行政院國家科學委員會專題研究計畫 成果報告

五氯酚的致癌分子機制及其可能的預防方法研究

計畫類別：個別型計畫
計畫編號：
執行期間：○○年○○月○○日至○○年○○月○○日
執行單位：國立成功大學工業衛生科暨環境醫學研究所

計畫主持人：王應然

報告類型：完整報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫可公開查詢

中華民國○○年○○月○○日
1. Introduction

Pentachlorophenol (PCP) is a general biocide which is extensively used in wood and lumber industry mainly as a wood preservative because of its strong fungicidal or antibacterial activity proven in 1936 (Greene et al., 1978; Jorens and Schepens, 1993). Nowadays, although PCP has been prohibited or restricted to use in many countries, previous overuse and improper disposal make PCP become a detectable environmental contaminants (Eduljee, 1999; Muir and Eduljee, 1999).

PCP is a member of polychlorophenols occupational exposure. Lymphoma was induced in mice by dermal exposure of PCP. However, the precise mechanism is still unclear. Thereby, this study was designed to investigate the effects of PCP and TCHQ on immune system, particularly spleen in mice by dermal exposure. The result indicated that PCP and TCHQ affect splenocytes and its immune function both in vivo and in vitro. In vivo, mice treated with PCP for 6 wks had increased spleen weight, S phase of splenocytes and mitogen-induced cell proliferation. Mice treated with PCP for 12 wks decrease spleen weight, increased apoptotic splenocytes and inhibited LPS-induced proliferation. Spontaneous apoptosis was found in normal primary culture of splenocytes. We found a significant inhibition of apoptosis in culture splenocytes treated with TCHQ. Whereas, pretreatment of TCHQ inhibited splenocytes proliferation and secreted cytokines, including IL-2 and TNF-αstimulated by mitogen. Uncontrolled cell proliferation and inhibition of apoptosis in cells could be associated with the process of carcinogenesis. We suggest that malfunction of splenocytes induced by PCP and TCHQ could be one of the possible mechanisms of PCP-induced lymphoma in mice. Nevertheless, the detail mechanisms need to be further investigated.

Key words: PCP, TCHQ, Dermal exposure, Splenocytes, Apoptosis

2. Introduction
family. Some epidemiologic studies have shown significant connections between several cancer, including non-Hodgkin’s lymphoma, multiple myeloma, soft-tissue sarcoma and kidney cancer and chlorophenols and related chlorophenoxy acid herbicides exposure (Hardell et al., 1984; Scherret al., 1992). A case-control study showed forestry and agricultural workers exposed to phenoxyacetic acids or chlorophenols which the most common type is pentachlorophenol have increased risk for non-Hodgkin’s lymphoma, soft-tissue sarcoma and Hodgkin’s lymphoma (Hardell et al., 1981). Another cohort study about British Columbia sawmill workers found dermal exposed to PCP is associated with non-Hodgkin’s lymphoma, multiple myeloma and kidney cancer (Demers et al., 2006). In spite of many studies suggested that PCP is associated with malignant lymphoma, the evidence for carcinogenicity of PCP in human was limited and still debatable. Furthermore there are few study investigates carcinogenicity of PCP through dermal exposure. In our previous study, Chang et al first indicated that repeated dermal applications of PCP could induce lymphoma in spleen, liver and kidney in mice (Chang et al., 2003).

Genetic and acquired immunodeficiencies are the strongest known risk factors in malignant lymphoma (Staudt and Wyndham, 2002). Occupational and household exposed to PCP has been linked to immune alterations in human. People lived in PCP-treated log houses had blood lymphocytes with elevated frequencies of CD25 and CD26 which was T-cell activation marker, depressed mitogen responses which was associated with functional immunodepression, and dysregulation of B cells (McConnachie and Zahalsky, 1991). Workers exposed to PCP in a wood factory resulted in decreased response to mitogen and the degree of reduction was correlated with PCP levels in plasma (Colosio et al., 1993). Daniel et al. also found that human exposed to PCP for more than six months with increased levels of PCP in blood can result in severe T lymphocytes dysfunction, including impaired lymphocytes stimulation, decreased CD4/CD8 ratio, and increased IL-8 serum levels (Daniel et al., 1995). With further investigation, the results indicated that long-term low-dose exposed to PCP increased blood levels of PCP and is relative to abnormalities of cellular and humoral immunodeficiencies (Daniel et al., 2001). Animal studies were revealed that the PCP has immunotoxicity to mice, rat, chicken, and cattle (McConnachie and Zahalsky, 1991). Dietary exposed to PCP is immunosuppressive to mice (Kerkvliet et al., 1985; Blakley et al., 1998). Rat exposed to PCP by oral administration for 28 days, resulted in decreased antibody production but enhanced T lymphocyte and B lymphocyte blastogenesis (Blakley et al., 1998). The purpose of the present study was to investigate the effects of PCP and TCHQ on spleen in mice by dermal exposure.

3. Results and discussion

3.1. In vivo

3.1.1. Effects on body and spleen weights

Regardless of applying PCP or acetone to mice skin for 6 weeks or 12 weeks, there are no significant changes on mice body weights of all groups (data not shown). PCP dermal treatment does not appear obviously harmful effects on body weight in this present study. However, PCP can affect the proportions of weight of the spleen and weight. Mice treated with PCP five times a week for 12 weeks at the exposure dose of 60 μg results in a decreased spleen/body weight ratio (Table 1) in stead of higher dose. And exposure PCP for 6 week would not alter spleen/body weight ratio whether at the lower or higher concentrations. Repeated exposed to low dose PCP could decrease spleen weight.

3.1.2. Cell cycle

To investigate the possibility of PCP altering the cell cycle of splenocytes,
Splenocytes were isolated and the cell cycle was measured by PI stain. More than 70% of all splenocytes measured are in G1 phase in cell cycle. Splenocytes from mice with dermal exposed to PCP for 6 week showed increased percentage of S phase compared with the group unexposed to PCP (Figure 1.A). But when exposed to PCP for 12 weeks, the percentage of apoptosis in splenocytes was increased instead of the percentage of S phase (Figure 1.B).

3.1.3. Proliferation response
Splenocytes proliferation response was altered by PCP exposure. Splenocytes separated from mice were challenged with PHA or LPS as T cell and B cell mitogen to measure the proliferation response of splenocytes, and the response was expressed using stimulated indices (SI) which corrects for background value. Mice with PCP administration at the concentration of 1000 μg for 6 weeks have about twice enhanced proliferation responses compared to untreated group whether stimulated with PHA or LPS (Figure 2.A and C). However, the proliferation responses to LPS was inhibited in splenocytes isolated from mice treated with PCP for 12 weeks (Figure 2.B), and splenocytes were unaffected to PHA (Figure 2.D).

3.2. Ex vivo
3.2.1. Cell viability and cell cycle
To evaluate the toxicity of PCP to spleen, splenocytes isolated from mice without PCP pretreatment were incubated with different doses of TCHQ, the metabolite of PCP, and trypan blue exclusion followed. The cell viability of untreated splenocytes is 33.44%, less than half, and splenocytes treated with 25 μM TCHQ has less viability than treated with 500 μM TCHQ after 24 hr (Figure 3). Groups excepting for TCHQ treatment were continued to decrease as treatment time increased. The cell viability of untreated splenocytes is almost 0% after incubating for 168 hr. However, splenocytes administrated to high dose TCHQ, 500 μM, had stable viability kept in about 30% even until 168 hr, and there are still 5% cells of splenocytes which were treated with 25 μM viable at 168 hr. Analyzing those cells using PI stain found that almost 90% of the untreated splenocytes were in G1 phase (data not shown) at 0 hr, and it was decreased with treatment time, accompanying increased apoptosis percentage. On the contrary, more than 80% splenocytes applied to 500 μM TCHQ were kept in G1 phase for at least 96 hr. TCHQ administration at the concentration of 500 μM inhibits splenocytes spontaneous apoptosis.

3.2.2. Proliferation response and cytokines secretion
Proliferation responses of splenocytes to both mitogen, PHA and LPS, were greatly inhibited by TCHQ, no matter in low or high dose (Figure 5.A and B). TCHQ inhibits T cells and B cells proliferation of mitogen stimulation. To investigate the effects of TCHQ on the secretion of cytokines related to proliferation in the spleens, the concentration of IL-2 and TNF-α in the splenocyte cultures were measured. As figure 6 shows, splenocytes were incubated with 10 μg/ml PHA for 72 h gave a significant increase of IL-2 and TNF-α (Figure 6.A and B). Whereas, IL-2 and TNF-α were almost completely inhibited by TCHQ at both low and high doses. Splenocytes were incubated with 50 μg/ml LPS for 72 h also gave a significant increase of TNF-α. As well as the effects on PHA stimulation, TCHQ inhibited TNF-α secreted by LPS stimulated (Figure 7).

4. Discussion
The present study indicated that mice exposed to PCP for 12 weeks results in decreased spleen/body weight ratio, increased percentage of apoptosis in cell cycle and decreased proliferation response to B cell mitogen. PCP may harm spleen through induced abnormal splenocytes apoptosis and dysfunction of B cells, and finally expressed as decreased spleen weight. Furthermore, mice administrate to
PCP for 6 weeks, the splenocyte S phase percentage in cell cycle was increased and proliferation responses to T cell and B cell mitogen were also increased. Although spleen/body weight ratio had no significant increased, the results and exposure time seems to be related. Whether PCP could stimulate splenocytes to proliferate, and therefore results in increased spleen weight in the earlier stage of exposure or induce cell death, and therefore results in decreased spleen weight in the later period of exposure still need further research.

To investigate whether PCP could affect on the immune function of spleen, splenocytes were separated from spleen and stimulated to PHA or LPS as T cell and B cell mitogen followed TCHQ treatment, the metabolite of PCP, and IL-2 and TNF-α, the cytokines related to lymphocytes proliferation were measured. It was proven that unstimulated murine spleen lymphocytes undergo spontaneous apoptosis when cultured in vitro. T cells are activated by PHA treatment for 72 hr. In that system, PHA stimulate T cells to enter G1 phase from G0 phase through activating a signal (Firpo et al., 1994). The percentage of apoptotic cells was 2% in fresh spleen T cells and B cells, and respectively increased to 21% and 32% at least until 16 hr in vitro (Yi et al., 1998; Perandones et al., 1993; Illera et al., 1993). This study showed similar outcomes, fresh cultured splenocytes undergo apoptosis from 2% to all the cells death at 168 hr. To be the most interesting, the results also indicated that TCHQ can prevent splenocytes undergoing apoptosis, but the viable cells are dysfunction in immune response. The immune system are considered to play a significant role in the prevention of malignancy, previous study indicate that immunodeficiency in humans is a risk factor in the development of malignancy (Alexander et al., 1980).

Diverse molecular abnormalities in lymphomas results in several common pathways, including proliferation promotion, blacked differentiation, cell death inhibition, or permit genetic instability (Staudt and Wyndham, 2002). The detail mechanisms of PCP-induced genetic alternation in splenocytes still need to be further investigated.

四、計畫成果自評
1. 原計畫內容在探討五氯酚在皮膚兩階段化學致癌過程中促癌能力之評估，以小鼠皮膚的兩階段動物致癌模式探討五氯酚及四氯對苯二酚在鼠體皮膚造成腫瘤及其相關的基因表現，應已達成原計畫之預期目標。
2. 五氯酚及四氯對苯二酚的毒性及致癌性在小白鼠的實驗中甚為明確，但其致癌的作用機轉及傷害指標的研究卻甚少。本研究內容已整理成可供學術期刊發表之格式。其中有部分內容已發表在 2006 年的 Toxicology. 223: 181-190 (2006)

5. References
Daniel V, Huber W, Bauer K, Suesal C,


<table>
<thead>
<tr>
<th>報告人姓名</th>
<th>王應然</th>
</tr>
</thead>
<tbody>
<tr>
<td>服務機構及職稱</td>
<td>國立成功大學醫學院環境醫學研究所</td>
</tr>
<tr>
<td>時間 會議地點</td>
<td>2005/12/15-12/20 Honolulu, Hawaii, U.S.A</td>
</tr>
<tr>
<td>本會核定補助文號</td>
<td>NSC94-2314-B-006-038</td>
</tr>
</tbody>
</table>

會議名稱:
(中文) 2005年太平洋化學會年會
(英文) International Chemical Congress of Pacific Basin Societies

發表論文題目:
(中文) 二氫硫辛酸透過抗發炎及抗氧化作用抑制皮膚之促癌反應
(英文) Dihydrolipoic acid inhibits skin tumor promotion through anti-inflammation and anti-oxidation.

報告內容應包括下列各項：(如附件)

一、參加會議經過

二、與會心得

三、考察參觀活動(無此项活動者省略)

四、建議

五、攜回資料名稱及內容

六、其他
2005 年太平洋化學會年會 心得報告

國立成功大學醫學院 環境醫學研究所 王應然 副教授

一、參加會議經過

此次和台北醫學大學何元順教授，高雄海洋大學潘敏雄教授，高雄醫學大學生化所何自銓教授同行參加今年的 2005 年太平洋化學會年會。該會選在美屬夏威夷群島舉行，此一盛會結合世界各地從事化學研究的學者，分享彼此的研究經驗，堪稱是這一領域研究人員一年一度的盛事。這次會議的早上照慣例均安排專家針對特定的主題作專題演講，題目涵蓋的領域非常廣且都與化學有關，除了同行間科學新知的交流外，對於研究者及博士後研究員而言，這也是一個很好的教育機會。此行認識一位在美國教書研究極其優秀的何其黨教授何教授目前在美國羅格斯特大學食品科學系擔任教授，何教授有非常前瞻地研究視野，是一位值得學習的長者。趁這個機會了解別人的研究室，聽聽大家最近的進展及對未來的想法，彼此交換一點訊息也是一种良性的互動。今年的會議選在島上的 Hilton Hotel 舉辦，為頗具規模的高級觀光渡假中心，乾淨整潔的陳設讓人身心舒暢。美屬夏威夷群島是一個相當美麗的觀光聖地，許多來自世界各地的遊客專程來這裡享受陽光和沙灘。因為緯度的關係，小島上終年的氣候都溫暖舒適，因此是歐美高緯度國家人士的避寒渡假聖地。由於飯店附近有潔淨的海灘，因此參加會議的學者在會議空檔時間如果不想去其它景點參觀，留在飯店附近的海灘游泳曬太陽就已經是很大的享受了。

二、與會心得

第一天早上的主題是血基質過氧酵素 (Heme-peroxidase) 在生物及醫學上的角色，Dr. Buettner 先介紹自由基的一些基本概念，再由 Dr. kettle 講解過氧酵素的生化特
性。從自由基的定義、命名到脂質過氧化作用及抗氧化劑的作用方式均在 Dr. Buettner 精彩的圖表中完整的將訊息傳達給聽眾。而有關過氧化酵素的研究則側重在白血球的防禦功能中過氧化酵素可能擔任的角色。重點放在嗜中性白血球 (neutrophils) 即嗜伊紅性白血球 (eosinophils) 在吞噬細菌及發炎有關反應中過氧化酵素可透過產生大量的自由基來殺死入侵的微生物。第二天的主題是壓化性壓力與心血管疾病 (oxidative stress and cardiovascular diseases)。由 Dr. Berliner 講解動脈粥狀硬化 (Atherosclerosis) 的前期病理反應中，一氧化氮 (NO) 及氧化脂質 (oxidized lipids) 所誘發的分子調控機轉。目前已有許多證據支持氧化性壓力與動脈粥狀硬化有關。舉例而言，動脈粥狀硬化的血管內活性氧物種 (ROS) 的含量會上升。損傷的血管產生過量的脂質過氧化產物，而這些產物會誘發進一步的發炎反應。抗氧化劑的攝取則會降低血管損傷的程度。另一個演講則由 Dr. Dewhirst 介紹缺氧 (Hypoxia) 在腫瘤的血管新生 (Angiogenesis) 過程中可能的角色。內容主要在探討缺氧如何引發訊息傳遞機轉 (Signal Transduction Pathways) 而進一步調控與血管新生有關的基因表現，缺氧與血管新生的相互關係可以在正常組織，病理狀態 (Pathological states) 及腫瘤中被詳細的研究。第三天的主題是活性氧及活性氮物種與發炎及癌症的研究 (ROS & RNS in Inflammation and Cancer)。Dr. Weitzman 訂了一個有趣的題目來介紹慢性發炎與癌症的關係，他的題目是 Your mother was right, stop picking at that。其假說根基於發炎反應活化的吞噬細胞 (phagocytes) 會產生有毒的氧化物質進而使 DNA 損傷，因此與吞噬細胞有關的氧化物質 (oxidants) 可能誘發腫瘤。這些氧化物質包含由 NADPH oxidase 及 myeloperoxidase 作用產生的 ROS 如 O2-, OH-, ONOO-, HOC1, H2O2 等。而其致癌的可能機制則包含：DNA 的股斷裂、突變 氧化修飾鹼基、染色體錯亂、促癌作用、促進基因表現，細胞死亡及組織新生、惡性腫瘤轉化等。另一個演講由 Dr. Marnett
介紹氧自由基導致的 DNA 損傷及癌症的關係。重點放在 DNA 鹼基的氧化修飾作用與突變及 DNA 修飾機轉的關係。DNA 內嘌呤及嘧啶鹼基的化學結構若受到氧自由基的攻擊會產生結構的改變而引起基因的突變，若修復系統無法有效且即時的修補，則突變的基因就有機會發展成腫瘤。兩場的演講均相當的豐富精彩。第四天的主題是細胞凋亡及增生中氧化還原的調控（Redox Regulation of Apoptosis and Proliferation）。第一場演講由 Dr. Davies 講解增生中的細胞受到氧化物質作用時可能有哪些反應產生，而這些不同的反應主要取決於氧化物質的濃度。不同的氧化物濃度可能讓增生中的細胞暫時或永久的停止生長，分裂加速，凋亡或壞死。第二場演講由 Dr. Griffith 紹 glutathione (GSH) 的重要性，由於 GSH 是體內非常重要的內生性抗氧化防禦機轉的一環，它的濃度高低便具有重要的生理意義。文中探討 GSH 的代謝及功能，如何抑制 GSH 的生成及降解並介紹 GSH 的分析定量方式，相當的實用有趣。

三、建議事項

此次會議令人相當深刻的是多數的華人科學研究者均來自中國大陸，台灣僅佔非常小的比例。由此可知大陸的科學研究已有大幅的進展，台灣似乎應更加強於基礎科學的投資和獎勵，以保持科技和產業的優勢。另一個值得重視的問題個人認為語言能力是台灣的科學工作者必須認真面對和克服的障礙。此次大會的口頭論文報告及專題演講幾乎是西方人的舞台。台灣學者在科學論文的寫作方面已無太大問題，也有許多科學論文寫作的課程教授及科學論文的修改。但在英語演講及人際溝通方面似乎仍有成長的空間。如何提升台灣科學研究人員在這方面的能力和競爭力應是一個重要的課題。此次大會另一個值得借鏡之處個人認為整體的氣氛營造相當溫馨自在。所有的活動安排都以參與人員最大的方便為主，舉例而言每天早上的演講，與會人員可以端著大會提供的早餐坐在有桌面的位子一面品嚐香淳的咖啡一面聆聽大師級科學家的演講，心靈與肉體同時得到
滿足。雖是小事一椿，但多少傳達了科學研究是一種心智活動，不必受到外在的束縛和牽伴。
Dihydrolipoic acid inhibits skin tumor promotion through anti-inflammation and anti-oxidation

Yuan-Soon Ho¹, Hsin-I Liu², Sheng-Yow Ho³,⁴, Chein Tai⁵, Min-Hsiung Pan⁶ and Ying-Jan Wang²

¹Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan;
²Department of Environmental and Occupational Health, and ³Institute of Basic Medical Sciences, National Cheng Kung University, Medical College, Tainan, Taiwan; ⁴Sinlau Christian Hospital, Tainan, Taiwan; ⁵National Science Council, Taipei, Taiwan; ⁶Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan.

Running title: Dihydrolipoic acid inhibits skin tumor promotion

Please send all correspondence to:

Dr. Ying-Jan Wang,
Department of Environmental and Occupational Health
National Cheng Kung University Medical College
138 Sheng-Li Road
Tainan 70428, Taiwan
Tel: 886-6-235-3535 ext. 5804
Fax: 886-6-2752484
E-mail: vjwang@mail.ncku.edu.tw

And

Dr. Min-Hsiung Pan

Department of Seafood Science,

National Kaohsiung Marine University

No. 142, Hai-Chuan Rd, Nan-Tzu, Kaohsiung, Taiwan.

Tel: 886-7-361-7141

Fax: 886-7-361-1261

E-mail: mhpam@mail.nkmu.edu.tw
Abstract:

α-lipoic acid (LA) has been intensely investigated as a therapeutic agent for several diseases, including hepatic disorder and diabetic polyneuropathy. However, the effects of LA or its reduced form dihydrolipoic acid (DHLA) on cancer chemoprevention has never been reported. In the present study, we examined the effects of DHLA/LA on the production of nitric oxide (NO) by inducible NO synthase (iNOS) and the formation of prostaglandin E2 (PGE2) by cyclooxygenase-2 (COX-2), two important mediators associated with inflammation. DHLA/LA significantly inhibited lipopolysaccharide (LPS)-induced NO and PGE2 formation in RAW 264.7 cells. Treatment with DHLA/LA also suppressed the expression of iNOS protein but, unexpectedly, didn’t affect or even increase the expression of COX-2 protein. The in vivo anti-inflammatory and antitumor promoting activities were evaluated by topical TPA application to mouse skin with measurement of edema formation, epidermal thickness and hydrogen peroxide production. DHLA significantly inhibited the priming and activation stages of skin inflammation induced by double TPA application by decreasing the inflammatory parameters. Furthermore, DHLA inhibited DMBA (0.3 µmol)/TPA (2.0 nmol)-induced skin tumor formation by reducing the tumor incidence and tumor multiplicity. These results suggest that DHLA could be a possible chemopreventive agent in inflammation-associated tumorigenesis.

Key words: α-lipoic acid (LA); dihydrolipoic acid (DHLA); cancer chemoprevention; oxidative stress; inflammation; tumor promotion
1. Introduction:

Lipoic acid (LA) (Fig. 1) is a thiol-compound naturally occurring in plants and animals [1]. It is consumed in the daily diet, and taken up and transformed in cells and tissues into dihydrolipoic acid (DHLA) [2]. Both LA and DHLA can serve as strong antioxidants through several mechanisms, including the scavenging of free radicals, chelation of metal ions, and regeneration of endogenous and exogenous antioxidants, such as ubiquinone, glutathione, and ascorbic acid [3, 4]. Therefore, it appears that LA could be a potential agent in the prevention of different diseases that may be related to an imbalance of the oxidoreductive cellular status. This occurs in cases of neurodegeneration, ischemia-reperfusion, polyneuropathy, diabetes, AIDS, and hepatic disorder status [5]. Nevertheless, little is known about the effects of LA/DHLA on cancer chemoprevention.

Reactive oxygen species (ROS) from both endogenous and exogenous sources can cause oxidative DNA damage and dysregulated cell signaling, which are involved in the multistage process of carcinogenesis, including tumor initiation, promotion and progression. A number of structurally different anticarcinogenic agents inhibit inflammation and tumor promotion as they reduce ROS production and oxidative DNA damage [6]. Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of nitric oxide (NO) by inducible NO synthase (iNOS) and the formation of prostaglandins (PGs) by cyclooxygenase-2 (COX-2) [7, 8]. Many cell types, especially macrophages, express iNOS upon stimulation, such as through exposure to bacterial LPS or cytokines. NO exerts its role in host defense as a result of its antibacterial and virustatic properties. However, if NO production gets out of control, damage to host cells occurs due to the cytotoxic potential of NO [9]. NO has been reported to cause mutagenesis, deamination of DNA bases and to form carcinogenic N-nitrosamines [10-12]. It is also involved in the
production of VEGF, the overexpression of which induces angiogenesis, vascular hyperpermeability, and accelerated tumor development [13, 14]. PGs are important for the initiation, promotion and progression of chemical carcinogenesis [15]. They can suppress the humoral and cellular immune action responsible for the killing of malignant cancer cells [15, 16]. The direct interactions of prostaglandins with their receptors through autocrine or paracrine pathways to enhance cellular survival or stimulate angiogenesis have been proposed as the molecular mechanisms underlying the pro-carcinogenic functions of COX-2 [17]. The topical application of TPA on mice led to edema and papilloma formation by enhancing COX-2 protein expression. Specific COX-2 inhibitors could counteract these biological events [18, 19]. Collectively, the suppression of enzyme induction and the activities of iNOS/COX-2 is a new paradigm for the prevention of carcinogenesis [20].

For more than 50 years, mouse skin has been used as a conventional model for studying the mechanisms of carcinogenesis, and the modulation of sequential steps involved in this process [21, 22]. Skin tumors can be induced by the sequential application of a sub-threshold dose of a carcinogen (initiation stage) followed by repetitive treatment with a non-carcinogenic promoter [21, 23]. The topical application of the classic tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) on mouse skin is shown to result in a number of biochemical alterations, changes in cellular functions, and histological changes leading to skin tumor promotion [24, 25]. Among these, the ones that correlate best with the skin tumor-promoting activity of TPA include skin edema, epidermal hyperplasia, inflammation, proliferation and oxidative stress [26, 27]. All these changes in the skin have been defined as possible markers of skin tumor promotion, and were used to evaluate the potency of novel chemopreventive agents against tumor promotion [28, 29].

In our previous study, we proved that two-stage skin tumorigenesis in the ICR mouse is a good model for determining the skin tumor-promoting potential of environmental toxicants
Here we applied the same model to examine the anti-tumor promotion effects of DHLA/LA on mouse skin. The *in vivo* anti-inflammatory activities were evaluated by a double application of TPA to mouse skin (priming and activation) with measurement of edema formation, epidermal thickness and H$_2$O$_2$ generation [20]. Due to the importance of iNOS and COX-2 in inflammatory skin damage, we first tested the hypothesis that the anti-tumor promotion effects of LA/DHLA might occur through modulating the expression of these two crucial inflammatory mediators, by using a LPS-induced NO and PGE$_2$ generation test in mouse macrophage RAW 264.7 cells.
2. Materials and Methods:

2.1. Chemicals and Animals

TPA, dimethylbenz(a)anthracene (DMBA), LA and DHLA were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained in the purest form available commercially. Six-week-old female CD-1 mice were acquired from the animal center of the National Cheng Kung University Medical College. The animals were housed five per cage at 24 ± 2°C and 50 ± 10% relative humidity and subjected to a 12-h light/12-h dark cycle. They were acclimatized for 1 week before use and fed a Purina chow diet and water *ad libitum*. Prior to study, the dorsal side of the skin was shaved, and the tested compound dissolved in 100 µl acetone was administered by micropipette. The test area was regularly shaved and the mice were observed daily.

2.2. Nitrite and PGE₂ Determination.

RAW 264.7 cells, grown confluent in 2 ml of DMEM on a 60-mm dish, were treated with LPS (100 ng/ml), and LA/DHLA (0, 10, 50, 100, 250 or 500 µM) dissolved in DMSO. To determine whether LA/DHLA inhibits LPS-induced nitric oxide synthesis, the sum of stable nitric oxide metabolites, nitrate and nitrite in culture media samples 20 h after exposure to LPS were measured. Culture media was harvested for analysis of nitrates using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) that involves the Griess reaction. Nitrate concentrations were measured after reducing all nitrates into nitrite using nitrate reductase. The absorbance was measured at a wavelength of 570 nm. The values measured were compared with the standard curves. The concentrations of PGE₂ in the media were measured by a commercially available Prostaglandin E₂ Enzyme Immunoassay Kit (Assay Designs Inc,
Michigan, USA). Each experiment was done in duplicate independently twice, and the data were expressed as mean ± SD values.

2.3. Western blotting

Cells were washed with PBS and lysed in an ice-cold RIPA buffer (Tris-HCl pH 7.2, 25 mM; SDS 0.1%; Triton X-100 1%; sodium deoxycholate 1%; NaCl 0.15 M; EDTA 1 mM) containing 1 mM of phenyl methyl sulfonyl fluoride (PMSF), 10 µg/ml of aprotinin, 1 mM of sodium orthovanadate and 5 µg/ml of leupeptin. Protein concentrations were determined with the BCA method (Pierce, Rockford, IL, U.S.A.). Protein (50 µg) was resolved by 12.5% polyacrylamide gel electrophoresis and blotted onto nitrocellulose sheets using the semidry blot system (TE 70; Hoefer Scientific Instruments, San Francisco, CA) at 2 mA/cm² for 60 min in 25 mM Tris-HCl, pH 8.3; 192 mM glycine; and 20% methanol. The membrane was blocked overnight at room temperature with a blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% Tween 20; 4% nonfat dry milk; and 0.1% sodium azide). Then it was incubated for 1 h with anti-iNOS mAb (Transduction Laboratories) and anti-COX-2 mAb (Santa Cruz Biotechnology). The proteins were washed three times, incubated with alkaline phosphatase-conjugated rabbit anti-mouse in PBS and 0.5% Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were made visible by the Bio-Rad NBT-BCIP color development system.

2.4. Double TPA Treatment of Mouse Skin.

The double TPA treatment experiment was performed as reported by Nakamura et al [31]. Briefly, the back of each mouse was shaved 2 days before each experiment; each experimental group consisted of five mice. DHLA (4050 nmol in 100 µl of acetone) was
applied topically to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 µl of acetone). In the double-treatment protocol, the same doses of TPA and DHLA or acetone were applied twice at an interval of 24 h. We divided the mice into five groups as follows: group 1 (acetone x 2/acetone x 2); group 2 (acetone-TPA/acetone-TPA); group 3 [DHLA (priming)-TPA/acetone-TPA]; group 4 [acetone-TPA/DHLA (activation)-TPA]; and group 5 (DHLA-TPA/DHLA-TPA).

2.5. Epidermal Hyperplasia Study

For the epidermal hyperplasia study, skin samples from different treatment groups were fixed in 10% formalin and embedded in paraffin. Vertical sections were cut, mounted on a glass slide, and stained with H&E. Epidermal hyperplasia was determined by measuring the mean vertical epidermal thickness and the mean number of vertical epidermal cell layers through microscopic examinations of each treated skin tissue section. For each section of the skin, the thickness of the epidermis from the basal layer to the stratum corneum was measured at several equidistant interfollicular sites using a Nicon light microscope (Japan) equipped with an ocular micrometer. For each individual value, a mean of 25 vertical epidermal measurements, equidistant from each other at 2 mm, in 50 mm (linear) of skin section was determined.

2.6. Measurement of H$_2$O$_2$ and Edema Formation in Mouse Skin

Measurement of the levels of H$_2$O$_2$ and edema formation was done as reported by Nakamura et al [31]. In brief, mice treated with the double-treatment protocol were sacrificed 1 h after the second TPA treatment. Skin punches (epidermis and dermis) were obtained with an 8-mm-diameter cork borer and were weighed with an analytical balance. The skin punches
were minced in 3 ml of 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide and then homogenized at 4°C for 30 s twice. The homogenate was centrifuged at 10,000 \( x \) g for 20 min at 4°C. The \( \text{H}_2\text{O}_2 \) content was determined by the phenol red-horseradish peroxidase method.

2.7. Two-Stage Carcinogenesis Experiment in Mouse Skin

The antitumor promoting activity of DHLA was examined by a standard initiation-promotion protocol with DMBA and TPA, as reported previously [30]. The mice were randomly divided into six groups (group I to group VI) of 10 animals each, and a single 300 nmol dose of DMBA in 100 \( \mu \)l of acetone as a tumor initiator was applied topically on the dorsal shaved skin of each mouse in all groups. One week later, group I was treated with 100 \( \mu \)l of acetone topically as negative controls for monitoring any spontaneous tumor induction and group II was treated with 2.0 nmol of TPA in 100 \( \mu \)l of acetone topically as positive controls. Groups III, IV, and V were treated with DHLA (300, 900 and 2700 nmol/100\( \mu \)l acetone) 30 min before each TPA treatment. Group VI were treated similarly to group II, but with a 0.2% LA diet supplement. Treatments of DHLA and TPA were repeated two times per week up to the termination of the experiment at 20 weeks from the treatment of DMBA. The antitumor-promoting activity was evaluated by both the ratio of tumor-bearing mice and the number of tumors that measured more than 1 mm in diameter, per mouse.

2.8. Statistical Analysis

The statistical significance of the differences between the means was evaluated using the Student's t test. A difference with a \( p \) value less than 0.05 was considered statistically significant. All statistical analyses were carried out using the SAS package.
3. Results

3.1. DHLA/LA reduced LPS-induced nitrite accumulation in RAW 264.7 cells

Stimulation of RAW 264.7 cells with LPS for 20 h resulted in NO generation and then nitrite and nitrate (NOx) accumulation in the media (Fig. 2). DHLA, at a concentration range of 100–500 µM, concentration-dependently suppressed NOx production by 21.2–59.8% (Fig. 2A). A similar result was found in LA with mild inhibitory effects. LA, at a concentration range of 100–500 µM, suppressed NOx production by 13.8–43.0% (Fig. 2B). iNOS protein expression was then detected using Western blotting. As also shown in Fig. 2, under control conditions RAW 264.7 cells do not express iNOS protein, whereas LPS induces a remarkable increase in this enzyme. Treatment with DHLA or LA suppressed the expression of iNOS significantly when compared with treatment with LPS alone. The reduced expression of iNOS protein was consistent with reductions in total nitrates (NOx) in culture media.

3.2. DHLA/LA reduced LPS-induced PGE2 production but not COX-2 protein expression in RAW 264.7 cells

Stimulation of RAW cells with LPS for 20 h led to PGE2 production in the media (about 6 ng/ml; Fig. 3). DHLA or LA suppressed PGE2 production at concentrations of 10 to 500 µM. Both DHLA and LA reversed completely the increased PGE2 production stimulated by LPS at concentrations higher than 50 µM. Stimulation of RAW 264.7 cells also led to a marked up-regulation of COX-2 protein expression, which was much less expressed in the controls. Interestingly, treatment with DHLA or LA didn’t suppress the expression of COX-2 protein when compared with treatment with LPS alone (Fig. 3). Thus, the unchanged or even increased expression of COX-2 protein was inconsistent with reductions in PGE2 production in culture media. We further analyzed the effects of DHLA or LA alone on the expression of COX-2 in RAW 264.7 cells, and found that both DHLA and LA can induce COX-2 protein
expression significantly (Fig. 4A). Notable cytotoxicity was not observed in any of the above experimental conditions (Fig. 4B).

3.3. Anti-inflammatory and antioxidative activities of DHLA in mouse skin

We examined whether DHLA inhibits the priming and/or activation stages using the double-TPA-application model. As shown in Table 1, double TPA applications, at a dose of 8.1 nmol each with a 24-h interval, led to marked edema formation (76.9 ± 9.7 mg/punch in group 2) as compared with the control (44.3 ± 3.6 mg/punch in group 1; \( P < 0.05 \) versus group 2). Pretreatment in the priming phase (group 3) was found to be more effective for edema suppression than in the activation phase (group 4; \( P < 0.05 \)). Double pretreatment with DHLA 30 min prior to each TPA application suppressed edema formation completely (group 5; \( P < 0.05 \)). In accordance with the TPA-induced enhancement of edema formation, an increase in epidermal thickness was observed with double TPA applications (Table 1 and Figure 4, A and B; 18.6 ± 3.8 µm in group 1 versus 31.0 ± 4.9 µm in group 2; \( P < 0.05 \)). Pretreatment with DHLA in the priming stage was also more suppressive than that in the activation stage (Table 1 and Figure 4). Double DHLA pretreatment showed the highest inhibition. In addition, the double TPA application also dramatically increased the level of \( \text{H}_2\text{O}_2 \) in the mouse epidermis and dermis 4.15-fold (Table 1; 1.17 ± 0.24 nmol/punch in group 1 versus 4.86 ± 0.32 nmol/punch in group 2; \( P < 0.05 \)). A higher inhibition by DHLA was again observed when applied in the priming stage (IR, 50.9% in group 3) than in the activation phase (IR 32.8% in group 4). Double pretreatment with DHLA showed the highest inhibition of \( \text{H}_2\text{O}_2 \) production among the three inhibitory groups (IR, 65.6% in group 3).

3.4. Antitumor-promoting activity of DHLA/LA in mouse skin

We then examined the inhibitory effects of a topical application of DHLA, at a dose
range of 300–4050 nmol, on tumor formation in DMBA (0.3 µmol)-initiated and TPA (2.0 nmol)-promoted mouse skin. As shown in Table 2, with treatment of DMBA and TPA, the tumor incidence in this positive control group was 70% 20 weeks after promotion. In contrast, administration of DMBA followed by repeated applications of acetone produced no tumors. When DHLA (4050 nmol) was pretreated before the initiator DMBA, the tumor incidence was completely inhibited. In the other three DHLA-pretreated groups (30 min before TPA), the incidence was reduced by 10-60%. The average number of tumors per mouse in the control was 3.7, and pretreatment with DHLA at 300 to 2700 nmol dose-dependently reduced them by 43.2%, 62.2% and 91.9%, respectively. Unfortunately, we didn’t observe any antitumor-promoting activities of LA in this model when supplemented 0.2% in the diet.
4. Discussion:

In the present study, we demonstrate that both DHLA and LA treatment inhibit the expression of iNOS and NO synthesis in mouse macrophages (RAW 264.7 cells) activated with LPS. LA has recently been suggested as a regulator of iNOS in RAW 264.7 macrophages [32, 33]. The inhibitory effects on iNOS expression and NO synthesis are mediated, in part, through the NF-κB signaling pathway [32]. In addition, previous work using RAW 264.7 cells has identified NF-κB enhancer elements in the iNOS promoter that permit iNOS induction by cytokines and LPS [34]. Thus, LA interacts with a crucial mediator in inflammation and cancer [35]. This potency is most likely linked to the antioxidative properties of LA. The activation of NF-κB is commonly seen as an indicator for oxidative stress and antioxidants are well-described inhibitors of NF-κB activation [5, 36, 37]. DHLA is a more potent antioxidant than LA. Herein, we have demonstrated, for the first time, a higher inhibitory effect of DHLA than LA on the induction of iNOS and NO in RAW 264.7 cells stimulated by LPS. It is important to note that LA not only attenuates NO production in vitro in cell cultures but also in vivo. LA was demonstrated to protect from hyperdynamic circulation in biliary cirrhosis, which was related to an LA-mediated decrease in bile duct ligation-induced NO formation [38].

COX-2 overexpression has been reported to be associated with carcinogenesis, tumor growth and metastasis. The contribution of COX-2 to these processes has been attributed to a COX-2-mediated production of prostaglandin, with subsequent conversion of procarcinogens to carcinogens, inhibition of apoptosis, promotion of angiogenesis, modulation of the inflammation and immune function, and increased tumor cells invasiveness [39]. Therefore, PGE$_2$ production caused by increased expression or activity of COX-2 in macrophages after
treatment with LPS or proinflammatory cytokines might increase the risk of tumorigenesis.

LPS and proinflammatory cytokines promote PGE2 production by increasing COX-2 expression in a manner dependent on NF-κB activation [40, 41]. NF-κB has been proposed to be a redox-sensitive transcription factor [42], and LA inhibited LPS-induced NF-κB by inhibiting the activities of IκB kinase [32]. However, in this study, DHLA and LA (10-500 µM) inhibited PGE2 synthesis by LPS without affecting the protein expression level of COX-2 in RAW 264.7 cells (Fig. 3). Furthermore, treatment of RAW 264.7 cells with high doses of DHLA/LA (250-500 µM) alone increased the COX-2 protein expression (Fig. 4). Thus, it is unlikely that DHLA/LA affects the LPS-induced signaling pathways, including NF-κB activation, required for the increases in the enzymes involved in PGE2 biosynthesis. In a recent report, the authors indicated that iNOS specifically binds to COX-2 and S-nitrosylates it, enhancing COX-2 catalytic activity. Selectively disrupting iNOS–COX-2 binding prevented a NO-mediated activation of COX-2 [43]. They established a physiologic binding interaction of iNOS and COX-2, bringing NO into proximity with COX-2 and facilitating its S-nitrosylation and activation, suggesting that the molecular synergism between iNOS and COX-2 may represent a major mechanism of inflammatory responses. Drugs that block the iNOS–COX-2 interaction may be anti-inflammatory, synergizing with COX-2 inhibitors and permitting lower doses. As the binding site on iNOS is in the catalytic domain, derivatives of iNOS inhibitors that also prevent binding to COX-2 may decrease both NO and prostaglandin formation [43]. Whether the inhibitory effect of DHLA/LA on PGE2 production in RAW 264.7 cells stimulated by LPS occurs through disrupting the iNOS–COX-2 interaction still requires further investigation.

As summarized in Table 1, DHLA inhibited double-TPA-application-induced biological and histological parameters relating to oxidative damage and inflammation. DHLA significantly suppressed all of the parameters in both the priming and the activation stages.
The inhibitory effects of double DHLA pretreatment appear to be caused by the additive effects of each application in both stages because the double pretreatments were most suppressive. TPA has recently been reported to produce NO and VEGF in human polymorphonuclear leukocytes [14, 44]. Overexpressed VEGF leads to the induction of vascular hyperpermeability [13]. On the other hand, PGE$_2$ is well known to increase vascular permeability. DHLA inhibited the release of PGE$_2$ from RAW 264.7 cells (Fig. 3). Although we have not examined the effects of DHLA on vascular permeability, the inhibition by DHLA of edema formation and the reduction of epidermal thickness (Table 1 and Fig. 5) may be partly attributable to the suppression of NO, VEGF, and PGE$_2$ production.

Double-TPA-application-induced H$_2$O$_2$ production \textit{in vivo} was markedly inhibited by the pretreatment(s) of DHLA. Decreased levels of H$_2$O$_2$ may be attributable to the inhibition of O$_2^-$ generation because H$_2$O$_2$ is mostly derived from O$_2^-$ as a function of O$_2^-$ dismutase or nonenzymatically. DHLA, a dual inhibitor of both O$_2^-$ and NO radical generation, can be recognized as a potent, naturally occurring anti-inflammatory agent, because peroxynitrite, a coupling product of O$_2^-$ and NO, enhances COX-2 activity [45], involving inflammatory processes and thereby leading to carcinogenesis.

Most clinical tumors have a long history of pathological development during which they pass through several preneoplastic and premalignant stages before becoming malignant. This situation offers the opportunity to interrupt or reverse tumor development at a still harmless stage, for instance by chemoprevention, i.e. by taking medicines acting on distinct molecular processes of tumorigenesis [46]. In recent year, considerable emphasis has been placed on identifying new cancer chemopreventive agents which could be useful for human populations [47]. As predicted by the suppressive efficacies of biochemical markers related to oxidative stress and inflammation, topical application of DHLA at doses of 300 to 2700 nmol inhibited the incidence and multiplicity of skin tumors in a dose-dependent manner (Table 2). We also
found a completely inhibitory effect of DHLA on tumor incidence when applied before the initiator DMBA in this two-stage mouse skin tumorigenesis model. However, the detailed mechanisms are still unclear and need to be further investigated. In any case, this is the first report demonstrating the chemopreventive ability of DHLA in an animal model.

In summary, the results presented here demonstrate that LPS-induced NO and PGE$_2$ generation in macrophages can be inhibited by DHLA and LA. Searching for NO and PGE$_2$ inhibitors in this model may be a promising strategy for discovering effective chemopreventive agents. DHLA/LA was found to be a functionally novel antitumor promoter by working in both the priming and activation stages in mouse skin. Our results also indicate the possibility that DHLA/LA could have beneficial effects in preventing several other diseases mediated by NO and PGE$_2$ overproduction.
Acknowledgments

This study was supported by the National Science Council (NSC 94-2314-B-006-038).
Figure legends

**Figure 1.** The chemical structures of α-lipoic acid and dihydrolipoic acid.

**Figure 2.** Effects of DHLA and LA on LPS-induced iNOS protein level and nitrite production in RAW 264.7 cells. The cells were treated with 100 ng/ml of LPS only or with different concentrations of (A) DHLA and (B) LA for 20 h. At the end of incubation time, 100 μl of the culture medium was collected for nitrite assay. The expression of iNOS protein was detected by Western Blot using specific antibody. The values are expressed as means ± SE of triplicate tests. *P* < 0.05 indicate statistically significant differences from the LPS-treated group.

**Figure 3.** Effects of DHLA and LA on LPS-induced COX-2 protein levels and PGE2 production in RAW 264.7 cells. (A) The cells were treated with DHLA and (B) LA for 20 h. At the end of incubation time, 100 μl of the culture medium was collected for PGE2 assay. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of COX-2 protein was detected by Western Blot using specific antibody. The values are expressed as means ± SE of triplicate tests.

**Figure 4.** Effects of DHLA and LA on COX-2 protein expression in RAW 264.7 cells. (A) The cells were treated with various concentrations of DHLA and LA for 20 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of COX-2 and GAPDH protein was detected by Western Blot using specific antibodies. Quantification of COX-2 protein expression was performed by densitometric analysis of the immunoblot. These experiments were repeated three times with similar results. (B) Cytotoxic effects of DHLA and LA in RAW 264.7 cells. Cytotoxicity was estimated by trypan blue exclusion using a...
hemocytometer chamber.

The values are expressed as means ± SE of triplicate tests.

**Figure 5.** Suppression by DHLA of TPA-induced skin morphological changes, observed by hematoxylin and eosin staining. DHLA (4050 nmol in 100 μl acetone) was topically applied to the shaved area of dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 μl acetone). After 24, the same doses of DHLA or acetone was applied 30 min prior to a second TPA application. Methods for histological staining are described in Materials and methods. Ac: acetone.
5. References:


[40] Di Mari JF, Mifflin RC, Adegboyega PA, Saada JI, Powell DW. IL-1alpha-induced


