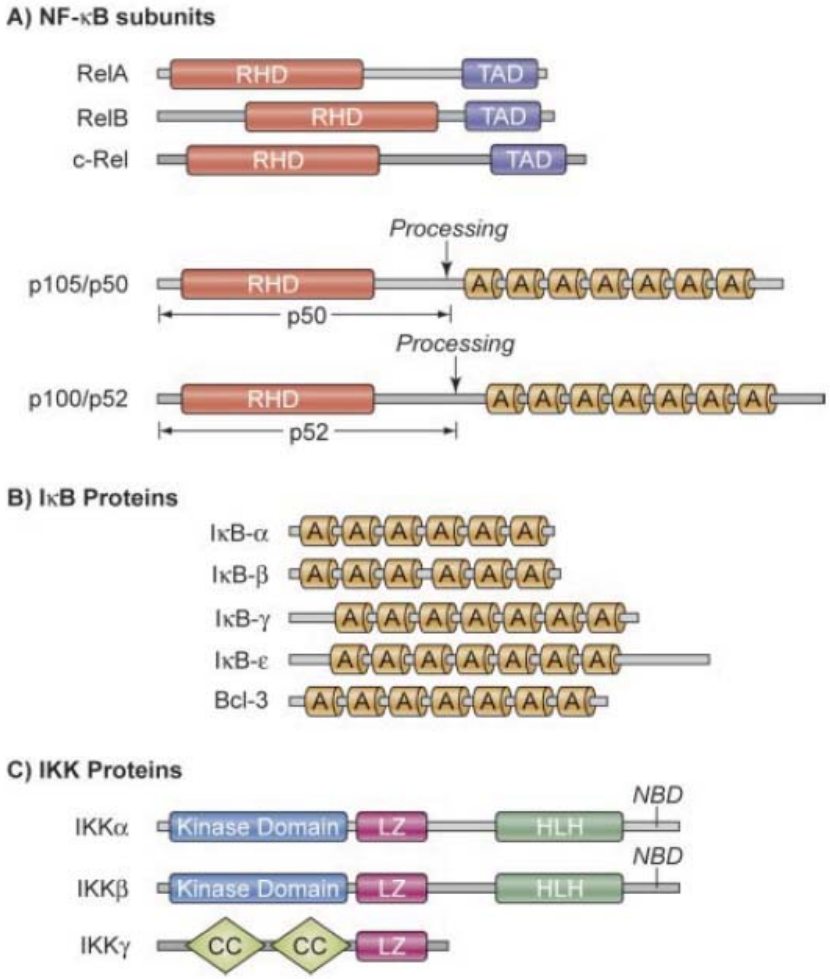


Figure 2.4 **Structural organization of NF-κB, IκB, and IKK proteins.** (A) NF-κB subunits. NF-κB comprises a group of 5 related transcription factors that share a highly conserved amino-terminal Rel homology domain (RHD), which is responsible for dimerization, nuclear translocation, DNA binding, and interaction with inhibitory IκB proteins. RelA, RelB, and c-Rel additionally possess a carboxy-terminal transactivation domain (TAD) that initiates transcription from NF-κB–binding sites in target genes. The ankyrin repeat (A) containing NF-κB1 and NF-κB2 precursor proteins p105 and p100 can be proteolytically processed to p50 and p52. (B) IκB proteins. The IκB proteins are characterized by the presence of 6 or 7 ankyrin repeats (A) to mediate protein-protein interactions. The ankyrin repeat motif can bind to the nuclear localization sequence of NF-κB proteins and is important for the retention of NF-κB in an inactive state in the cytoplasm. The mammalian IκB family members are IκB-α, IκB-β, IκB-γ, IκB-ϵ, and BCL-3. In addition, NF-κB1 and NF-κB2 precursor proteins p100 and p105 can also function as IκBs. (C) IKK proteins. The IKK complex contains the catalytic kinase subunits IKKα and IKKβ, as well as a regulatory subunit IKKγ (NEMO). IKKα and IKKβ possess a helix-loop-helix region (HLH) and a leucine zipper (LZ), which are responsible for both homodimerization and heterodimerization of IKKα and IKKβ. The catalytic subunits interact through their NEMO-binding domain (NBD) with IKKγ, which contains a coiled coil (CC) domain and a leucine zipper (LZ). Illustration by Kenneth Probst.

圖 2.4 NF-κB、IκB 及 IKK 結構(Jost & Ruland, 2007)

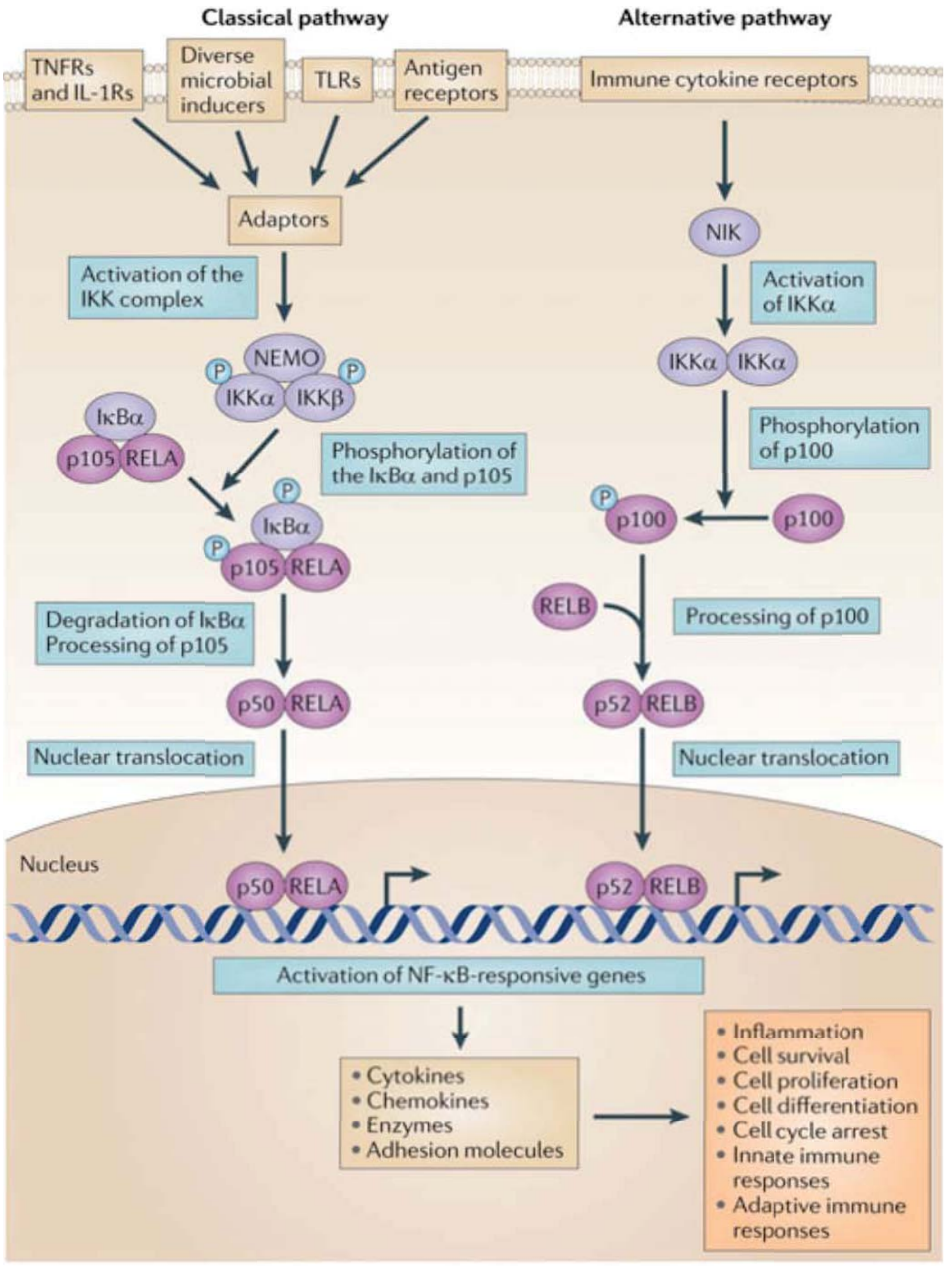


圖 2.5 NF-κB 訊息傳遞路徑(Rahman & McFadden, 2011)

表 2.3 活化 NF-κB 的刺激(Barnes et al., 1997)

Table 2.3 STIMULI THAT ACTIVATE NF-κB.

Cytokines
Tumor necrosis factor α
Interleukin-1β
Interleukin-17
Protein kinase C activators
Phorbol esters
Platelet-activating factor
Oxidants
Hydrogen peroxide
Ozone
Viruses
Rhinovirus
Influenzavirus
Epstein–Barr virus
Cytomegalovirus
Adenovirus
Immune stimuli
Phytohemagglutinin
Anti-CD3 antibodies (by means of T-lymphocyte activation)
Antigen
Other
Lipopolysaccharide
Ultraviolet radiation

表 2.4 受 NF-κB 調控的蛋白質(Barnes et al., 1997)

Table 2.4 PROTEINS REGULATED BY NF-κB.

Proinflammatory cytokines
Tumor necrosis factor α
Interleukin-1β
Interleukin-2
Interleukin-6
Granulocyte–macrophage colony-stimulating factor
Macrophage colony-stimulating factor
Granulocyte colony-stimulating factor
Chemokines
Interleukin-8
Macrophage inflammatory protein 1α
Macrophage chemotactic protein 1
Gro-α, -β, and -γ
Eotaxin
Inflammatory enzymes
Inducible nitric oxide synthase
Inducible cyclooxygenase-2
5-Lipoxygenase
Cytosolic phospholipase A_2
Adhesion molecules
Intercellular adhesion molecule 1
Vascular-cell adhesion molecule 1
E-selectin
Receptors
Interleukin-2 receptor (α chain)
T-cell receptor (β chain)

4-2. IKK

NF-κB 訊息傳遞過程是由上游的 NIK 磷酸化 IKK，IKK complex 是由 IκB kinase α (IKKα)、IκB kinase β (IKKβ)和 IκB kinase γ (IKKγ，又稱為 NEMO, NF-κB essential modulator)及 casein kinase Ⅱ (CKⅡ)所組成。IKKα / IKKβ 和 IKKγ 形成複合物，IKKα 和 IKKβ 屬於 serine-specific kinase，分子量分別為 85 kDa 及 87 kDa，且具有 52%之相似性(homology)。IKKα 由 745 個胺基酸所組成，IKKβ 則由 756 個胺基酸所組成，其結構為 N 端 Kinase domain、Leucine zipper (LZ) region 及 C 端 Helix-loop-helix domain (HLH) (圖 2.4)。在 NF-κB 活化過程中，IKKα / IKKβ 和 IKKγ 複合物會使 IκBα 於 N 端 serine 32 和 36 的位置磷酸化，導致 IκBα 構型改變並蓄積足夠的能量，誘導 26S-proteasome 於 IκBα 的 lysine 21 與 22 進行降解作用；而 IκBβ 則於 ser-19 和 ser-23 位置被磷酸化，但 IκBβ 的分解速度較 IκBα 緩慢；因此，IκBα 對 NF-κB 活化是快速而短暫的，而 IκBβ 則較能夠維持

長久。IKKα / IKKβ 不會對其它 IκB isoform (IκBε)之 serine resides 進行磷酸化。casein kinase Ⅱ (CKⅡ)可將 IκB 蛋白質 C 端 PEST 序列磷酸化，一些學者認為：必須先有 IκB 蛋白質 C 端持續性磷酸化，才能誘發 IκB 蛋白質 N 端磷酸化(May & Ghosh, 1998; Karin & Delhase, 2000; Jost & Ruland, 2007)。

4-3. IκB

IκB 蛋白質的成員包括 IκBα、IκBβ、IκBγ、IκBε 和 Bcl-3(或稱 IKAP) (圖 2.4)，IκB 蛋白質藉由 Ankyrin domain 和 C 端 PEST (Proine-, Glutamio acid-, Serine- and Threonine-rich)序列與 NF-κB 的 RHD 結合。雖然這些蛋白質都能阻止 NF-κB 進入細胞核，但是不同的 IκB 有其特定之作用對象，例如：IκBα 可以特定結合到 p50/p65 和 p65/p65，但不能有效地結合到 p50/p50；而 IκBβ 會專一性的結合到 p50/c-Rel 異質複合體;IκBε 則會結合到 p65 及 c-Rel 的同質複合體;IκBγ 和 Bcl-3 則是專一性結合到 p50 及 p52 同質複合體上。就 NF-κB 存在型式比例最高的 p50/p65 異質複合體而言，IκBα 結合到 p50/p65 的親和力(affinity)比結合到 p65/p65 的親和力高 27 倍，比結合到 p50/p50 的親和力高 60 倍。IκBα 基因的啟動子上具有 κB site 調控序列，因此 NF-κB 活化將導致 IκBα 大量合成，初合成的 IκBα 會進入細胞核中，結合 NF-κB 將其帶回細胞質。但 IκBβ 並不受活化的 NF-κB 所調控，因此 IκBβ 之降解可以給予 NF-κB 較長的活化時間(May & Ghosh, 1998; Christman et al., 2000; Wertz & Dixit, 2010)。

二、魚油與心血管疾病之關係

1. 魚油概述

海洋浮游生物含有 n-3 系列的 ALA (α-linolenic acid，次亞麻油酸)、EPA (eicosapentaenoic acid，二十碳五烯酸)及 DHA (docosahexaenoic acid，二十二碳六烯酸)，由小型魚類吃下後，再被大型魚所捕食，在形成食物鏈的過程中，被魚攝取的 ALA 會再轉變成其他 n-3 系列的形式，積存在魚體內，即是魚油的有效成份。而魚油中的 n-3 脂肪酸，主要為 EPA 和 DHA。雖然植物油中的 ALA 亦可在人體內轉換成其他 n-3 脂肪酸，但轉換比例小於十分之一(Siddiqui et al., 2008)。天然食物中的深海魚類，如鮭魚、鮪魚、鯖魚等，其油脂含有較高量的 EPA 及 DHA，所以一般如果要補充 EPA 及 DHA，還是以魚油為最佳來源。

2. n-3 多元不飽和脂肪酸

多元不飽和脂肪酸(polyunsaturated fatty acids, PUFAs)按 n 編號系統，根據第一個雙鍵所在的位置可將不飽和脂肪酸分為四種類型，即 n-3、n-6、n-7 和 n-9 系列，但具有重要生物學意義的是 n-3 和 n-6 PUFAs。人類無法自行合成 n-3 和 n-6 不飽和脂肪酸，必須從食物中直接攝取，因此又被稱為必需脂肪酸，尤其是 α-次亞麻油酸(n-3, α-linolenic acid, ALA)和亞麻油酸(n-6, linoleic acid, LA) (SanGiovanni & Chew, 2005)。目前較普遍的 n-3 脂肪酸有 ALA、EPA 和 DHA 三種，除了 ALA，EPA 和 DHA 通常存在於深海魚油中。

2-1. 結構

n-3 多元不飽和脂肪酸(n-3 PUFAs)是包含數個以上不飽和鍵的脂肪酸，因為第一個雙鍵出現在碳鏈距甲基端的第三個碳原子上，所以稱之為 n-3 脂肪酸，也叫作 ω-3 脂肪酸。從圖 2.6 中可以看到，DHA 有六個雙鍵、二十二個碳原子。

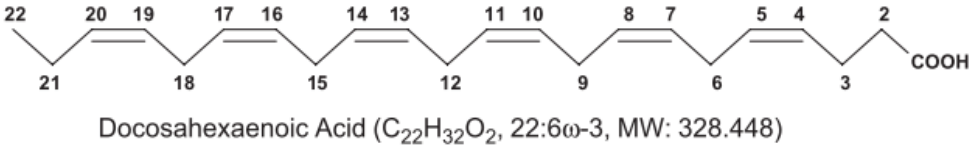


圖 2.6 DHA 結構(SanGiovanni & Chew, 2005)

2-2. 生合成作用

α-次亞麻油酸(α-linolenic acid, ALA)便是 n-3 脂肪酸的典型代表，它有三個雙鍵。ALA 是對人體健康非常重要的一種脂肪酸，但人體不能正常合成，因而被視為是一種必需脂肪酸(EFA)。ALA 可以透過去飽和酶(desaturase)和碳鏈延長酶(elongase)的催化作用，最後合成 EPA 和 DHA，統稱為 n-3 系列脂肪酸。

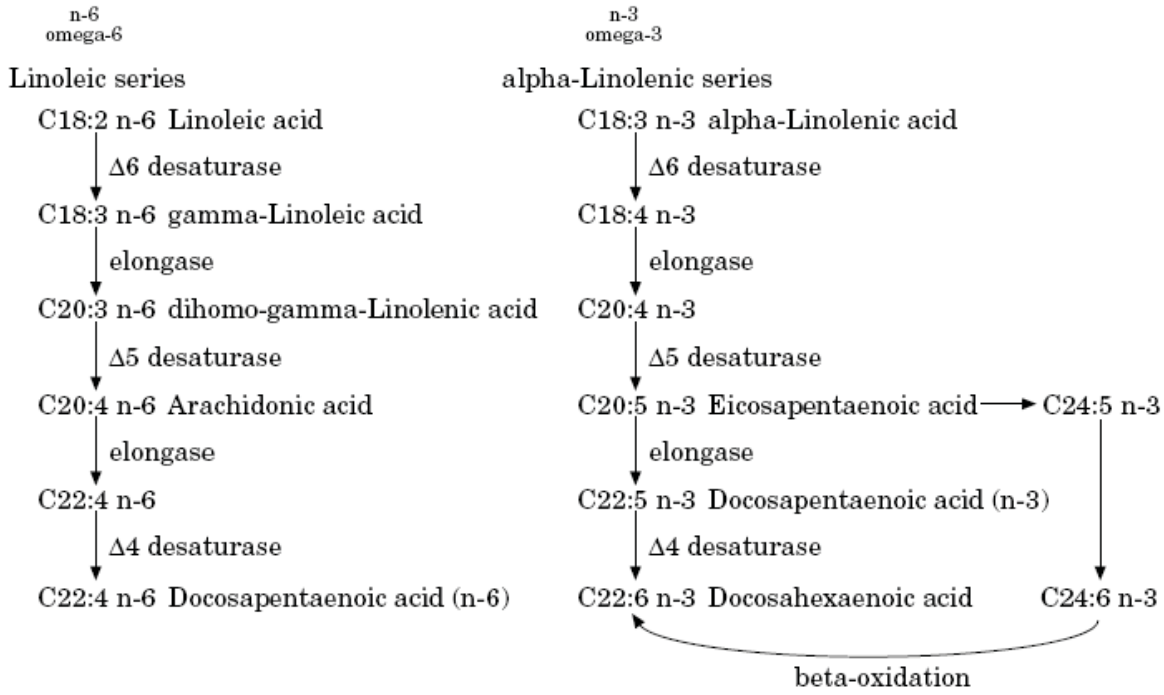


圖 2.7 多元不飽和脂肪酸的生合成作用(De Caterina & Basta, 2001)

3. DHA 的生理作用/功能

魚油中富含 n-3 多元不飽和脂肪酸(n-3 PUFAs)，例如 EPA 和 DHA，為魚油的活性成分，具有各種生物功能，會影響人類健康和疾病的發生，具有延緩動脈粥狀硬化，抗心律失常及改善血液流動等多種心血管效應(Kris-Etherton et al., 2002)。研究顯示，增加 n-3 PUFAs 的攝取可以透過以下途徑降低心血管疾病的發生率：(1)減少膽固醇堆積，舒緩動脈粥狀硬化斑的生長；(2)降低血清三酸甘油酯濃度，並能改善代謝症候群的情況(Schmidt et al., 1992; Jiménez-Gómez et al., 2010)；(3)預防血栓的形成，降低血液黏稠度，具有抑制血小板凝集等作用，攝入適當劑量的魚油可使 TXA2 平衡向有利方向轉變(Umemura et al., 1995)；(4)預防心律不整的發生，攝取魚油可降低心臟猝死的風險(Bucher et al., 2002; Leaf et al., 2003)；(5)降低血壓：DHA 可以透過影響血管結構從而產生降低血壓的作用(Diep et al., 2000)；(6)調節發炎反應，可能包括：①影響二十碳烷類化合物(eicosanoids)的合成，例如 n-6 PUFAs 攝入過多會生成前列腺素(PGI2)、白三烯素(LTB4)和血栓素(TXA2)，總體表現為較強的血小板凝集性和發炎反應，從而引起關節炎等，如果增加膳食中的 n-3 PUFAs 則能抑制這些相關的發炎反應(Calder, 2006)。②使膜成分發生改變，影響膜流動性(Chen et al., 2007; Chapkin et al., 2008)。③影響細胞激素的分泌(von Schacky, 2007)。④調控基因的表達：PUFAs 可以直接進入細胞核與核受體(nuclear receptor)或轉錄因子結合，進而影響許多發炎相關基因的表現；例如：n−3 PUFAs 可抑制 NF-κB 活性，由於 NF-κB 直接或間接調控一些發炎反應，減少黏附分子的生成(SanGiovanni & Chew, 2005; Chen et al., 2005; Chapkin et al., 2009)；n−3 PUFAs 也是 peroxisome proliferators activated receptors (PPARs)的自然配位子(ligands)，PPAR 也會透過影響 NF-κB 路徑而抑制發炎反應(Delerive et al., 2000, 2001; Moraes et al., 2006)，因此 n−3 PUFAs 對於調節 NF-κB 訊息路徑扮演著重要的角色。⑤調控某些酵素活性：富含 n-3 PUFAs 之魚油可透過誘發抗氧化酵素之表現，來抑制 ApoE 剃除小鼠心臟血管中動脈粥狀硬化斑之形成(Wang et al., 2004)。(7)改善血管內皮細胞功能(De

Caterina et al., 2000; Brown & Hu, 2001)。

表 2.5 n-3 脂肪酸降低心血管疾病風險的可能機制(Kris-Etherton et al., 2002)

Table 2.5 Potential Mechanisms by Which Omega-3 Fatty Acids May Reduce Risk for Cardiovascular Disease

Reduce susceptibility of the heart to ventricular arrhythmia
Antithrombogenic
Hypotriglyceridemic (fasting and postprandial)
Retard growth of atherosclerotic plaque
Reduce adhesion molecule expression
Reduce platelet-derived growth factor
Antiinflammatory
Promote nitric oxide-induced endothelial relaxation
Mildly hypotensive

表 2.6 n-3 脂肪酸與黏附分子之關係(Brown & Hu, 2001)

Table 2.6
In vitro studies of n−3 fatty acids and endothelial cell adhesion properties[1]

Reference	Year	Fatty acid and concentration	Methods	Outcome
De Caterina et al (57)	1994	DHA, EPA, oleate, and AA: 10 μmol/L	Adhesion molecule surface and mRNA expression in cytokine-stimulated HSVEC	DHA reduced VCAM-1 cell surface and mRNA expression
			Monocyte adhesion assays	DHA decreased monocyte adhesion
Weber et al (58)	1995	DHA, EPA, and AA: 20 μmol/L	Adhesion molecule surface and mRNA expression in cytokine-stimulated HUVEC	DHA reduced VCAM-1 cell surface and mRNA expression
			Monocyte adhesion assays	DHA reduced monocyte adhesion
Khalfoun et al (59)	1996	DHA, EPA, and AA: 100 mg/L	Adhesion molecule surface expression in cytokine-stimulated HUVEC	DHA and EPA decreased VCAM-1 cell surface expression
			PBL adhesion assays	DHA and EPA reduced PBL adhesion
De Caterina et al (60)	1998	DHA and ricinoleic, oleic, palmitoleic stearic, and palmitic acids: 25 μmol/L	Adhesion molecule surface and mRNA expression in cytokine-stimulated HSVEC	DHA reduced VCAM-1 cell surface and mRNA expression

[1] DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; HSVEC, human saphenous vein endothelial cells; HUVEC, human umbilical vein endothelial cells; PBL, peripheral blood lymphocytes; VCAM-1, vascular cell adhesion molecule 1; mRNA, messenger RNA.

美國心臟學會(American Heart Association)建議成年人應該攝取適量魚類。哈佛醫學院學者也認為，每週至少兩次攝入魚肉，如：鮪魚、金槍魚、沙丁魚等能阻止突發性猝死，因為魚肉中的不飽和脂肪酸可減少心律不整、預防心臟病復發、延緩心臟病患細胞老化(Kris-Etherton et al., 2002; Hu et al., 2002; Farzaneh-Far et al., 2010)。

表 2.7 n-3 脂肪酸建議攝取量(Kris-Etherton et al., 2002)

Table 2.7 Summary of Recommendations for Omega-3 Fatty Acid Intake

Population	Recommendation
Patients without documented CHD	Eat a variety of (preferably oily) fish at least twice a week. Include oils and foods rich in α-linolenic acid (flaxseed, canola, and soybean oils; flaxseed and walnuts)
Patients with documented CHD	Consume $\approx$1 g of EPA+DHA per day, preferably from oily fish. EPA+DHA supplements could be considered in consultation with the physician.
Patients needing triglyceride lowering	Two to four grams of EPA+DHA per day provided as capsules under a physician's care

三、血基質氧化酶(Heme oxygenase, HO)

1. 血基質氧化酶之分類

目前已知血基質氧化酶(Heme oxygenase, HO)有三種同功異構酶，分別為第一型血基質氧化酶(HO-1)，分子量約為 32 kDa；第二型血基質氧化酶(HO-2)，分子量約為 36 kDa；以及第三型血基質氧化酶(HO-3)，分子量約為 33 kDa (Farombi & Surh, 2006)。HO-1 屬於誘導表現型的酵素，會受到許多不同的刺激因素所誘發，如發炎反應、局部缺血(ischemia)、過氧症(hyperoxia)、缺氧(hypoxia)、高熱(hyperthermia)和某些重金屬(如鎘、銅、砷) (Farombi & Surh, 2006; Idriss et al., 2008)等。HO-1 廣泛分佈於脾臟、肝臟及網狀內皮系統中；HO-2 和 HO-3 則是持續表現於各組織細胞中，如腦、神經系統、肝臟、脾臟、睪丸以及心血管組織等(Siow et al., 1999)。HO-2 是生理狀態下的主要存在型式，能被腎上腺皮質素所調節(Raju et al., 1997)，但 HO-3 為單一轉錄產物，其活性顯著低於 HO-1 和 HO-2 (Hayashi et al., 2004)，目前對於 HO-3 相關的研究並不多，其在生理上所扮演的角色尚未清楚。HO-1 與 HO-2 的胺基酸序列有 43%的相似性，而 HO-3 是一種與 HO-2 非常近似的同功酶，其胺基酸序列與 HO-2 約有 90%的相似性(Siow et al., 1999; Hayashi et al., 2004)。

第一型血基質氧化酶(Heme oxygenase-1, HO-1)是體內重要的抗氧化酵素。HO-1 最早在 1964 年由 Wise 等人從細胞中將其分離並發現它可在體外將血基質(heme)分解產生膽綠素(biliverdin)。1968 年 Tenhunen 等人於大鼠肝臟、脾、腎的微粒體(microsome)中證實了血基質氧化酶的存在。HO-1 在正常生理狀態下只有少量表達，人類 HO-1 基因(hmox-1)位於染色體 22q12 位置，基因全長 6.8 kb (Lavrovsky, 1993)，主要可受到氧化壓力、發炎激素、重金屬等刺激，透過調控 antioxidant response element (ARE)而誘發其大量表現，由於 HO-1 可受到熱休克

反應活化，因此又稱為熱休克蛋白 32 (heat shock protein 32, Hsp32)，是一種內源性保護蛋白質(Farombi & Surh, 2006)。

2. HO-1 之生理角色

HO-1 基因被認為是一種因應體內環境變化的基因，也是目前發現受到最多因素誘導的壓力反應蛋白，HO-1 基因表達的調控主要發生在轉錄階段(Alam & Cook, 2003)。許多研究指出，當組織或細胞處於氧化壓力或損傷等情況時，均可誘導 HO-1 表現，這是身體的一種防衛性反應，調控組織或細胞來因應生理變化以維持其動態平衡的關鍵(Gruber et al., 2010)。另有文獻指出，天然存在的植化物(如 quercetin、resveratrol、curcumin 與 sulforaphane 等)亦會透過調控 HO-1 表現，改善細胞內的氧化壓力(Balogun et al., 2003; Lin et al., 2004; Juan et al., 2005; Farghali et al., 2009)。

HO-1 具有相當多的生理作用，已知有(1)抗發炎：增加 HO-1 表現已被證實與降低發炎反應有關(Takahashi et al., 2007; Kim et al., 2007; Lee et al., 2009)，透過降低內皮細胞之黏附分子與趨化物質的表現，直接或間接抑制發炎反應(Vachharajani et al., 2000; Soares et al., 2004; Lin et al., 2005; Yu et al., 2010)。這個概念可經由兩項遺傳發現來呈現，第一：缺乏 HO-1 之小鼠會增加其發炎狀況(Kapturczak et al., 2004; Tracz et al., 2007)，第二：HO-1 缺乏患者的發炎症狀是造成其死亡的原因之一(Kawashima et al., 2002; Koizumi, 2007)。(2)抗細胞凋亡：在小鼠初代肝細胞損傷模式下，增加 HO-1 表現有助於抑制細胞凋亡(Zuckerbraun et al., 2003)。(3)抗細胞增生(Morse & Choi, 2002)。(4)作為心血管疾病治療之標的：HO-1 的產物 CO 可能透過降低 p38 MAPK 磷酸化表現，並抑制 calcineurin/NFAT 途徑的活化來減少心肌肥大的發生(Tongers et al., 2004)；Ishikawa 等人(2001)發現在動脈粥狀硬化血管中，誘導 HO-1 會抑制血漿中脂質過氧化物的生成，說明高脂血症誘導的 HO-1 對動脈粥狀硬化的形成具有保護作用，並可能透過影響 NO

途徑來發揮作用。(5)避免器官移植排斥反應：增加 HO-1 活性可防止心血管受到局部缺血/再灌流(ischemia/reperfusion)的傷害，例如 HO-1 可以透過調節 NOS (nitric oxide synthase)表現及活性來降低糖尿病大鼠心肌缺血再灌流之損傷 (L'Abbate et al., 2007; Abraham & Kappas, 2008)。(6)調控細胞週期(cell cycle)：HO-1 會抑制血管平滑肌細胞(vascular smooth muscle cells, VSMC)的細胞週期，造成細胞停滯在 G1/S 期，同時也會調控 cyclin kinase inhibitor $p21^{Cip}$ (Duckers et al., 2001)；在 VSMC 中 CO 的增加，會抑制 E2F-1 的生成，E2F-1 在細胞週期中扮演調節 c-myc、cyclin 和 DNA polymerase 的角色(Morita & Kourembanas, 1995)。(7)抑制癌細胞侵襲和轉移：研究證實 HO-1 能抑制乳癌細胞侵襲和轉移的能力，其作用機轉與抑制 MMP-9 (Matrix metallo- proteinase-9)基因活化有關 (Lin et al., 2008)。(8)除了上述生理功能之外，最受到注目的則是其抗氧化能力，藉由調控 HO-1 表現及其代謝產物之作用，維持體內氧化還原狀態之恆定，降低細胞氧化壓力和調節多種細胞保護作用，在許多疾病發展過程中扮演重要的角色 (Slebos et al., 2003; Hwang & Jeong, 2008; Lee et al., 2009)。

3. HO-1 代謝產物對細胞的保護作用

HO-1 在氧分子(O_2)、NADPH、細胞色素 P450 還原酶(cytochrome P450 reductase)的參與下，可催化血基質(heme)降解為膽綠素(biliverdin)、一氧化碳 (carbon monoxide, CO)、游離鐵(Fe^{2+})，是血基質代謝過程中的速率限制酵素，它廣泛分佈於生物體內各種組織和器官，有著重要的生理功能(圖 2.8) (Farombi & Surh, 2006)。許多研究發現，HO-1 及其代謝相關產物可共同發揮抗發炎、抗氧化、抑制細胞凋亡和改善組織微循環等作用(Ryter et al., 2007)。HO-1 代謝相關產物的生理功能如下：

3-1. 膽綠素(biliverdin, BV)/膽紅素(bilirubin, BR)

HO-1 的代謝產物膽綠素(biliverdin)可進一步經膽綠素還原酶(biliverdin

reductase)的作用，轉變為膽紅素(bilirubin)。近年的研究證實，BR 是一種重要的內源性抗氧化分子，有強大的抗氧化能力，能有效地清除氧自由基，降低氧化壓力(Baranano et al., 2002)；保護心血管免於局部缺氧的傷害(Clark et al., 2000; Ollinger et al., 2007)；抑制 LPS 誘發黏附分子的作用，降低發炎反應(Vachharajani et al., 2000)；在以 LPS 誘發大鼠休克的模式下，發現 BV 可降低血清前發炎細胞激素濃度(Sarady-Andrews et al., 2005)；BR 也可透過抑制 E-selectin 和 VCAM-1 表現來降低內皮細胞的活化作用(Soares et al., 2004)；另外，BV/BR 的抗發炎作用可能與它們導致 NF-κB 失活有關(Soares et al., 2004; Sarady-Andrews et al., 2005)。

3-2. 一氧化碳(carbon monoxide, CO)

許多研究指出，內源性一氧化碳(CO)可作為一種訊息傳遞分子，活化可溶性鳥苷酸環化酶(soluble guanylate cyclase, sGC)，sGC 會進一步將 GTP 活化而形成環磷酸鳥苷(cGMP) (Morita et al., 1995)，繼而發揮廣泛的生理調節功能，包括調節血管舒張、支氣管擴張、抑制血小板凝集、減少血栓形成、減輕缺血再灌注損傷及抑制血管平滑肌細胞增生等作用(Slebos et al., 2003; Piantadosi, 2008)及維持微血管循環的平衡(Suematsu & Ishimura, 2000)。CO 被認為是負責 HO-1 大部分抗發炎作用的因子(Ryter et al., 2006)：在巨噬細胞中，CO 透過調控 p38 來抑制前發炎細胞激素 TNF-α 的產生(Otterbein et al., 2000)；亦有文獻指出，CO 可透過 MAPK 途徑(Otterbein et al., 2003)抑制纖維母細胞(fibroblast)或內皮細胞(endothelial cells)凋亡，細胞凋亡會加劇發炎反應，尤其是凋亡的血管內皮細胞會刺激血栓形成，因此 CO 的抗凋亡作用在細胞保護作用中十分重要。在人類 T 細胞中，CO 透過抑制 ERK 路徑來降低 IL-2 分泌及細胞大量增生(Pae et al., 2004)；在巨噬細胞中，CO 會透過調控 C/EBP 和 NF-κB 來抑制前發炎酵素 iNOS 和 COX-2 的表現(Suh et al., 2006)；在人類結腸上皮細胞，CO 透過調控 NF-κB、AP-1、C/EBP、和 MAPK 路徑來抑制 iNOS 表現以及 IL-6 分泌(Megías et al., 2007)。

3-3. 鐵離子(Fe^{2+})/鐵蛋白(ferritin)

鐵蛋白(ferritin)與 HO-1 的表現增加具有一致性(Balla et al., 2005)，雖然尚未完全證實鐵蛋白是否與 HO-1 的抗發炎作用相關，但是鐵蛋白的確是一種有效的抗氧化分子(Arosio et al., 2009)。二價鐵是一種易氧化的金屬離子，容易引起發炎反應；而當 HO-1 代謝血基質時會釋出二價鐵，二價鐵一旦被釋出，會被鐵蛋白快速地接收，增加鐵儲存的效率，因此大幅地限制它的促氧化/前發炎能力，以維持細胞內鐵離子濃度之恆定，故細胞內鐵蛋白含量提高時可抵抗氧化傷害，並產生壓力調適(stress adaptation)達到保護細胞之作用(Balla et al., 2005)；根據資料顯示，誘發 HO-1 表現會增加鐵蛋白合成，抑制發炎反應產生(Schaer et al., 2006)。

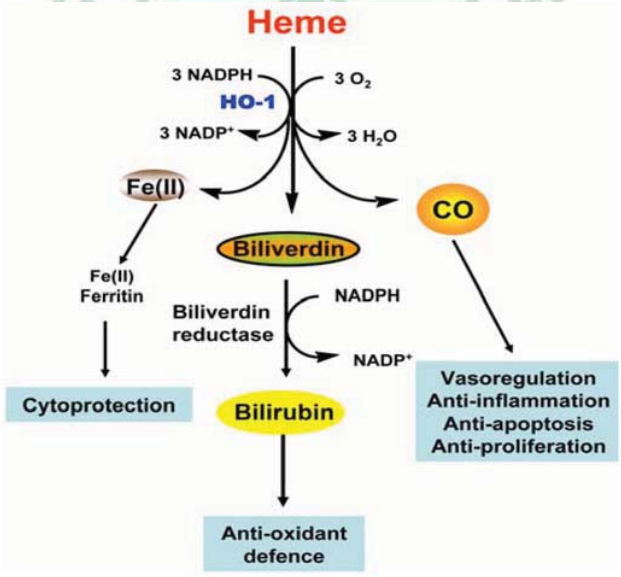


圖 2.8 HO-1 之作用及其代謝產物(Farombi & Surh, 2006)

4. 調控 HO-1 基因表現之訊息傳遞路徑

過去文獻指出，參與誘導 HO-1 基因表現之訊息傳遞路徑(signal transduction pathways)，主要包括 MAPKs (mitogen-activated protein kinase)、PI3K (phosphoinositide 3-kinase) /Akt、PKC 等(Owuor & Kong, 2002; Lee & Johnson,

2004 ; Xu et al., 2006; Ryter et al., 2006; Paine et al., 2010)；此外，分析 HO-1 基因的啟動子(promoter)區域，發現具有許多重要轉錄因子的結合位置，包括 NF-E2 (nuclear factor-erythroid 2)、AP-1、NF-κB 等，以調控 HO-1 的表現(圖 2.9) (Farombi & Surh, 2006; Alam & Cook, 2007; Gruber et al., 2010)。

4-1. 轉錄因子 Nrf2

Nrf2 (nuclear factor erythroid 2-related factor 2)為其重要調控者之一，因 Nrf2 會與相關抗氧化酵素基因上的一段調控序列 Antioxidant Response Element (ARE) 結合，故被證實與 HO-1 的表現有密切關係(Xu et al., 2006; Kim et al., 2007; Johnson et al., 2009)。Nrf2 屬於 Cap'n'Collar / basic leucine zipper (CNC-bZIP)轉錄因子家族的一個成員。在沒有刺激的情況下，Nrf2 藉由與 Keap1 (Klech-like ECH-associated protein 1)結合，被隔離在細胞質中；一旦受到活化，這個複合物將被瓦解，被釋放出的 Nrf2 得以轉移進入細胞核內，進而與 small Maf 家族的成員(i.e., MafK、MafG、MafF)組合成異質二聚體(Motohashi et al., 2002, 2004; Katsuoka et al., 2005)。除了 small Maf 家族的成員，Nrf2 也可能與 c-Jun 或 activating transcription factor 4 (ATF4)形成異質二聚體(heterodimers)，增強 ARE/EpRE (electrophile response element)-driven 報導基因活性，促進 HO-1 的轉錄(Venugopal & Jaiswal, 1998; He et al., 2001; Mann et al., 2007)。

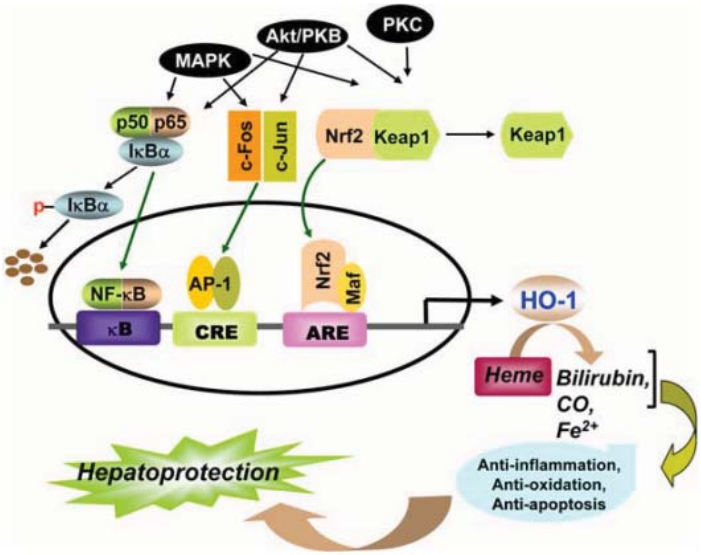


Figure 2.9

Regulation of HO-1 by transcription factors and their upstream kinases. Under normal conditions, transcription factors, such as NF-κB, AP-1 and Nrf2, are located in the cytosol. Upon external stimuli, the active forms of these transcription factors translocate to the nucleus where they bind to the specific DNA sequence leading to the transcription of HO-1 gene.

圖 2.9 調控 HO-1 表現之訊息傳遞路徑(Farombi & Surh, 2006)

4-2. 其它路徑(PI3K/Akt、MAPKs、PKC)

已有許多研究探討參與Nrf2活化的訊息傳遞路徑，例如PI3K和PKC可使Nrf2發生磷酸化，而PI3K抑制劑(LY-294002)或PKC抑制劑(Ro-32-0432)也可降低ARE luciferase報導基因的活性；由此推論，Nrf2之活化與PI3K和PKC的活性有關(Lee & Surh, 2005; Farombi & Surh, 2006; Keum et al., 2008)。此外，MAPKs也被證實參與Nrf2活化作用(Kong et al., 2001; Lee & Johnson, 2004; Xu et al., 2006)。目前已知多種具有生理活性的植化物(phytochemicals)，如異硫氰酸塩類(isothiocyanates)、引朵(indoles)、二烯丙基硫化物(diallyl sulfides)、黃酮類化合物(flavonoids)與薑黃素(curcuminoids)等，都有促進或抑制Nrf2結合至標的基因promoter上的作用(Jeong et al., 2006)。在HepG2細胞株模式下，辣椒素(capsaicin)可透過活化PI3K/Akt訊息傳遞路徑，增加Nrf2與ARE的結合，進而正向調控HO-1基因表現(Joung et al., 2007)；Carnosol誘發HO-1的表現也被認為與PI3K訊息路徑有關(Martin et al., 2004)。

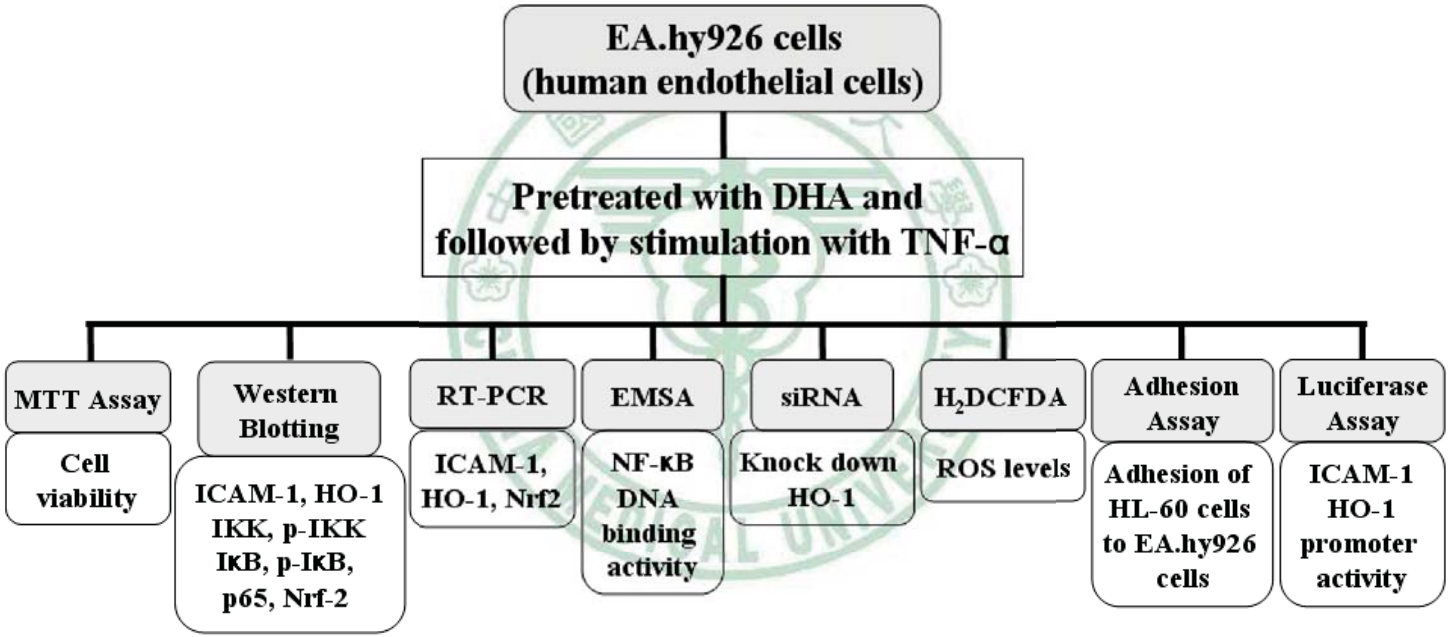
雖然很多的 phytochemicals 被證實可以誘發 HO-1 表現且具有保護細胞作用(cytoprotection)，但目前為止，DHA 誘發 HO-1 表現的研究相對較少。研究發現魚油中重要成分 DHA 可誘發 BV-2 microglia 表現 HO-1 (Lu et al., 2010)，其機制可能與 Akt 和 ERK 有關。另外，DHA 可透過 Nrf2-dependent 訊息傳遞來誘發 mouse peritoneal macrophages 表現 HO-1，進而抑制 LPS 誘發的發炎反應(Wang et al., 2010)。Gao 等人(2007)發現 DHA 需經過氧化作用後所產生的產物才具有誘發 Nrf2 表現和活化 ARE 序列的作用，而且他們推測 DHA 氧化產物也可能會與 Keap1 作用進而活化 Nrf2。

第三章 研究目的

根據衛生署的統計，心血管疾病一直佔居國內十大死因的前幾名，動脈粥狀硬化是心血管疾病的一種。臨床醫學證據顯示，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的形成；此外，研究發現細胞黏附分子的生成對於動脈粥狀硬化疾病的發展扮演重要的角色，黏附分子之一 ICAM-1 可作為一種發炎的生理指標，來預測白血球在內皮細胞中的黏附情形。TNF-α 是一種已知的前發炎細胞激素，會透過活化轉錄因子 NF-κB 來刺激黏附分子的表現，所以常被用來作為誘發細胞產生發炎反應的模式。

了解動脈粥狀硬化的形成並加以預防是目前預防醫學的重要課題之一，許多文獻指出魚油及其重要活性成分 DHA 具有抗發炎作用，並且會透過許多途徑來降低心血管疾病的發生率；而 HO-1 是體內重要的抗氧化酵素，主要會受到氧化壓力、發炎、化學物、重金屬等刺激誘發而大量表現，這是一種防衛性反應，調控組織或細胞來因應生理變化以維持其動態平衡的關鍵。本實驗室先前研究發現以穿心蓮內酯預處理 HUVECs 及 EA.926 細胞，可以抑制 TNF-α 所誘發的 ICAM-1 表現(Chao et al., 2011)。因此本實驗將利用 TNF-α 誘發內皮細胞 EA.hy926 產生發炎反應的模式，探討 DHA 是否可以藉由影響 NF-κB 訊息傳遞路徑來抑制 TNF-α 所誘發的 ICAM-1 表現，並且探討 DHA 所誘發的 HO-1 是否參與抑制發炎的機制，進而達到預防發炎疾病的功效。

實驗架構


EA.hy926 cells
(human endothelial cells)
Pretreated with DHA and
followed by stimulation with TNF-α
MTT Assay
Cell viability
Western Blotting
ICAM-1, HO-1
IKK, p-IKK
IκB, p-IκB,
p65, Nrf-2
RT-PCR
ICAM-1,
HO-1, Nrf2
EMSA
NF-κB DNA binding activity
siRNA
Knock down HO-1
H2DCFDA
ROS levels
Adhesion Assay
Adhesion of HL-60 cells to EA.hy926 cells
Luciferase Assay
ICAM-1
HO-1
promoter activity

第二部份

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

1. Introduction

Fish oils, rich in long-chain n-3 polyunsaturated fatty acids (n-3 PUFAs), especially eicosapentanoic acid (EPA, 20:5) and docosahexanoic acid (DHA, 22:6), are well known for their anti-inflammatory (Mullen et al., 2010), immunoregulatory (Simopoulos, 2002), anti-aging (Jicha et al., 2010), and anti-tumor (Ghosh-Choudhury et al., 2009) properties. Additionally, EPA and DHA were shown to possess anti-arrhythmic effect (Leaf et al., 2005). Epidemiological studies have provided evidence indicating that n-3 PUFAs supplementation regulates inflammation partially via improvement of endothelial functions (Brown & Hu, 2001). DHA was shown to significantly decrease the cytokine-induced adhesion molecule expression (Chen et al., 2003), diminish the adhesion of leukocytes to the activated endothelial cells (De Caterina et al., 2000; Mayer et al., 2002), and inhibit production of cytokines by endothelial cells (Novak et al., 2003; von Schacky, 2007). It has been demonstrated that treatment with n-3 PUFAs suppressed ICAM-1 and VCAM-1 expressions in TNF-α, IL-1, and VEGF-stimulated endothelial cells (Chen et al., 2005), with DHA being more potent than EPA (Weldon et al., 2007). It is reported that DHA affects several target genes via inhibition of the NF-kB activation (Chapkin et al., 2009; Wang et al., 2011). Dietary intake of n-3 PUFAs is associated with a reduced risk of

atherosclerosis (Kris-Etherton et al., 2002; Paulo et al., 2008), and this is considered to play a pivotal role in the prevention of cardiovascular disease (CVD).

In recent years, it has been recognized that inflammation is a major contributing factor to many cardiovascular events (Blake, 2001). Atherosclerosis, a chronic inflammatory disease of the vasculature, is characterized by infiltration of leucocytes (Blankenberg et al., 2003), deposition of lipids and thickening of the vascular wall in response to cytokines (Ross, 1999; Lusis, 2000), and it increasingly threatens human health worldwide (Hansson & Libby, 2006). Leukocyte recruitment is a multistep process and this process is predominantly mediated by cellular adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and selectins, which are expressed on the surface of epithelial and endothelial cells in response to several inflammatory stimuli, including oxidized LDL, free radical species, lipopolysaccharide (LPS), and cytokines, such as tumor necrosis factor- alpha (TNF-α), interleukin-1β (IL-1β), and interferon-gamma (INF-γ) (Roebuck & Finnegan, 1999; Blankenberg et al., 2003). Studies have shown that TNF-α, the pro-inflammatory cytokine, is commonly found in atherosclerotic lesions and can induce expression of ICAM-1 and VCAM-1, which are critically dependent on the activation of nuclear factor-κB (NF-κB) (Liu, 2005; Oh et al., 2010). NF-κB is an important transcription factor regulating the expression of many inflammatory response genes such as adhesion molecules and cytokines (Luo et al., 2005). In quiescent cells, NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory kappa B (IκB) family (Sun & Karin, 2008). In response to stimulation, IκB-α is phosphorylated at Ser32 and 36 by the IκB kinase (IKK) complex (May & Ghosh, 1998; Karin & Delhase, 2000) and subsequently degraded by the ATP-dependent 26S proteasome complex (Chen et al., 1995; Wertz & Dixit, 2010).

IκB degradation frees NF-κB and allows NF-κB translocation to the nucleus, where it can bind to the κB element of promoter of target genes (Rahman & McFadden, 2011).

Heme oxygenase (HO)-1 is an inducible enzyme responsible for the rate-limiting step of heme degradation and produces carbon monoxide (CO), free iron and biliverdin (BV), which is further converted into bilirubin (BR) via biliverdin reductase (Farombi & Surh, 2006; Abraham & Kappas, 2008). HO-1 can be triggered by a variety of stress-related cellular stimuli, including its substrate heme, heavy metals, oxidative stress, UV radiation, inflammatory cytokines, hypoxia, and ischemia-reperfusion (Farombi & Surh, 2006; Idriss et al., 2008). The physiological relevance of the HO-1 expression has been reported in several pathological states such as atherosclerosis and inflammation, wherein it confers cytoprotection (Morita, 2005; Idriss et al., 2008; Lee et al., 2009; Paine et al., 2010; Kim et al., 2010). HO-1 induction reduces atherosclerotic lesion size in Watanabe heritable hyperlipidemic rabbits (Ishikawa et al., 2001a) and in LDL-receptor knockout mice (Ishikawa et al., 2001b). Moreover, transgenic mice deficient in HO-1 of an apolipoprotein E null background (Yet et al., 2003) exhibited accelerated and more advanced atherosclerotic lesion formation in response to a Western diet. Nevertheless, recent evidence suggests that by-products of HO-1, alone or in concert, mediate the protective effects of HO-1 (Kirkby & Adin, 2006; Ryter et al., 2006, 2007). Bilirubin is an endogenous radical scavenger with recently recognized antioxidant, anti-inflammatory, anti-proliferative properties (Ollinger et al., 2007). The release of free iron is rapidly sequestered into the iron storage protein, ferritin, leading to additional antioxidant and anti-apoptotic effects (Arosio et al., 2009). CO exerts several biological functions, including anti-apoptotic, anti-inflammatory, and vasodilatory effects (Kirkby & Adin, 2006; Ryter et al., 2006, 2007). HO-1 expression is primarily regulated at the transcriptional

level (Alam & Cook, 2003), and its inducibility by diverse inducers is linked to the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf-2) (Shan et al., 2006; Kim et al., 2007). Under basal conditions, Nrf2 is sequestered in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al., 2004; Kaspar et al., 2009). When disrupted by electrophilic antioxidants, Nrf2 is released from Keap1 and translocates to the nucleus, dimerizes with Maf, and activates transcription of genes containing the antioxidant response element (ARE) sequences in the promoter regions (Owuor & Kong, 2002; Katsuoka et al., 2005; Kobayashi & Yamamoto, 2005; Kensler et al., 2007).

Although anti-inflammatory effect of DHA (n-3, 22:6) has been studied before, the molecular mechanism underlying DHA-mediated inhibition of TNF-α-induced ICAM-1 expression in human vascular endothelial cells still remains unclear. The aim of this study was to evaluate the effect of DHA on the adhesion of monocytes to TNF-α-activated endothelial cells which is mediated by adhesion molecules such as ICAM-1, as well as the molecular mechanisms underlying DHA inhibition of ICAM-1 expression.

2. Materials and Methods

2.1 Chemicals

Dulbecco's modified Eagle medium (DMEM), RPMI 1640, RPMI-1640 (without phenol red), OPTI-MEM, and penicillin/streptomycin were from GIBCO/BRL (Grand Island, NY); 0.25% trypsin-EDTA was from BioWest (Miami, FL); fetal bovine serum (FBS) was from HyClone (Logan, UT); docosahexaenoic acid (DHA) was from Cayman Chemical (Ann Arbor, MI); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium bicarbonate, human tumor necrosis factor-alpha (TNF-α) and anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO); Z-Leu-Leu-Leu-CHO (MG-132) was from Boston Biochem (Cambridge, MA); 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) was from Molecular Probes (Eugene, OR); H_2DCFDA and TRIzol reagent were from Invitrogen (Carlsbad, CA); antibody against HO-1 was obtained from Calbiochem (Darmstadt, Germany); antibodies against Nrf2, IκBα, IKKα/IKKβ, JNK, phospho-JNK, ERK, and p38 were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against ICAM-1, phospho-IκBα (Ser32/36), phospho-IKKα (Ser180)/IKKβ (Ser181), PARP, phospho-ERK, and phospho-p38 were from Cell Signaling Technology (Boston, MA); antibody against p65 was from BD Bioscience (San Jose, CA).

2.2 Cell cultures

The human endothelial cell line EA.hy926 was a kind gift from Dr. T. S. Wang, Chung Shan Medical University, Taichung, Taiwan, and was cultured in DMEM supplemented with 3.7 g/L $NaHCO_3$, 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37^{o}C in a 5% CO_2 humidified incubator. Human leukemia promyelocytic cells (HL-60) were obtained from Bioresources Collection and

Research Center (BCRC, Hsinchu, Taiwan). The HL-60 cells were cultured in T-75 tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/L streptomycin at 37°C in a 5% CO_2 humidified incubator.

2.3 Fatty acid preparation

DHA samples were prepared and complexed with fatty acid-free bovine serum albumin at a 6:1 molar ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

2.4 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 DHA (0-100 μM) for 24 h followed by incubation with 1 ng/mL TNF-α for an additional 6 h. Finally, the 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 14,000×g 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 control group was regarded as 100% cell viability.

2.5 Nuclear extracts preparation

After each experiment, cells were washed twice with cold PBS and were then scraped from the dishes with 1,000 μL of PBS. Cell homogenates were centrifuged at 2,000×g 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 (10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5% NP-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM PMSF). After centrifugation at 6,000×g for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer (10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2 mM PMSF, and 10% glycerol) at 4^{o}C for 30min. The samples were then centrifuged at 10,000×g for 15min. The supernatant containing the nuclear proteins was collected and stored at -80^{o}C until the Western blotting and electrophoretic mobility shift assays.

2.6 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 14,000×g for 20 min at 4^{o}C. The resulting supernatant was used as a cellular protein for Western blotting analysis. The total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL). Equal amounts of cellular proteins were electrophoresed in a sodium dodecyl sulfate (SDS)-polyacrylamide gel, and proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Nonspecific binding sites on the membranes were blocked with 5% nonfat milk in 15 mM Tris/150 mM NaCl buffer (pH 7.4) at room temperature for 2 h.

Membranes were probed with 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 scanned by a luminescent image analyzer (LAS-4000, FUJIFILM, Japan). The bands were quantitated with ImageGauge software (FUJIFILM).

2.7 RNA isolation and RT-PCR

Total RNA of EA.hy926 cells was extracted by using TRIzol reagent. After treatment, cells were washed twice with cold PBS and scraped with 500 μL of TRIzol reagent. Cell samples were mixed with 100 μL of chloroform and centrifuged at 11,000×g for 15 min. The supernatant was collected and mixed with 250 μL of isopropyl alcohol. After centrifuged at 11,000×g for 15 min, the supernatant was discarded and the cell pellet was stored in 70% ethanol or dissolved in deionized water for quantification. We used 0.2 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’-CCTGCTTCACCACCTTCTTG-3’) were designed on the basis of published

sequences (Meagher et al., 1994). Amplification of ICAM-1 and GAPDH were achieved when samples were heated to 95^{o}C for 5 min and then immediately cycling 32 times through a 1-min denaturing step at 94^{o}C, a 1-min annealing step at 56^{o}C, and a 1-min elongation step at 72^{o}C. Amplification of HO-1 and GAPDH were achieved when samples were heated to 95^{o}C for 5 min and then immediately cycling 39 times through a 1-min denaturing step at 95^{o}C, a 1-min annealing step at 55^{o}C, and a 2-min elongation step at 72^{o}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 a Digital Image Analyzer (Alpha Innotech) and quantitated with ImageGauge software.

2.8 Electrophoretic mobility shift assay (EMSA)

EMSA was performed according to our previous study (Cheng et al., 2004). The LightShift Chemiluminescent EMSA Kit and synthetic biotin-labeled double-stranded NF-κB consensus oligonucleotides (forward, 5'-AGTTGAGGGGACTTTCCCAGGC -3'; reverse, 5'-GCCTGGGAAAGTCCCCTCAACT-3') were used to measure the NF-κB nuclear protein-DNA binding activity. Nuclear extract (4 μg), poly (dI-dC), and biotin-labeled double-stranded NF-κB oligonucleotides were mixed with the binding buffer (to a final volume of 20 μL) and were incubated at 27^{o}C for 30 min. In addition, the unlabeled and mutant double-stranded NF-κB oligonucleotides (5'-AGTTGAGGCGACTTTCCCAGGC-3') were used to confirm the protein binding specificity, respectively. These oligonucleotide primers were synthesized by MDBio Inc. (Taipei, Taiwan). The nuclear protein-DNA complex was separated by electrophoresis on a 6% TBE-polyacrylamide gel and then were transferred to Hybond-N^{+} nylon membranes (Amersham Pharmacia Biotech, Inc., Pisscataway, NJ). Next, the membrane were cross-linked by UV light for 10 min and treated with

streptavidin-horseradish peroxidase, and the nuclear protein-DNA bands were developed with Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). The bands were scanned by a luminescent image analyzer.

2.9 Plasmids, transfection, and luciferase assay

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. (2001) was introduced into the pGL3 promoter plasmid. The ICAM-1 promoter-luciferase construct (pIC339, -339 to 0) was a gift from Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). pIC339 contains NF-κB (-187/-178), AP-1 (-84/-279), AP-1 (-48/-41), and Sp1 (-59/-53, -206/-201) binding sites (van de Stolpe et al., 1994). All subsequent transfection experiments were performed by using nanofection reagent (PAA, Pasching, Austria) according to the manufacturer's instructions. EA.hy926 cells were transiently transfected with 0.4 μg of pIC339 or pGL3 plasmid and 0.2 μg of β-galactosidase plasmid by using 1 μL of nanofectin in OPTI-MEM medium for 8 h. After transfection, cells were changed to DMEM medium and treated with DHA for 16 h before being challenged with TNF-α for an additional 6 h. Cells were then washed twice with cold PBS, scraped with lysis buffer, and centrifuged at 14,000×*g* for 3 min. The supernatant was collected for the measurement of luciferase and β-galactosidase activities by using a Luciferase Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions, and the luciferase activity was measured by a microplate luminometer (TROPIX TR- 717, Applied Biosystems). The luciferase activity of each sample was corrected on the basis of β-galactosidase activity, which was measured at 420 nm with *O*-nitrophenyl-beta-D-galactopyranoside as a substrate.

2.10 RNA interference by small interfering RNA of HO-1 and Nrf2

Predesigned small interfering RNA (siRNA) against human HO-1, Nrf2, and non-targeting control-pool siRNA were purchased from Dharmacon Inc. (Lafayette, CO). EA.hy926 cells were transfected with HO-1 and Nrf2 siRNA SMARTpool by using DharmaFECT1 transfection reagent (Thermo) according to the manufacturer's instructions. The four siRNAs against the human HO-1 gene are (1) AUGCUGAGUUCAUGAGGAA, (2) ACACUCAGCUUUCUGGUGG, (3) CAGUUGCUGGUAGGGCUUUA, and (4) AGAUUGAGCGCAACAAGGA. The 4 siRNAs against the human Nrf2 gene are (1) UAAAGUGGCUGCUCAGAAU, (2) GAGUUACAGUGUCUUAAUA, (3) UGGAGUAAGUCGAGAAGUA, and (4) CACCUUAUAUCUCGAAGUU. Non-targeting siRNA construct (NC) was used as negative control. Specific silencing was confirmed by at least three independent Western blotting assays with cellular extracts 8 h after transfection.

2.11 Peroxide measurement

Detection of intracellular oxidative states was performed by using the probe 2,7-dichlorofluorescin diacetate (H_2DCF-DA) (Molecular Probes Inc., Eugene, OR) (Bae et al., 1997). Briefly, cells were grown to 60-70% confluence and then serum-starved in DMEM supplemented with 0.5% (v/v) FBS for an additional 2 days. The cells were then stabilized in serum-free DMEM without phenol red for at least 30 min before exposure to DHA or TNF-α for the indicated time periods. Cells were then incubated for 10 min with the ROS-sensitive fluorophore H_2DCF-DA (10 μM). Cells were immediately observed under a laser-scanning Confocal microscope (Leica TCS SP2). DCF fluorescence was excited at 488 nm using an argon laser, and the evoked emission was filtered with a 515-nm long pass filter.

2.12 Monocyte adhesion assay

EA.hy926 cells in 12-well plates were allowed to grow to 80% confluence and were then pretreated with 50 and 100 μM DHA for 16 h followed by incubation with 1 ng/mL TNF-α 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 acetoxymethyl ester (BCECF-AM). At the end of the DHA and TNF-α treatment, a total of 1×10^6 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 100×g 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 3,000-80,000 cells/well. The results are reported on the basis of the standard curve obtained.

2.13 Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference among mean values was determined by one-way analysis of variance followed by Tukey's test and the difference between mean values was determined by student's t-test; *P* values <0.05 were taken to be statistically significant.

3. Results

3.1 Cell viability

The MTT assay was used to test whether the concentration of DHA used in the presence of TNF-α caused cell damage. As shown in Fig. 1, there were no adverse effects on the growth of EA.hy926 cells up to a concentration of 100 μM DHA in the presence of 1 ng/ml TNF-α, which was used to induce the expression of ICAM-1. The highest concentration of DHA used in the present study was 100 μM, and thus the effects of DHA observed were not due to its cytotoxicity.

3.2 DHA inhibits TNF-α-induced ICAM-1 expression, promoter activity and HL-60 cell adhesion

To examine whether the TNF-α-induced ICAM-1 protein and mRNA expressions were affected by DHA in EA.hy926 cells, 50 and 100 μM DHA were used. The protein expression of ICAM-1 was significantly suppressed by DHA pretreatment in dose-dependent as well as time-dependent manners (Fig. 2A). In parallel, DHA also inhibited TNF-α-induced ICAM-1 mRNA expression (Fig. 2B). To investigate the role of DHA in TNF-α-induced ICAM-1 gene transcription, promoter activity assays were performed with a human ICAM-1 promoter-luciferase construct, pIC339 (-339 to 0). TNF-α-induced ICAM-1 promoter activity was inhibited by 50 and 100 μM DHA, and a significant effect was observed at 100 μM (Fig. 2C). Also, DHA decreased the adhesion of HL-60 cells to TNF-α-stimulated endothelial cells (Fig. 2D).

3.3 DHA inhibits TNF-α-induced NF-κB signaling pathway

Previous studies have shown that TNF-α induces ICAM-1 and VCAM-1

expressions through NF-κB signaling pathway in human epithelial and endothelial cells (Kim et al., 2001; Zhou et al., 2007; Oh et al, 2009, 2010; Wang et al., 2011). There are several putative recognition sequences for a variety of transcriptional activators, including AP-1, retinoic acid-response element (RARE), C/EBP, NF-κB, Ets-1, interferon-stimulated response element (IRE), Sp1, and AP-2, in the human proximal ICAM-1 promoter-enhancer region (-346 to -24) (Huang et al., 2005). Among these, NF-κB binding to the κB binding site in the promoter plays an important role in TNF-α-induced ICAM-1 expression by activating IKKα/β. Accordingly, we next determined whether NF-κB activation was inhibited by DHA. As shown in Fig. 3A, TNF-α induced both IKKα and IKKβ phosphorylation, and the activation of IKKα and IKKβ was significantly attenuated by DHA pretreatment. In addition to the activation of IKKα/β, TNF-α caused the phosphorylation and degradation of IκBα at 5, 10, and 15 min, respectively. However, the phosphorylation induced by TNF-α was attenuated by pretreatment with DHA at 5 and 10 min, and the degradation was decreased by pretreatment with DHA or MG132 (a proteasome inhibitor) (Fig. 3B). To investigate the effect of DHA on TNF-α-induced NF-κB activation, the nuclear translocation of p65 was determined by Western blotting. Nuclear translocation of p65 was induced by TNF-α, and this effect was attenuated by 50 μM DHA pretreatment (Fig. 3C). A significant effect was observed for pretreatment 8 h and thereafter. EMSA further revealed that TNF-α increased NF-κB nuclear protein-DNA complex formation, and that pretreatment with DHA resulted in the inhibition of NF-κB nuclear protein-DNA binding activity (Fig. 3D).

3.4 DHA increases HO-1 expression in the presence of TNF-α

The stress-responsive gene encoding HO-1 has been recognized to be a protective gene in EC (Soares et al., 2004). To evaluate whether the inhibition of the

TNF-α induced expression of ICAM-1 by DHA was associated with the up-regulation of HO-1, EA.hy926 cells were pretreated with DHA for 8 h before exposure to TNF-α for an additional 6 h. As shown in panels A and B of Fig. 4, TNF-α did not affect either protein or mRNA expression of HO-1. However, DHA pretreatment significantly enhanced both protein and mRNA levels of HO-1 in a concentration-dependent manner.

Furthermore, to investigate whether the induction of HO-1 expression by DHA arises from increased mRNA or protein synthesis, actinomycin D (Act D), an inhibitor of transcription or cycloheximide (CHX), a protein synthesis inhibitor, was added to the cells before the addition of DHA. The results revealed that Act D blocked both HO-1 mRNA and protein levels, whereas CHX attenuated HO-1 protein expression (Fig. 4C). This suggested that DHA induced HO-1 expression at the transcriptional level, which was in agreement with previous studies (Hill-Kapturczak et al., 2000; Lin et al., 2008; Hwang & Jeong, 2008) showing the induction of HO-1 expression including both de novo transcription and translation.

3.5 DHA induces Nrf2 protein accumulation, nuclear translocation and the ARE-luciferase reporter activity

The majority of genes encoding phase II xenobiotic detoxifying and antioxidant enzymes have an enhancer region that contains an ARE sequence (Lee & Johnson, 2004). Nrf-2 is the major transcription factor that regulates ARE-driven gene expression (Nguyen et al., 2004). Thus, we attempted to verify whether DHA could activate Nrf2 in association with HO-1 up-regulation. The results showed that DHA increased protein levels of Nrf2 in a dose-dependent manner; however, Nrf2 mRNA expression was not affected by DHA (Figs. 5A and B). This indicated that the increased Nrf2 accumulation by DHA was possibly due to decreased Nrf2 degradation

instead of de novo synthesis of Nrf2. In order to clarify the decreased Nrf2 degradation by DHA, we assayed the effect of DHA on Nrf2 ubiquitination and proteasome activity. In addition, DHA treatment increased Nrf2 accumulation in the nucleus as early as 0.5 h, and this accumulation was sustained until 6 h (Fig. 5C).

Afterward, we used cells transfected with luciferase reporter vectors carrying the ARE sequence of HO-1 to ascertain the specificity of DHA for this activation. DHA (25-100 μM) potently induced the ARE-luciferase activity in a dose-dependent manner (Fig. 5D). This demonstrated that DHA-stimulated HO-1 gene expression through Nrf2/ARE signaling. Zhang and Gordon (2004) substantiated that an increase in the Nrf2 nuclear level is required for the activation of ARE.

3.6 HO-1 siRNA alleviates the inhibition of TNF-α-induced ICAM-1 expression and p65 translocation by DHA

The role of HO-1 in the inhibition of TNF-α-induced ICAM-1 expression by DHA was confirmed by using the siRNA system to create a HO-1 knockdown model. EA.hy926 cells were transfected with siHO-1 plasmids for 8 h, followed by treatment with 100 μM DHA for 16 h and TNF-α for an additional 6 h. Control cells were transfected with nontargeting siRNA constructs (NTC). The efficiency of the siRNA system to silence HO-1 was ascertained by Western blot and RT-PCR assay. As shown in Figs. 6A and B, HO-1 siRNA partially abolished the inhibition of TNF-α-induced ICAM-1 expression by DHA. These findings implicate that the induction of HO-1 by DHA plays a role in the suppression of TNF-α-induced ICAM-1 expression. To further identify whether HO-1 inhibited ICAM-1 expression is through suppressing NF-κB activation, we took the advantage of HO-1 siRNA to determine its effect on NF-κB activation. The results indicate that DHA-mediated suppression of p65 nuclear translocation was partially reversed (Fig. 6C).

3.7 PI3K/Akt and ERK1/2 pathways are involved in DHA-induced HO-1 expression

To further elucidate the upstream signaling pathways involved in DHA-mediated induction of HO-1, we examined the activation of PI3K/Akt and MAPKs in EA.926 cells. Cells were incubated with 100 μM DHA for the indicated times. As shown in Fig. 7A, induction of Akt and phosphorylation of p38 and ERK were detected in DHA-treated cells by Western blot analysis.

To corroborate the role of individual Akt and MAPKs pathways in HO-1 expression by DHA, we examined the effects of LY294002, PD98059 and SB203580, specific inhibitors for the PI3K/Akt, ERK and p38 pathways, respectively, on DHA-induced HO-1 expression. Inhibitor of p38 had no effect on DHA-induced HO-1 expression; however, the inhibitor of the PI3K/Akt and ERK pathways significantly reduced DHA-induced HO-1 expression (Fig. 7B). The results suggest that HO-1 induction by DHA is via PI3K/Akt and ERK pathways.

3.8 DHA triggers early-phase reactive oxygen species (ROS) production in EA.hy926 cells

Electrophile responsive element (EpRE)/ARE-mediated gene induction plays a critical role in cellular defense against the toxicity of electrophiles and reactive oxygen species (ROS). Nrf2, which belongs to the cap-'n'-collar family of basic region-leucine zipper transcription factors, has been recognized as an essential component of an EpRE/ARE-binding transcriptional complex (Itoh et al., 2004). With regard to the induction of HO-1 expression which is regulated by Nrf2, we next testify whether this would be the result of oxidative stress imposed by DHA. An increase of cellular ROS began at 5 min of cells treated with 100 μM DHA, and a

peak increase was observed at 20 min as measured by the DCF probe (Fig. 8). These results imply that short-term exposure to DHA leads to oxidative stress which may be involved in the activation of Nrf2.

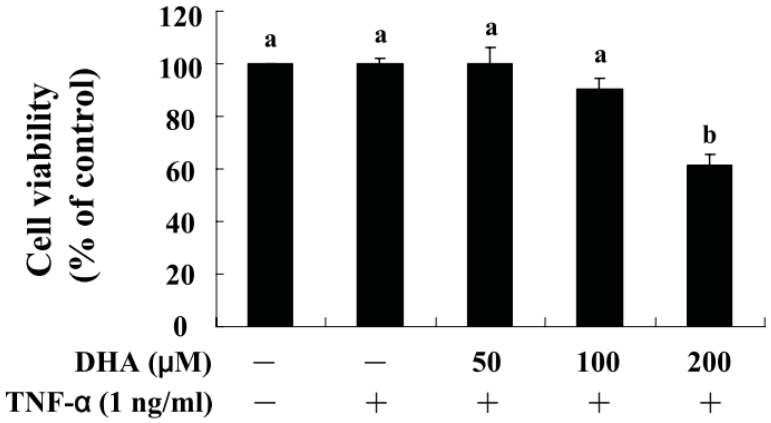
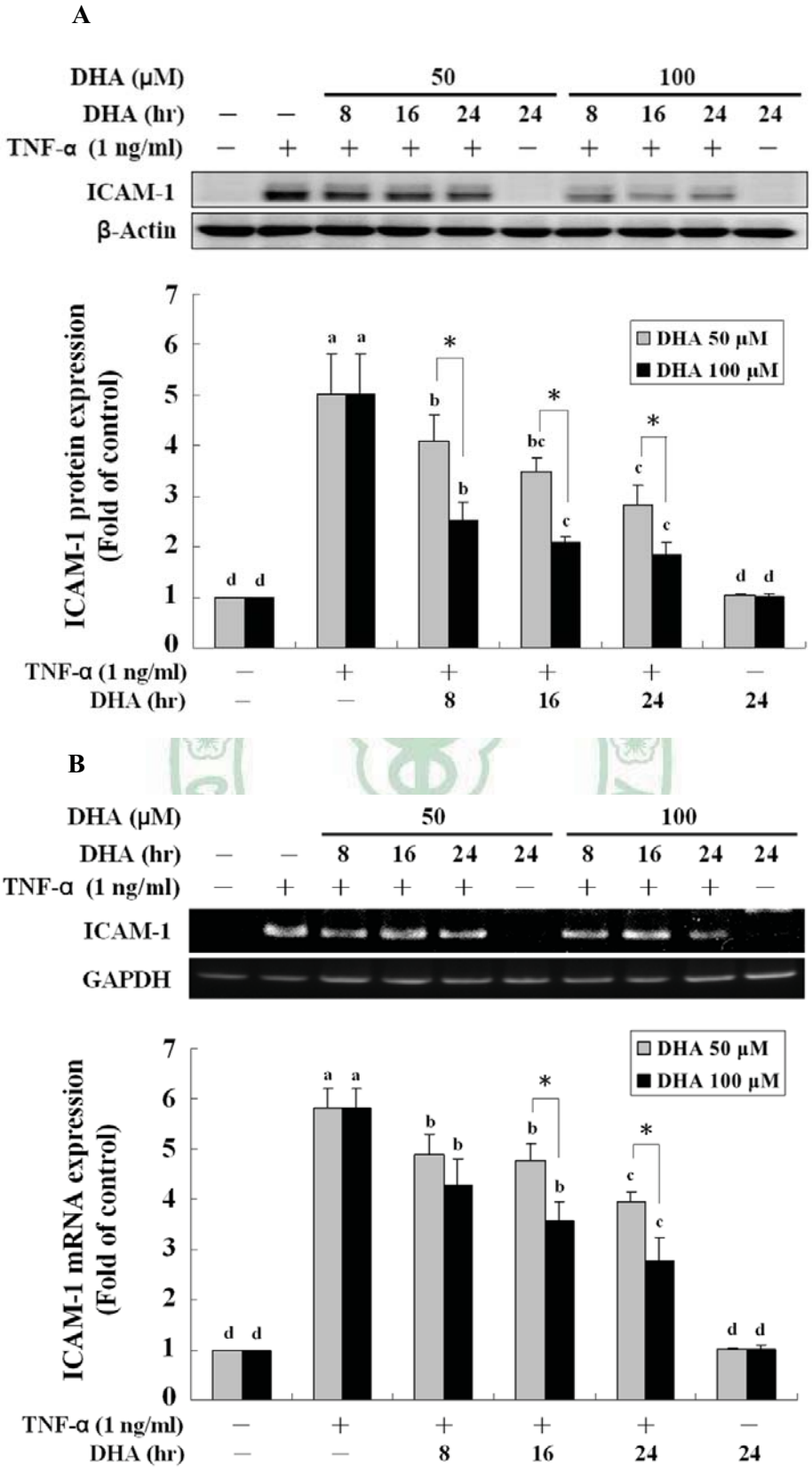


Figure 1. Effect of DHA on the cell viability of EA.926 cells in the presence of TNF-α.

Cells were pretreated with 50-200 μM DHA for 24 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 expressed as mean ± SD of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$).


A
DHA (μM)
50
100
DHA (hr)
– – 8 16 24 24 8 16 24 24
TNF-α (1 ng/ml)
– + + + + – + + + –
ICAM-1
β-Actin
ICAM-1 protein expression (Fold of control)
DHA 50 μM
DHA 100 μM
TNF-α (1 ng/ml)
DHA (hr)
B
DHA (μM)
50
100
DHA (hr)
– – 8 16 24 24 8 16 24 24
TNF-α (1 ng/ml)
– + + + + – + + + –
ICAM-1
GAPDH
ICAM-1 mRNA expression (Fold of control)
DHA 50 μM
DHA 100 μM
TNF-α (1 ng/ml)
DHA (hr)

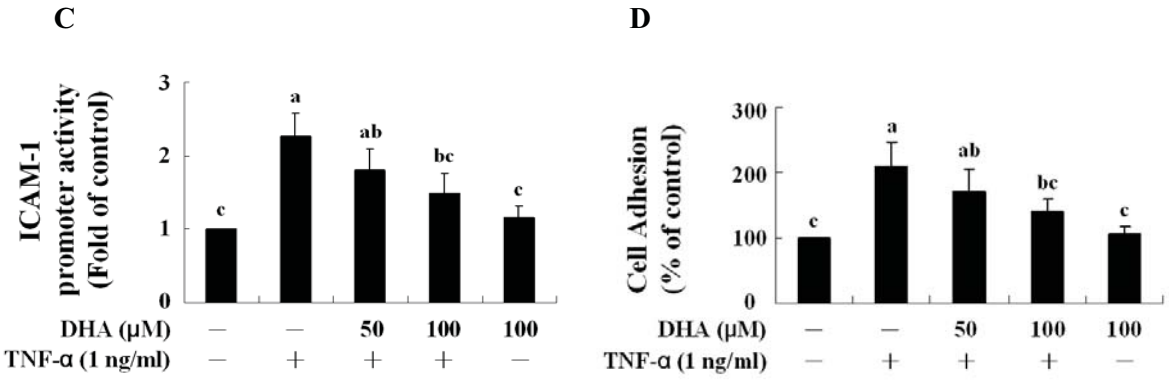
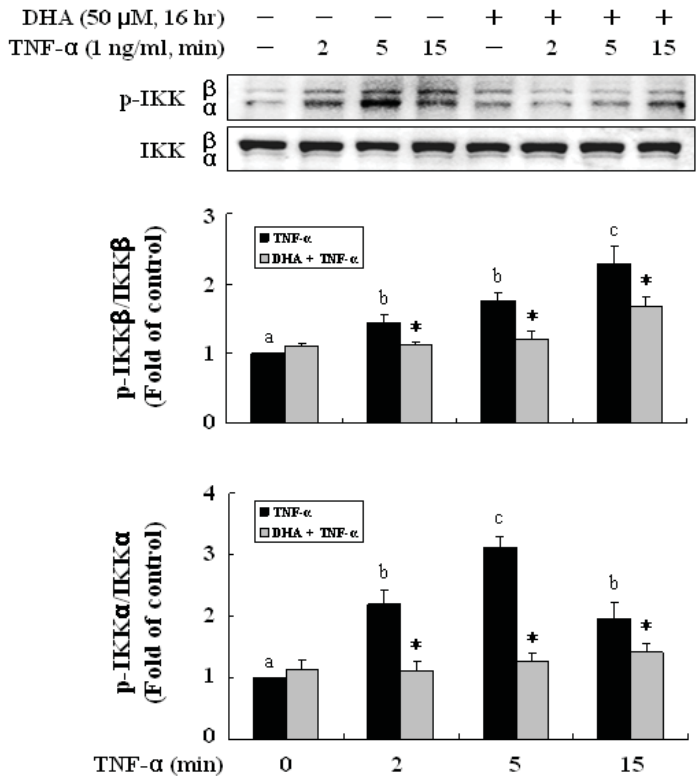


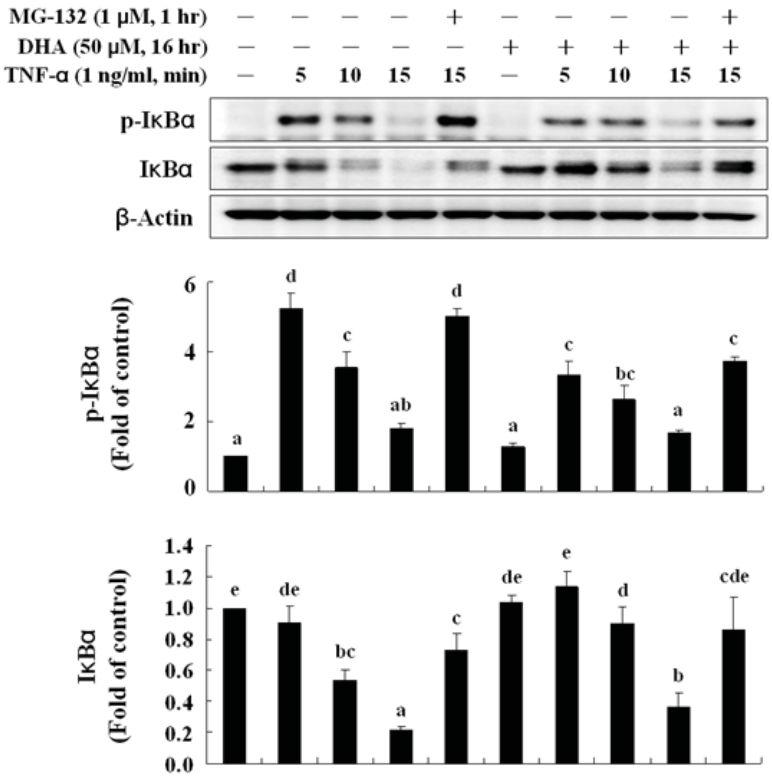
Figure 2. DHA decreases TNF-α-induced ICAM-1 expression, promoter activity and HL-60 cell adhesion.

(A) Cells were pretreated with 50 and 100 μM DHA for the indicated times and then stimulated with 1 ng/ml TNF-α for another 6 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (B) Total RNA was isolated from cells and subjected to RT-PCR with specific ICAM-1 and GAPDH primers as described in Materials and Methods. (C) Cells transfected with the pIC339 luciferase expression vector were pretreated with 50 and 100 μM DHA for 16 h followed by incubation with 1 ng/ml TNF-α for an additional 6 h. Cells were then lysed and analyzed for luciferase activity. Luciferase activity was assayed as described in Materials and Methods. Induction is shown as an increase in the normalized luciferase activity in the treated cells relative to the control. (D) Cells were pretreated with 50 and 100 μM DHA for 16 h before being challenged with 1 ng/ml TNF-α for an additional 6 h. Values are mean ± SD of three independent experiments. Values not sharing a letter or symbol are significantly different ($p < 0.05$). One representative picture out of three independent experiments is shown. Asterisk represents significant difference between 50 and 100 μM DHA treatments at the same indicated time.

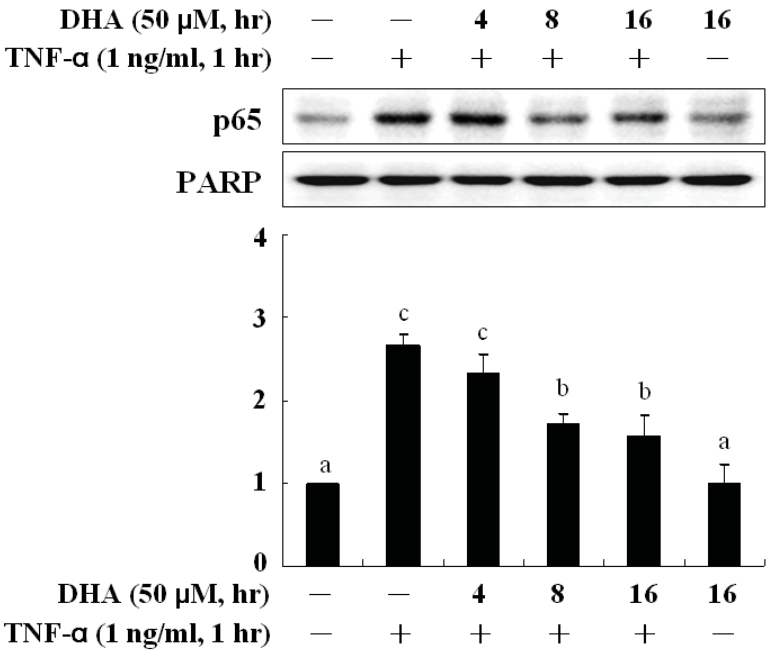
A


DHA (50 μM, 16 hr) – – – – + + + +
TNF-α (1 ng/ml, min) – 2 5 15 – 2 5 15
p-IKK β α
IKK β α
p-IKKβ/IKKβ (Fold of control)
TNF-α
DHA + TNF-α
p-IKKα/IKKα (Fold of control)
TNF-α (min) 0 2 5 15

B


MG-132 (1 μM, 1 hr) – – – – + – – – – +
DHA (50 μM, 16 hr) – – – – – + + + + +
TNF-α (1 ng/ml, min) – 5 10 15 15 – 5 10 15 15
p-IκBα
IκBα
β-Actin
p-IκBα (Fold of control)
IκBα (Fold of control)

C


DHA (50 μM, hr) — — 4 8 16 16
TNF-α (1 ng/ml, 1 hr) — + + + + —
p65
PARP
4
3
2
1
0
a
c
c
b
b
a
DHA (50 μM, hr) — — 4 8 16 16
TNF-α (1 ng/ml, 1 hr) — + + + + —

D

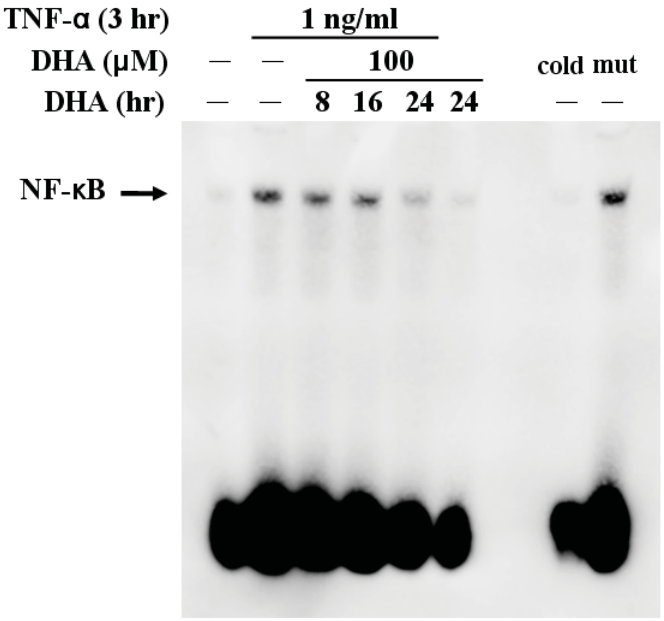

TNF-α (3 hr)
1 ng/ml
DHA (μM) — — 100
cold mut
DHA (hr) — — 8 16 24 24 — —
NF-κB

Figure 3. DHA inhibits TNF-α-induced activation of NF-κB.

(A) Cells were pretreated with 50 μM DHA for 16 h followed by incubation with 1 ng/ml TNF-α for the various time periods. Aliquots of total protein (20 μg) were used for Western blot analysis, and the expression of IKK was assessed. Fold activation is shown as an increase in the normalized phosphorylation in the treated cells relative to the control. (B) Cells were pretreated with 50 μM DHA for 16 h or 1 μM proteasome inhibitor, MG132, for 1 h followed by incubation with 1 ng/ml TNF-α for the various time periods. Aliquots of total protein (20 μg) were used for Western blot analysis, and the expression of IκBα was assessed. (C) Nuclear extracts (10 μg) were used for Western blot analysis. (D) Aliquots of nuclear extracts (10 μg) were used for EMSA. One representative picture out of three independent experiments is shown. From (A) to (C), the levels in control cells were set at 1. Values are mean ± SD of three independent experiments. Values not sharing a letter are significantly different ($p < 0.05$).

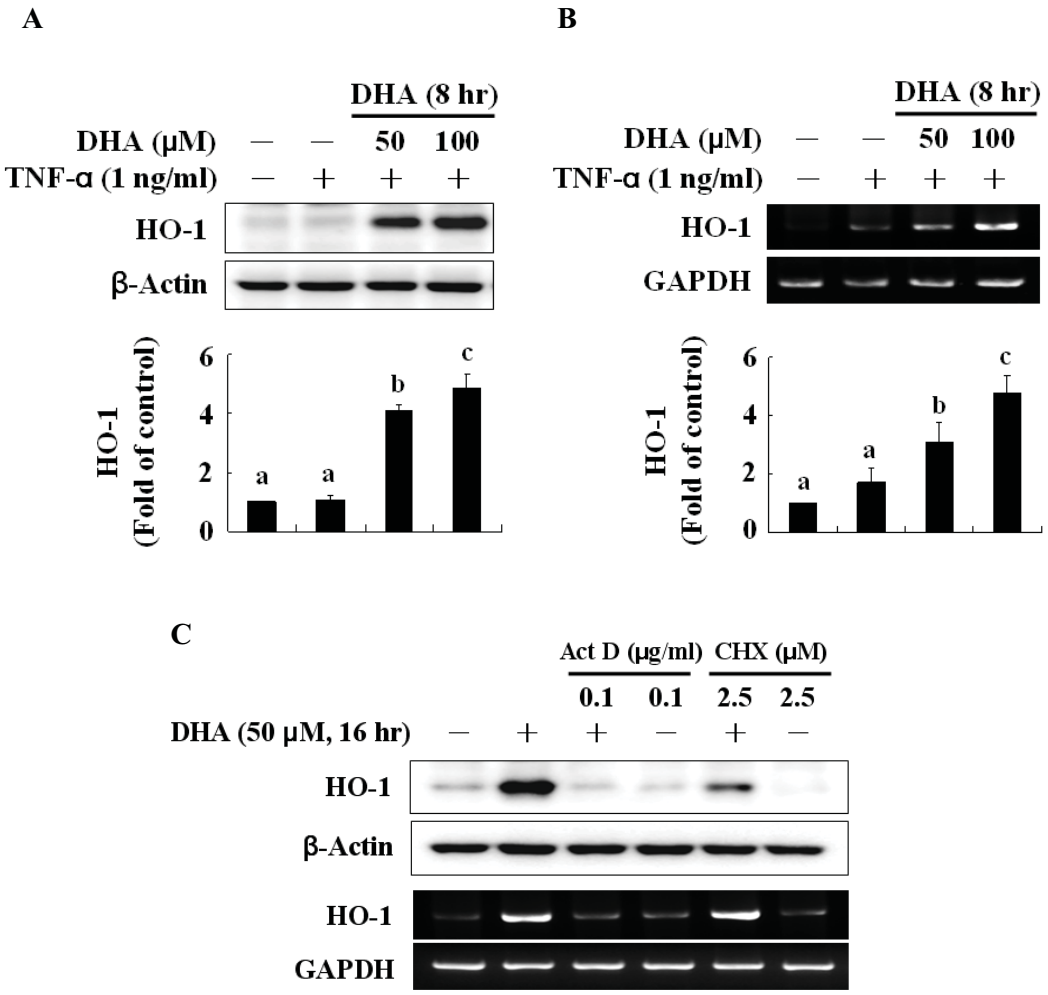


Figure 4. DHA induces HO-1 expression in EA.hy926 cells.

(A) Cells were pretreated with 50 and 100 μM DHA for 8 h followed by incubation with 1 ng/ml 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. (C) Cells were pretreated with either 0.1 μg/ml actinomycin D (Act D) or 5 μM cycloheximide (CHX) for 4 h followed by incubation with 50 μM DHA for an additional 16 h. One representative picture out of three independent experiments is shown. Values are mean ± SD from three independent experiments. Values not sharing a letter are significantly different ($p < 0.05$).

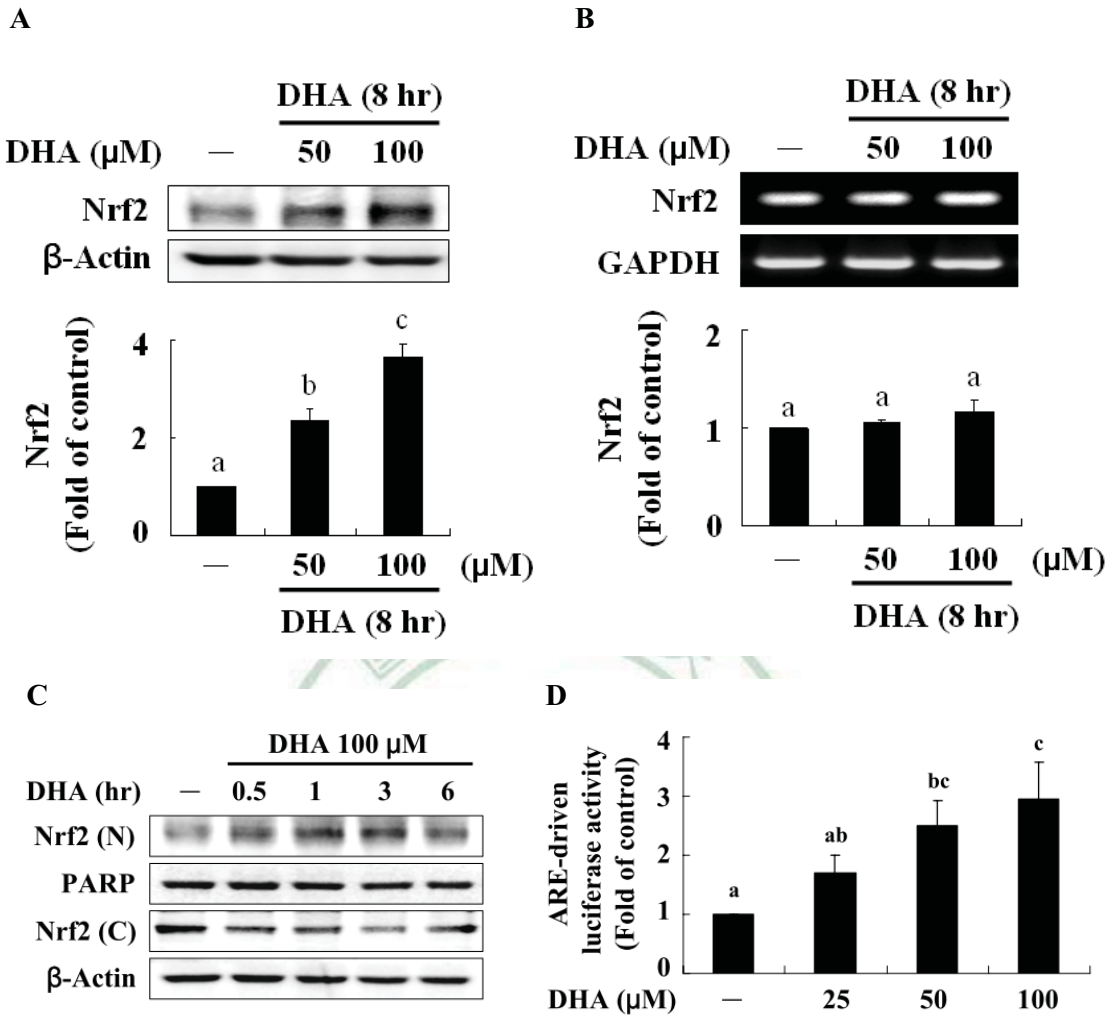


Figure 5. Effect of DHA on Nrf2/ARE signaling pathway.

Cells were treated with 50 and 100 μM DHA for 8 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 Nrf2 and GAPDH primers as described in Materials and Methods. One representative picture out of three independent experiments is shown. (C) Nuclear extracts [N] and cytosolic extracts [C] from cells were prepared after treatment with 100 μM DHA for the indicated time periods. Immunoblots of nuclear extracts from treated cells were then probed with Nrf2-specific antibody. PARP is a housekeeping gene which was used as internal

nuclear loading control. (D) Cells were transfected with the ARE-luciferase construct (ARE) for 8 h and were then stimulated with 25-100 μM DHA for an additional 16 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 are mean ± SD from three independent experiments. Values not sharing a letter are significantly different ($p < 0.05$).

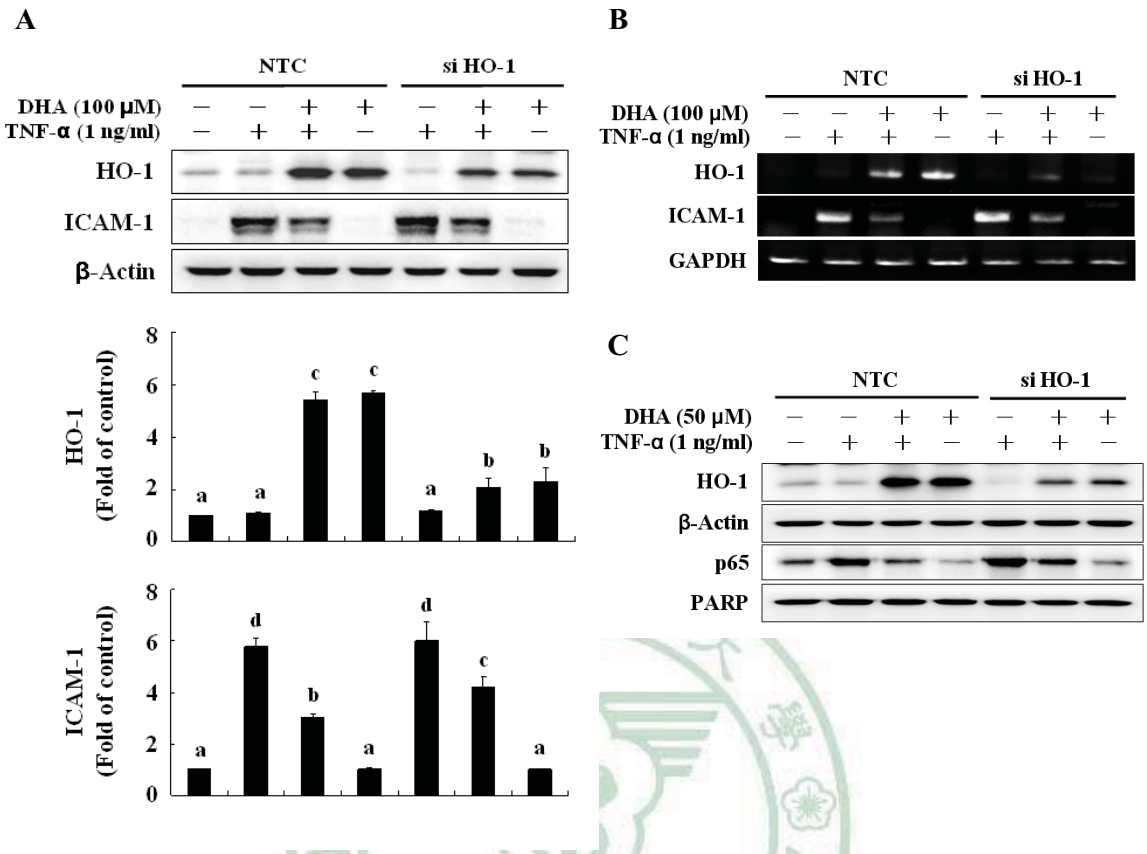


Figure 6. Effect of HO-1 siRNA on the DHA-mediated inhibition of ICAM-1 expression and p65 translocation.

An HO-1 siRNA system was used to silence HO-1 mRNA and to create an siRNA knock-down model in EA.hy926 cell. Cells were transfected with HO-1 siRNA for 8 h, and then treated with 100 μM DHA for 16 h before being challenged with 1 ng/ml TNF-α for an additional 6 h. (A) Aliquots of total protein (20 μg) were used for Western blot analysis, and expressions of HO-1 and ICAM-1 were assessed. (B) Total RNA was subjected to RT-PCR with specific HO-1, ICAM-1 and GAPDH primers. (C) Aliquots of nuclear extracts (10 μg) were used for Western blot analysis, and the expression of p65 was assessed. One representative immunoblot of three independent experiments is shown. Values are mean ± SD of three independent experiments. Values not sharing a letter are significantly different ($p < 0.05$).

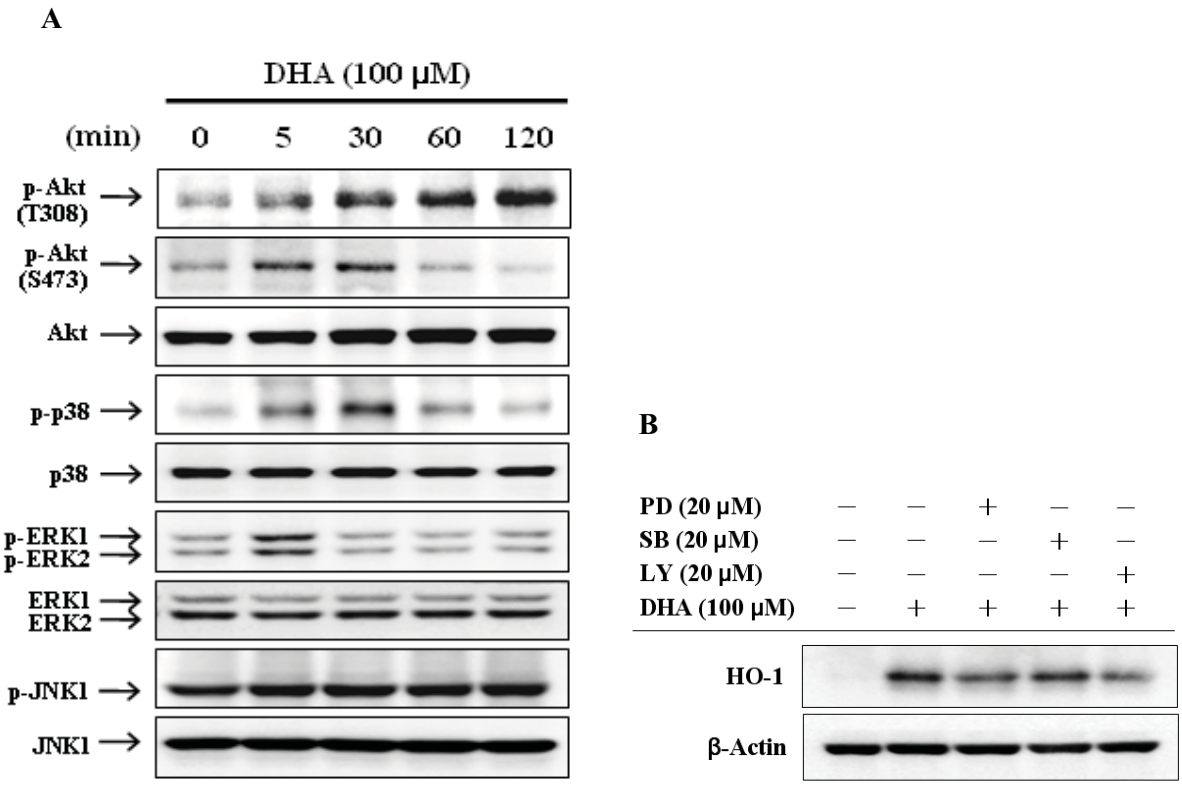


Figure 7. PI3K/Akt, p38 and ERK1/2 pathways are involved in DHA-induced HO-1 expression.

(A) Cells were incubated with 100 μM DHA for the indicated time periods. Immunoblots of total protein extracts from treated cells were then probed with Akt and MAPKs specific antibodies. One representative picture of three independent experiments is shown. (B) Cells were pretreated with 20 μM PD (PD98059), SB (SB203580) and LY (LY294002) for 1 hr before induced by 100 μM DHA for another 16 hr. Immunoblots of total protein extracts from treated cells were then probed with HO-1 specific antibody.

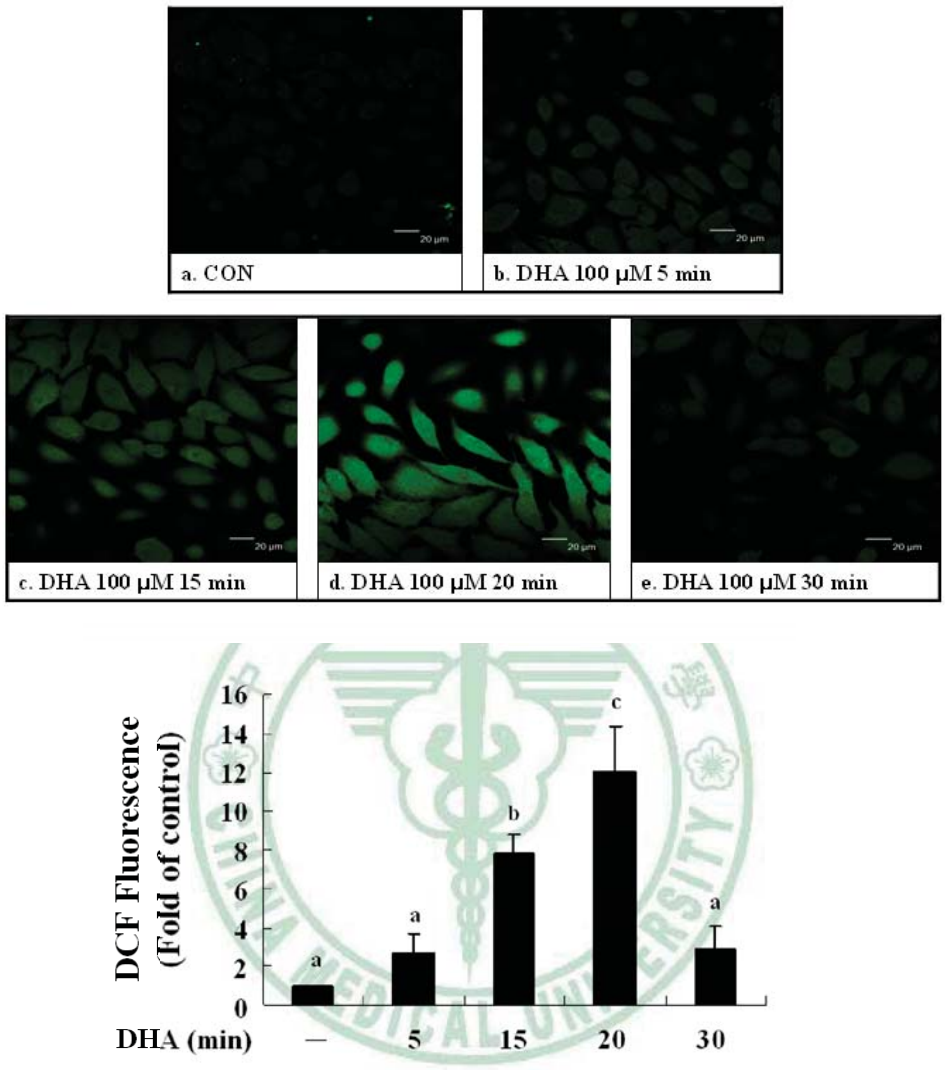


Figure 8. Effect of DHA on ROS generation.

EA.hy926 cells were treated with 100 μM DHA for the indicated time periods, and followed by incubation with 10 μM ROS-sensitive fluorophore H_2DCF-DA. DCF fluorescence, reflecting the relative levels of ROS, was imaged with a confocal laser scanning fluorescence microscope. Quantitative induction is shown as an increase in the treated cells relative to the control. Data are expressed as mean ± SD of three independent experiments. Values not sharing a letter are significantly different ($p <$ 0.05).

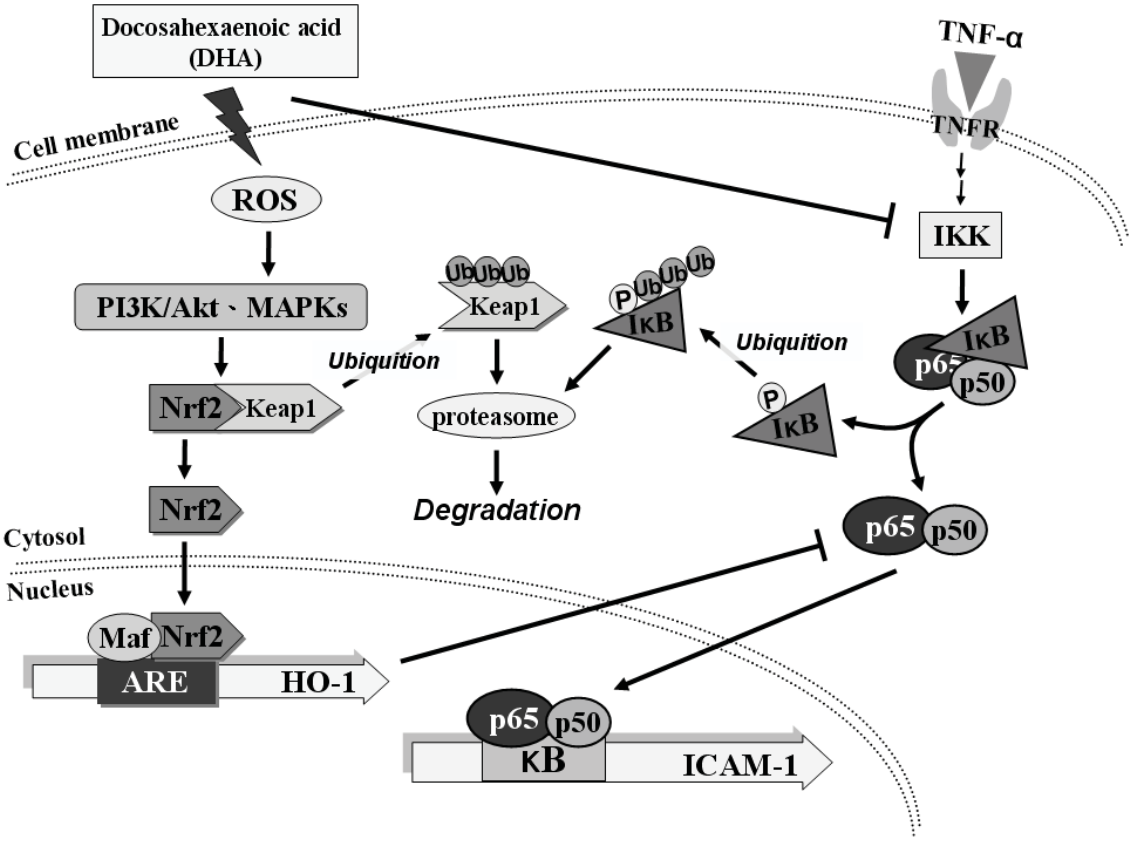


Figure 9. Model showing pathways that mediate the inhibition of expression of ICAM-1 and adhesion of HL-60 cells to EA.hy926 cells by DHA under inflammatory conditions.

DHA 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. DHA also inhibits TNF-α-induced IKK/NF-κB activation, ICAM-1 expression and eventual HL-60 cell adhesion. There is a crosstalk between HO-1 and NF-κB.

4. Discussion

In this study, we demonstrated that DHA effectively inhibited TNF-α-induced inflammatory responses in EA.hy926 cells and this suppression was likely associated with an up-regulation of Nrf2-dependent HO-1 and a down-regulation of IKK/NF-κB signaling pathway.

The specific mechanisms underlying the anti-inflammatory effects of n-3 PUFAs have been intensively sought for decades. Several possible mechanisms have been suggested, including the displacement of the major substrate for the synthesis of proinflammatory eicosanoids, arachidonic acid (20:4 n-6), from the sn-2 position in membrane phospholipids; direct activation of the nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) (Delerive et al., 1999, 2000; Li et al., 2005); or modification of specific plasma membrane domains called lipid rafts or caveolae (Chen et al., 2007). Meanwhile, the anti-atherogenic properties of fish oil (FO) were thought due to its capacity to inhibit the adhesion molecule expression and cytokine production induced by inflammatory stimuli (Novak et al., 2003; Chen et al, 2005). Furthermore, Casós et al. (2008) reported that administration of an FO-rich diet to apoE−/− mice, the reduction of atherosclerotic lesions was associated with decreased expression of endothelial adhesion molecules and NF-κB activation. Some clinical studies have shown that intake of FO decreases plasma levels of soluble ICAM-1 and P-selectin in patients with atherosclerosis (Paulo et al., 2008). Accumulating evidence indicates that dietary n-3 PUFAs may help reduce atherogenesis (Jung et al., 2008; Harris et al., 2008); however, the molecular mechanisms underlying the anti-atherosclerotic effect of n-3 PUFAs were not fully clarified. In this study, we used DHA to represent the FO source and explored the

possible mechanisms involved in the inhibition of TNF-α-induced ICAM-1 expression in EA.hy926 cells.

It is suggested that TNF-α can induce the expression of adhesion molecules including VCAM-1, ICAM-1 and E-selectin, and this is responsible for transendothelial migration of leukocytes, which is a central feature underlying the inflammatory process (Lawson & Wolf, 2009). Inflammation plays a key role in the pathogenesis of atherosclerosis; the increased interaction between activated vascular endothelial cells and circulating monocytes via adhesion molecules (Lusis, 2000), is a critical initial step in atherosclerosis. Increased expression of adhesion molecules has emerged as an important mediator in vascular inflammation (Glass & Witztum, 2001). The importance of adhesion molecules in atherosclerosis has been examined in many studies; for example, knockout of ICAM-1 results in attenuation of atherosclerotic plaques in the apoE knockout mice (Collins et al., 2000; Bourdillon et al., 2000), and levels of soluble adhesion molecules have been implicated to be risk predictors for cardiovascular events (Paulo et al., 2008). These observations collectively indicate that regulation of adhesion molecule expression (e.g. ICAM-1) is therefore a potential target for the development of new therapeutics in the prevention and treatment of atherosclerosis. The present study showed that TNF-α-induced ICAM-1 expression and ICAM-1 luciferase reporter activity were inhibited by DHA pretreatment (Figs. 2A, B, and C). Moreover, the inhibition of adhesion of HL-60 cells to activated EA.hy926 cells by DHA pretreatment was demonstrated in the present study (Fig. 2D). The results indicate that DHA has the potential to prevent vascular inflammation. Data of the current study are consistent with the findings of previous studies showing that DHA is an anti-inflammatory agent (Saw et al, 2010; Mullen et al., 2010).

It has been reported that transcriptional regulation of ICAM-1 gene by TNF-α in human endothelial cells is dependent on NF-κB activation (Zhou et al., 2007; Oh et al., 2010). The essential role of the NF-κB pathway in TNF-α-induced expression of adhesion molecules has been convincingly demonstrated by several studies (Jiang et al., 2004; Kuldo et al., 2005). NF-κB is a redox-sensitive transcription factor that activates inflammation through regulating the gene expression of a large number of cytokines and adhesion molecules (Karin & Greten, 2005). Disruption of NF-κB activation has been shown to delay or prevent atherogenesis (Lopez-Franco et al., 2006). As described earlier, the dissociation of NF-κB from IκB requires phosphorylation of IκB, which results in rapid and ubiquitous degradation of IκB. A study by Denk et al. (2001) showed that overexpressing dominant negative IκB, which is resistant to proteolysis, completely blocked TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin. Numerous natural components and therapeutic agents have been shown to inhibit NF-κB activation by preventing IκBα phosphorylation and degradation (Kim et al., 2006). Kaileh et al. (2007) recently reported that withaferin A inhibited TNF-α-stimulated NF-κB activation through blocking the activity of IKKβ kinase. Our data indicated that DHA not only inhibited the upstream IKK and IκBα phosphorylation but also IκBα degradation (Figs. 3A and B). In addition, we found that TNF-α-induced NF-κB and DNA binding activity and nuclear translocation of p65 were attenuated in response to DHA pretreatment (Figs. 3C and D). This provides evidence that DHA antagonizes adhesion molecule expression by attenuating NF-κB signaling pathway in TNF-α-activated EA.hy926 cells.

The cytoprotective capacity of compounds has been partially attributed to their induction of antioxidant enzymes. Among the antioxidant enzymes, HO-1 expression

was considered to be an adaptive and beneficial response to oxidative stress in a wide variety of cells (Alam and Cook, 2003; Takahashi et al., 2004). Quercetin and theaflavin have been reported to provide specific HO-1-dependent anti-inflammatory protection in an apoE knockout mouse model of arteriosclerosis (Loke et al., 2010). In this study, our results revealed that DHA dose-dependently up-regulated HO-1 mRNA and protein expression in EA.hy926 cells (Figs. 4A and B). Induction of the HO-1 gene by DHA is primarily regulated at the transcriptional level (Fig. 4C).

Recent evidence has implicated that transcription factor Nrf2 is required for the activation of the HO-1 gene (Johnson et al., 2009). Nrf2 is a basic leucine zipper (bZIP) transcription factor (Kaspar et al., 2009), which is involved in cellular protection against oxidative stress through ARE-mediated expression of various phase II detoxifying and antioxidant enzymes, including HO-1 (Zhang & Gordon, 2004). Nrf2 is negatively regulated by association with the cytosolic inhibitor Keap1 (Kobayashi et al., 2005; Kaspar et al., 2009). Keap1/Nrf2/ARE signaling is thought to play a significant role in protecting cells from endogenous and exogenous stresses (Kensler et al., 2007). A previous study indicated that puerarin increased HO-1 mRNA and protein expression through Nrf2-mediated ARE activation in Hepa1c1c7 cells (Hwang & Jeong, 2008). In this study, DHA enhanced Nrf2 nuclear translocation and ARE-luciferase activity (Figs. 5C and D). Moreover, DHA increased protein levels of Nrf2 in a dose-dependent manner (Fig. 5A). The increased Nrf2 protein accumulation by DHA can be due to increased Nrf2 protein synthesis or decreased its degradation. As shown in Fig. 5B, the Nrf2 mRNA expression was not affected by DHA.

HO-1 has been shown to possess the anti-inflammatory endothelial protective action via reduction of TNF-α-induced expression of various adhesion molecules

(Soares et al., 2004). Brunt et al. (2009) found that overexpression of HO-1 reduced NF-κB promoter activity and NF-κB and DNA binding activity in response to H_2O_2 in HL-1 cardiomyocytes. Other studies suggest the major transcription factor Nrf2 mediating HO-1 expression suppresses inflammation by inhibiting NF-κB activation through regulation of redox balance (Thimmulappa et al., 2006). Several anti-inflammatory or anti-carcinogenic phytochemicals suppress NF-κB signaling and activate the Nrf2-ARE pathway (Li et al., 2008), suggesting that NF-κB and Nrf2 may crosstalk with each other. On the basis of these findings, the possibility that DHA inhibits TNF-α-induced ICAM-1 expression is via suppression of NF-κB activation, and that is regulated by HO-1 induction cannot be excluded. 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 DHA was partially abolished (Figs. 6A and B), suggesting a pivotal role of the antioxidant enzyme. We also ascertained that HO-1 inhibited ICAM-1 expression through attenuation of NF-κB activation by transfection with HO-1 siRNA. The results show that DHA-mediated suppression of p65 nuclear translocation was partially reversed as excepted (Fig. 6C). The cytoprotective actions of HO-1 were at least partially attributed to the attenuation of ICAM-1 expression, and NF-κB appeared to be the target. In this study, DHA inhibits TNF-α-induced ICAM-1 expression is via two distinct mechanisms. One is inhibition of IKK/NF-κB signaling pathway, and the other is activation of Nrf2/HO-1 pathway.

Previous studies have suggested that several signaling pathways, including mitogen-activated protein kinases (MAPKs) (Gong et al., 2004; Kong et al., 2001), protein kinase C (PKC) (Numazawa et al., 2003; Kim et al., 2010), and phosphatidylinositol 3-kinase (PI3K) pathways (Nakaso et al., 2003; Hwang & Jeong,

2008), are involved in Nrf2 activation and subsequent HO-1 induction. Among these, contribution of PI3K/Akt and MAPKs in the regulation of HO-1 in various cell types is well appreciated (Wu et al., 2006; Alam & Cook, 2007; Choi & Kim, 2008). Our results supported the role of PI3K/Akt and ERK pathways in DHA-induced HO-1 expression (Fig. 7). The Keap1-Nrf2 complex is a closest ROS receptor in mammals, modification of Keap1 is proposed to affect Nrf2 stability and nuclear accumulation (D'Autréaux & Toledano, 2007). ROS causes Keap1 modification is through different pathways including cysteine oxidation, Zn release and phosphorylation by PKC, PI3K, and ERK (D'Autréaux & Toledano, 2007). In this study, 100 μM DHA was found to increase cellular ROS and peak effect was observed at 20 min after DHA treatment (Fig. 8). This result suggests that the early-phase generation of ROS by DHA might be involved in activation of signaling pathways that led to Nrf2 nuclear translocation and eventual HO-1 induction.

In summary, as depicted in Fig. 9, DHA significantly inhibits TNF-α-induced ICAM-1 mRNA and protein expression and the subsequent adhesion of HL-60 cells to activated EA.hy926 cells. Two working mechanisms by which DHA inhibits ICAM-1 expression are identified, one is inhibition of IKK/NF-κB pathway, and the other is activation of Nrf2/HO-1 pathway. Taken together, these results support the beneficial role of DHA in cardiovascular-protection as an anti-inflammatory agent.

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附錄

過氧化物的生成(Peroxide measurement)

◎ **實驗原理：**

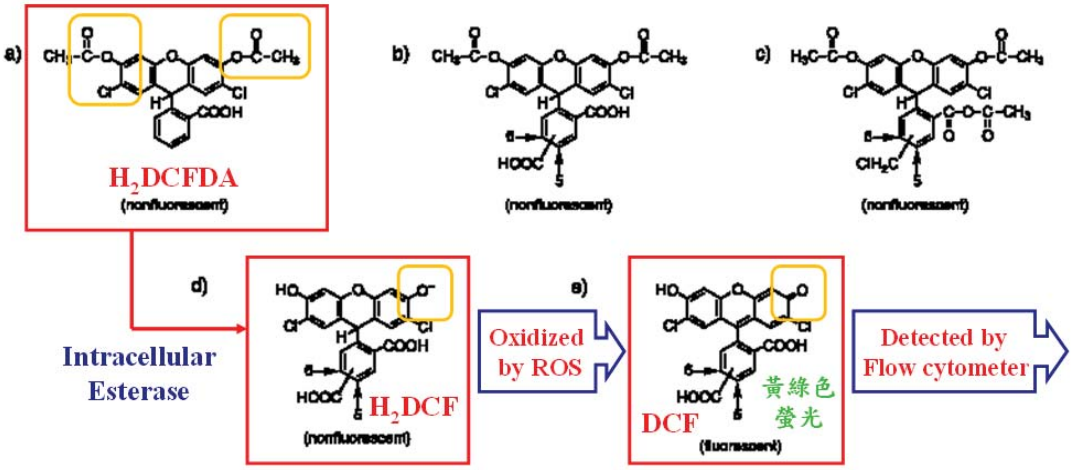

Figure 2. The structures of (a) H_2DCFDA, (b) carboxy-H_2DCFDA, (c) chloromethyl-H_2DCFDA, (d) deacetylated H_2DCF, and (e) the deacetylated, oxidized product, DCF.

DCFH-DA 本身沒有螢光，可以自由穿過細胞膜，進入細胞內後，可以被細胞內的酯解酶(esterase)分解生成 DCFH。而 DCFH 不能通透細胞膜，細胞內的活性氧可以氧化 DCFH 生成有螢光的 DCF。檢測 DCF 的螢光就可以知道細胞內活性氧的含量。

◎ **實驗操作：**

舉例：EA.hy926 cells

Day 1 分盤 ― 6 cm dish

(1) 0.5 百萬(10^7)/盤 細胞 → 正常組 (10% FBS)

(2) 1.5 百萬(10^7)/盤 細胞 → Starvation 組 (0.5% FBS)

分盤後仍先用正常 medium (DMEM＋10% FBS)培養一天(24hr)。

※ 因為之後處理情況(FBS)不同，所以分盤的細胞數也不同；正常組(10% FBS)細胞的生長速度會比 Starvation 組(0.5% FBS)略快。

※ 原則：在正常培養情況下，以 medium (DMEM＋10% FBS)培養細胞至 9 分滿即可進行實驗。

Day 2、Day 3

(1) 正常組換新的 medium (DMEM＋10% FBS)
(2) 但將 Starvation 組換成 DMEM＋0.5% FBS

} 培養兩天。

Day 4 對細胞預處理 H_2DCFDA 10μM 10 min (or H_2DCFDA 5μM 30 min)。
再給予 TNF-α 刺激(視時間點而定)，時間到後，用 4℃ PBS wash 兩次，於雷射掃描式共軛聚焦顯微鏡 (Laser Scanning Confocal Microscope)下觀察(綠色)螢光亮度。

實驗結果之判定：螢光亮度愈多，則代表誘發 ROS 產生量愈多。
※ 若 TNF-α (藥品)處理的時間較短，則 H_2DCFDA 需預先加入。

◎ 準備物品

1.回溫 PBS
2. H_2DCFDA
3.處理之藥品 (ex: DHA, TNF-α, H_2O_2 .....)
4. pipet / tip (10, 100)
5. Timer ×2
6.紙巾
7.垃圾桶
8.光碟(CD-RM)

舉例：(時間點)

Date：

14:50 加 H_2DCFDA 10 μM 10 min
到 7F 貴重儀器室 Stand by.

	加藥	上機
D5	15:00	→ 15:05
D15	15:05	→ 15:20
D20	15:10	→ 15:30
D30	15:15	→ 15:45
H20	15:35	→ 15:55
CON	15:55	→ End

◎ 觀察順序：

1. 先用光學(鎢絲燈)找焦距。
2. 再用 Laser 488 調焦距，找到清楚的切面。
3. 調整至適當的能量強度並固定，以 Laser 488 拍，放 scale (比例尺)。

備註：

※ EA.hy926 cells 常用強度約 PMT 2：500~600 → 可再調整。
※ 光學－鎢絲燈、螢光－汞燈；
光電倍增管老舊或 Dish 材質不同→ 會造成視野的光學亮度不同。
(塑膠光學折射差、玻璃或 confocal 專用 well 較佳)
※ 上機時，若用汞燈去找焦距，會因為汞燈的光激發訊號(很強)，但在 10s 後就變弱了(degrade)，30s 後幾乎消失；只在 Laser 488 激發下，隨時間會使螢光訊號(微微)增強(變化不大)，至少到 200s 都不會降解掉。→ 所以一開始不能開汞燈去找焦距。汞燈波長(480~495 nm)：包含 488 nm 以外部份的波長。