行政院國家科學委員會專題研究計畫　成果報告

磷脂質在氧化低密度脂蛋白抑制鹼性纖維細胞生長素(bFGF)基因表達的角色

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-002-185-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立臺灣大學醫學院內科

計畫主持人：張博淵

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中　華　民　國 92 年 11 月 10 日

行政院國家科學委員會補助專題研究計畫成果報告

計畫名稱：

磷脂質在氧化低密度脂蛋白抑制鹼性纖維細胞生長素（bFGF）基因表達的角色

Role of phospholipids in oxidized LDL (Ox-LDL)-mediated suppression of basic fibroblast growth factor (bFGF) gene expression

計畫類別：■個別型計畫 □整合型計畫
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研究成果自評

這個一年期的研究計劃進行順利，我們的結果顯示 lysophosphatidylcholine（LPC）對血管內皮細胞的影響，無論是在 DNA synthesis，bFGF suppression，或 bFGF promoter activity 方面均與氧化態低密度脂蛋白(OxLDL)相當。LPC 對 bFGF mRNA 的 downregulation 也是因為減低 bFGF mRNA stability 而與 bFGF 之 transcriptional activity 無關，這些發現與我們已經發表的 oxLDL 的結果一致。

雖然短短一年時間的研究，我們已經可以相當程度的確定 OxLDL 對血管內皮細胞的傷害是源自其中一個重要的氧化磷脂質 LPC。這些實驗的結果是一個全新的發現，我們也開始著手寫成 manuscript，相信這個研究對 oxLDL 所造成之內皮細胞功能缺損進而引起冠狀動脈硬化疾病的一些機轉有更深入的瞭解，進而藉由這些認識幫助我們發展預防及治療冠狀動脈硬化疾病的新方針。

主要研究成果說明

Endothelial dysfunction is an early manifestation of atherosclerosis but its underlying mechanism is not fully understood. In the past 4 years, under the financial support of two NSC grants, first NSC89-2314-B-002-029 (1998-2000) followed by NSC89-2316-B-002-017-M52 (1999-2002) [Principal Investigator: Yuan-Teh Lee], we have demonstrated that cytotoxic effects of oxidized low-density lipoproteins (oxLDL) on endothelial cells (EC) are attributable in part to suppression of basic fibroblast growth factor (bFGF) expression. The "oxLDL-bFGF suppression-EC dysfunction" hypothesis was first brought up by co-Investigator Dr. Chu-Huang Chen in Baylor College of Medicine (USA) in 1996, and worked out by Dr. Yuan-Teh Lee's group in National Taiwan University Hospital. We have been working intensively on this hypothesis and have finally made the concept of this novel oxLDL-bFGF pathway recognized by publishing the results in the accredited ***Circulation* Journal**.

The first grant concluded by demonstrating in cultured human and bovine aortic EC that ox-LDL reduced bFGF mRNA levels in a time- and concentration-dependent manner. Suppression of mRNA was accompanied by commensurate reductions in intra and extracellular bFGF concentrations, total DNA and RNA syntheses, and cell replication. Reduced syntheses of total DNA and RNA were completely restored by bFGF but not by vascular endothelial growth factor. We also showed, through RNase protection assay, that bFGF mRNA is destabilized by oxLDL at the posttranscriptional level. The conclusion of this project is twofold. First, cytotoxic effects of oxLDL on EC are attributable in part to suppression of bFGF expression (**published in *Circulation* Journal 2000**) **(參考文獻-1)**. This report is the first proposal of a novel mechanism underlying oxLDL-induced EC dysfunction—through downregulation of bFGF gene expression. Second, data obtained above describe bFGF gene regulation at the RNA level by oxLDL and help subsequent delineation of the signal transduction pathways mediating the effects of oxLDL, which led to our next NSC grant 1999-2002 (part of a program project directed by Dr. Yuan-Teh Lee).

The second grant of 1999-2002 NSC project concluded by delineating the lipid components of oxidized low-density lipoprotein (oxLDL) and determining their signal transduction pathways in inhibiting down regulation of basic fibroblast growth factor (bFGF) in cultured aortic endothelial cells. We found that incubating bovine aortic endothelial cells with copper-oxidized low-density lipoprotein (oxLDL) down-regulated bFGF and markedly suppressed concomitant DNA synthesis. The inhibitions were sensitive to pertussis toxin (PTX), but were not attenuated by the Ca^{2+} chelator or by inhibitors of protein-activated protein kinase kinase, cGMP- and cAMP-dependent protein kinase, or protein kinase C. Whereas platelet-activating factor (PAF) failed to produce oxLDL-like effects, WEB 2086—an antagonist of the PTX-sensitive G protein–coupled PAF receptor—blocked the action of oxLDL. Effects of oxLDL were abolished after degradation of oxLDL phospholipids with PAF acetylhydrolase. Thus, oxLDL down-regulates endothelial bFGF through a PTX-sensitive heterotrimeric G-protein pathway involving mediator phospholipids similar, but not identical, to PAF. An abstract on oxLDL-bFGF signaling was presented in the **1999 American Heart Association Scientific Sessions (參考文獻-2)**, and a comprehensive manuscript has been published in ***Circulation* Journal 2001 (參考文獻-3)**.

The main goal of this 2002-2003 NSC project is to further characterize the role of phospholipids in oxLDL-mediated suppression of basic fibroblast growth factor (bFGF) gene expression. In this one-year project, we have constructed the bFGF promoter constructs for

reporter gene assay, and identified phospholipid lysophosphatidylcholine (LPC) as an important lipid mediator for oxLDL-induced bFGF downregulation. In these experiments, we have found that 1) LPC dose- and time-dependently decreased bFGF protein in cultured bovine endothelial cells, 2) bFGF mRNA levels were decreased in parallel, 3) the bFGF promoter activity was not affected by LPC, 4) the bFGF mRNA stability was decreased by LPC, indicating a post-transcriptional regulation of bFGF. A comprehensive manuscript is in preparation for submission to *Circulation* Journal for review **(参考文献-4)**.

Meanwhile, several collaborative projects related to bFGF regulation were carried out with fruitful results, including the investigation of apoptotic and signal transduction mechanisms involved in oxLDL-bFGF pathways. The data have been either published or presented in several AHA scientific meeting **(参考文献-5,6)**, and a comprehensive manuscript is under review of the *Journal of Biological Chemistry* **(参考文献-7).** We also executed a side project under this grant support, demonstrating that homocysteine, another important cardiovascular risk factor, inhibited endothelial survival by reducing bFGF expression, which is similar to oxLDL-bFGF regulatory pathways. These results were presented in a 2002 AHA meeting **(参考文献-8)** and a comprehensive manuscript has been submitted to Circulation Journal for review **(参考文献-9)**.

In summary, we have demonstrated that

1) LPC reduces bFGF mRNA and protein levels in cultured EC in a time- and concentration-dependent fashion, which is similar to oxLDL;
2) LPC, like oxLDL, destabilize bFGF mRNA post-transcriptionally;
3) Neither oxLDL nor LysoPC suppresses bFGF promoter activity in cultured EC;
4) The inhibition of DNA synthesis by LPC can be reversed by bFGF but not VEGF.

These results and publications are listed in the following.

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8. Chang PY, Huang WH, Lu SC, Lee YT (2002). Homocysteine down-regulates basic fibroblast growth factor expression in endothelial cells. *Circulation.* 2002;105:e86-e119 [Abstract].

9. Chang PY, Lu SC, Lee CM, Huang WH, Chen YJ, Chen CH, Lee YT. Homocysteine inhibits arterial endothelial cell survival by selectively reducing endogenous fibroblast growth factor 2. *Circulation.* 2003 Under review.

Preliminary Results

1. LPC inhibits bFGF expression and DNA synthesis in cultured human aortic EC.

Cells exhibited a biphasic response when incubated with LPC in human aortic endothelial cells (HAEC). At lowers concentrations (20 μM), LPC induced a mild increase in both DNA synthesis and bFGF content. At higher concentrations (50 to 60 μM), LPC induced a concentration-dependent reduction in both DNA synthesis and bFGF content (Table).

Table. Effects of LPC and LDL Preparations on Intracellular bFGF Peptide Levels and DNA Synthesis in Cultured HAEC at 24 Hours

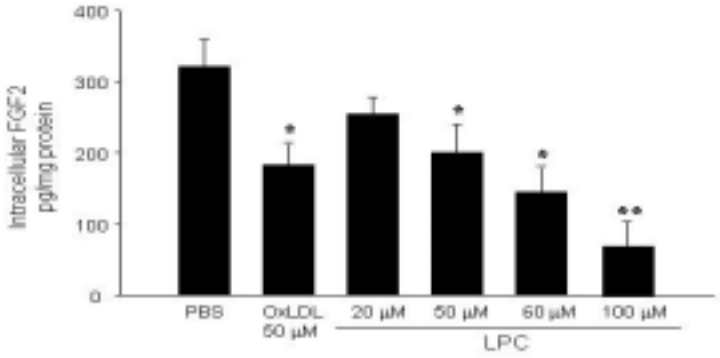
Assessment	NLDL 100 μg/mL	Copper-oxLDL 50 μg/mL	LPC 20 μM	LPC 50 μM	LPC 60 μM
Intracellular bFGF levels pg/mg protein x 10^{-4}/dish	311 ± 22	167 ± 58*	345 ± 30	215 ± 44*	184 ± 55*
^{3}H-thymidine incorporation cpm x 10^{-4}/dish (DNA)	90 ± 8	64 ± 9*	98 ± 10	78 ± 11	65 ± 12*

*P<.05 vs NLDL (normal control); n = 3 in all treatments.

2. LPC effects on ECs are species-independent.

LPC induced a dose-dependent decrease in bFGF protein assayed by ELISA in bovine aortic endothelial cells (BAEC). Between 20 and 100 μM, LPC caused linear reductions in bFGF protein levels comparable to those induced by oxLDL (Figure 1).

Figure 1



3. LPC down-regulates bFGF mRNA in BAEC. A dose-response effect was noted with LPC concentrations between 25 and 60 μM. OxLDL 50 μM was used as a comparison (Figure 2).

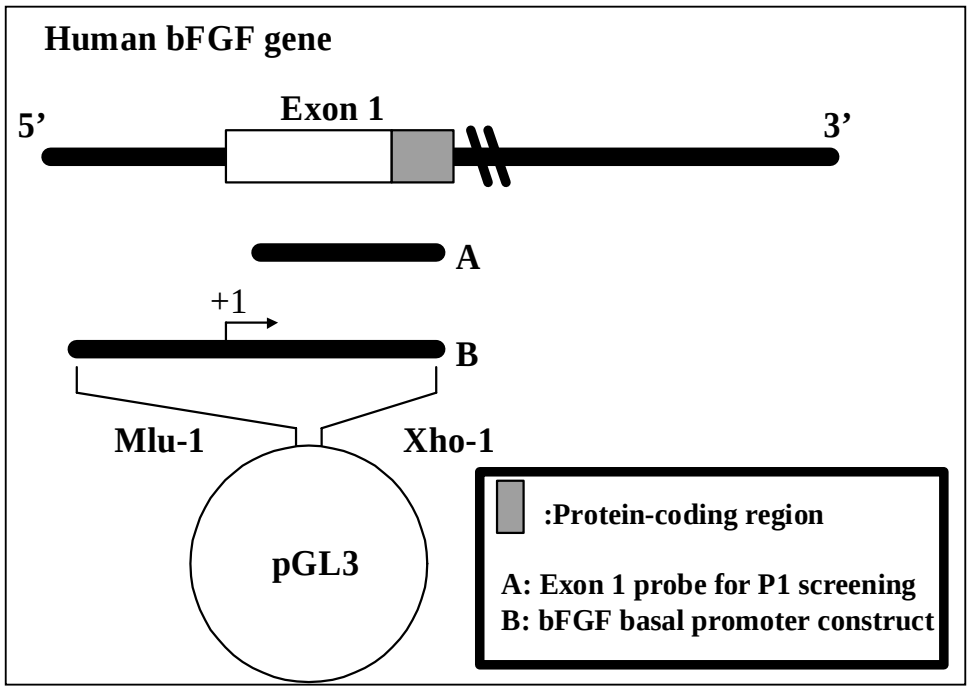
Figure 2

4. bFGF promoter-reporter construct

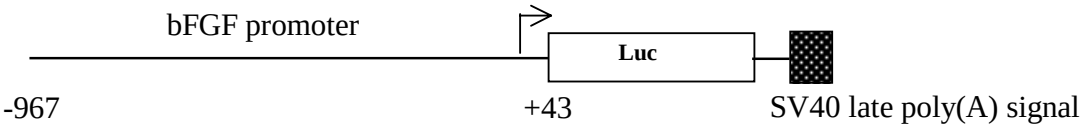
A human bFGF genomic clone constructed in P1 vector has been pulled out by hybridization screening. Initial sequence analysis demonstrates that this bFGF genomic clone encompasses at least the second intron and the third exon of human bFGF gene. Intensive sequencing of this clone and plasmid construction for reporter gene assays are underway.

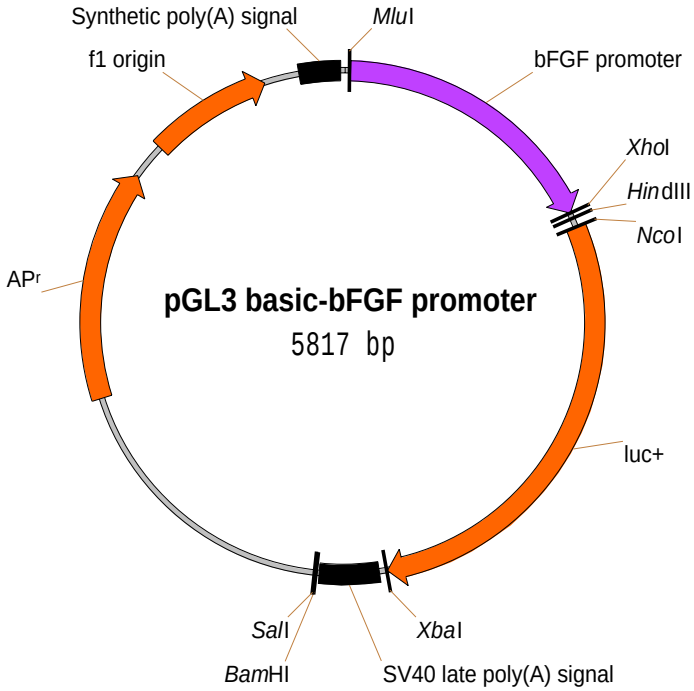
Recombinant PCR and reporter Gene constructs. To isolate the human bFGF 5'-flanking region, further P1 genomic library screening is underway using a 170-bp recombinant bFGF exon 1 DNA fragment as probe. This 170-bp exon 1 fragment was synthesized by recombinant PCR using 4 oligonucleotides as depicted in **Figure 3** (fragment A). While the P1 genomic library screening is in process, similar recombinant PCR technique as described above is used to construct a reporter gene vector containing the basal promoter of human bFGF (**Figure 3**, fragment B). This promoter construct was cloned into the pGL3 Luciferase expression vector (Promega) and sequenced, followed by transient expression and reporter gene assay. The remaining reporter gene constructs will be made either by recombinant PCR or by subcloning from a human bFGF P1 clone when available.

Figure 3



5. A reporter construct containing 1.0-kb bFGF promoter sequences was created by recombinant PCR. Reporter gene assay was carried out using the following procedures.



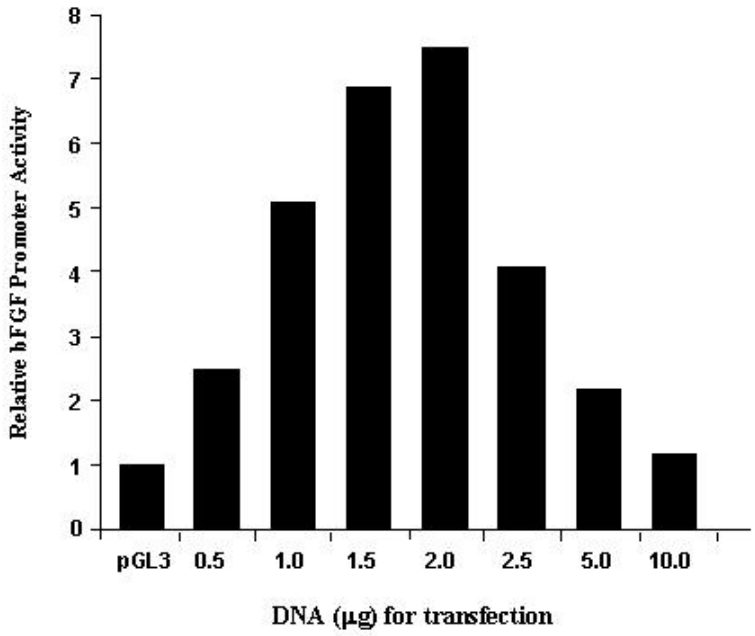


Cells were transfected with pGL3-bFGF by lipofection using Superfect according to the manufacturer's instructions. Cells were 80% confluence on the day of transfection in a 12-well plastic dish. For use in transfection, Superfect was incubated with plasmids (3.75µl of Superfect/ 0.75 µg of total plasmids) in 400µl serum-free medium for 15 min at room temperature. One milliliter of culture medium (with 5% FBS) was added to the DNA/Superfect mix, then added dropwise to the cells, and then incubated at 37°C in a humidified atmosphere of 5 % CO_2 for 2 h. After 2h, replace the DNA/Superfect medium to 2 ml of fresh culture medium. The day after transfection, cells were treated with various agents and LDL preparations for 24 h, unless stated otherwise. The cell lysates were then prepared for luciferase assays.

6. The promoter activity of bFGF vs. transfected DNA (μg) in cultured BAECs.

Figure 4. Dose-dependent bFGF promoter activity in transient gene expression assay. BAECs were co-transfected with varying amounts of pGL3-3 promoter construct (as described above in Figure 4) (horizontal axis) plus 0.5 μg of pRL-TK as transfection efficiency control using Lipofectamine. About 24h after transfection, cells were deprived of serum for another 24 h and then cell lysate was prepared for luciferase assay. Relative bFGF promoter activity (vertical axis) was expressed after normalization with pRL-TK.

Figure 4



7. The bFGF promoter is not regulated by LPC.

Figure 5. BAECs were co-transfected with pGL3-bFGF promoter construct plus 0.5 μg of pRL-TK as transfection efficiency control using Lipofectamine. About 24h after transfection, cells were deprived of serum and LPC or LDL preparations (horizontal axis) were added to the medium. After 24 h of incubation, cell lysates were isolated for luciferase assay. Relative bFGF promoter activity (vertical axis) was expressed after normalization with pRL-TK.

Figure 5