# *IN VITRO* AND *IN VIVO* ANTI-INFLAMMATORY EFFECTS OF ROSMANOL AND CARNOSOL ISOLATED FROM ROSEMARY

**by**

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## ABSTRACT OF THE DISSERTATION

*In vitro* and *In vivo* Anti-inflammatory Effects of Rosmanol and Carnosol Isolated from Rosemary

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Rosemary (*Rosmarinus officinalis L.*) leaves are widely used as food ingredient, and have been focused on their strong antioxidant, anti-inflammatory, and anti-cancer activities. Rosmanol is transformed from carnosol, one of strong antioxidant in rosemary extract, in the presence of oxygen and demonstrated that it has a potential for anti-inflammatiory activities.

Our study consists of two parts: Anti-inflammatory activity of rosmanol on LPS induced iNOS and COX-2 in RAW 264.7 cells and inhibition activity of rosmanol and carnosol on TPA inflammation in mouse ear.

In first study, rosmanol markedly inhibited LPS-stimulated iNOS and COX-2 protein and gene expression, as well as their regulated products, NO and $PGE_2$. Treatment with rosmanol also reduced translocation of NF-κB through preventing degradation and phosphorylation of IκB. Western blot analysis showed that rosmanol significantly inhibited translocation and phosphorylation of NF-κB and STAT3, and the protein expression of C/EBPβ and C/EBPδ. Our results showed that rosmanol down-

regulates iNOS and COX-2 gene expression by inhibiting the activation of NF-κB through interfering with the activation of PI3K/Akt and MAPK signal pathway.

Based on the positive results of *in vitro* activity of rosmanol and the similarity of chemical structure with carnosol, we designed and performed *in vivo* study with rosmanol and carnosol. In inhibitory activity of rosmanol and carnosol on TPA-induced persistent inflammation in mouse ear, we examined inhibitory activity of rosmanol and carnosol on production of pro-inflammatory cytokines such as Il-1β and Il-6. The expression of inflammatory COX-2 and its upstream regulators such as NF-κB and IKK-β, and its product, $PGE_2$ were also examined. A large amount of COX-2 expression and its up-stream regulators were detected in LPS induced mouse ear. In rosmanol and carnosol treated group, expression of pro-inflammatory cytokines and $PGE_2$ were decreased markedly. The expression of COX-2 was also reduced, which was resulted from inhibiting up-stream regulators such as NF-κB and IKK-β. In histology of rosmanol and carnosol treated groups their all inflammatory parameters are shown to be much milder condition. Also, there is significant reduced in degree of inflammation index.

Taken together, rosmanol and carnosol, abundant ingredient in rosemary might be a potent anti-inflammatory reagent.

**Acknowledgement**

There are so many people who played an essential role in helping me to complete this dissertation. First, I would like to thank my advisor, Dr. Chi-Tang Ho, for his support and guidance throughout my graduate school career. I have to thank my committee members, especially, Dr. Mou-Tuan Huang. I joined his lab last year and did my *in vivo* research with his support. I also would like to thank Dr. Henryk Daun and Dr. Qingrong Huang for their insightful advices and sharing experience as committee member.

I would like to thank Yue Liu in MT's lab. With her help I could continue to experiment. In second floor of Laboratory of Cancer Research, there are also many people whom I have to thank: Dr. Nanjoo Suh and her lab members, Dr. Hong Jin Lee and Jae Young So. Without their help I couldn't set up immunohistochemistry. Also Dr. Suh and Dr. Lee advised with valuable discussion. Dr. Qwang Xun Li in Dr. Yang's lab taught me how to evaluate and score histology.

What I learned during writing my dissertation is this result is not my own. Even though it is very fundamental, the research is definitely cooperative work.

## Dedication

I would like to dedicate this dissertation to my family, especially my mother and father, who gave me their unwavering support throughout my life. Also I dedicate my dissertation to my wife, Na Yeon. She quit her career and followed me to U.S. to support me. “I love you”. I promised her that I would give the whole dedication to her on my next book. That is the one reason I have to go through and publish another book. My daughter, Chloe J., gave me the power to stay on this research field.

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## Abbreviations

AP-1: activator protein-1

AREs: AU-enriched elements

C/EBPs: CCAAT/enhancer binding proteins

CAPE: caffeic acid phenethyl ester

CBP: CREB binding protein

Cdk: cyclin-dependant kinase

CHX: cycloheximide

COX-2: cyclooxygenase-2

cPLA2: phospholipase A

CRE: cAMP response element

CSF: colony-stimulating factors

DIM: 1,1-bis(3'indolyl)-1-(p-substituted phenyl) methanes

DMBA: dimethyl-benz(a)anthracene

DMSO: dimethylsulfoxide

EGCG: epigallocatechin-3-gallate

EGF: epidermal growth factor

eNOS: endothelial nitric oxide synthase

Epo: erythroprotein

ERK: extracelluar signal rugulated kinase

FGF: fibroblast growth factor

GAS: γ-activated sites

GRD: GTPase-responsive domain

GSK-3: glycogen synthase kinase-3

HER2: human epidermal growth factor receptor 2

IB: inhibitory kappa B alpha

IFN: interferone

IGF: insulin-like growth factor

IKK: IB kinase

Il-1: interleukine-1

iNOS: inducible nitric oxide synthase

JAK: janus activated kinase

JNK: Jun N-terminal kinase

LDL: low-density lipoprotein

LOX: lipoxygenase

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

MEK: MAPK kinase

MMP: matrix metalloproteinase

NF-IL6: nuclear factor interleukin 6

NF-kB: nuclear factor-kaapa B

nNOS neuronal nitric oxide synthase

p27KIP1: p27 kinase inhibitor portein 1

PARP: polyadenosine-5'-diphosphate-ribose polymerase

PDGF: platelet-derived growth factor

PDK: phosphoinositide-dependent kinase

PDTC: pyrrolidine dithiocarbamate

PEA3: polyomavirus enhancer activator 3

PH: pleckstrin homology

PI3K: phosphatidylinositol 3-kinase

PKB: protein kinase B

PLA2: phospholipase A2

PTK: protein tyrosine kinase

RNA Pol II: RNA polymerase II

SAPKs: stress-activated protein kinases

STAT: signaltransducer and activator of transcription

TGF: transforming growth factor

TLR: toll-like receptor

TNF: tumor necrosis factor

TPA: phorbol 12-O-tetradecanoate-1-acetated

TPB: TATA-binding protein

uPA: urokinase-type plasminogen activator

UTR: 3′-untranslated region

VEGF: vascular endothelial growth factor

# 1. Chapter I. Introduction, Hypothesis, and Objectives

## 1.1. Introduction

### 1.1.1 Health Benefits of Polyphenols

All human being has 99.1% of same genetic sequence, thus the incidence of illness cannot be attributed to the variation in their DNA sequence. In fact, if one twin is identified with breast cancer, the chance that the other twin will be diagnosed with breast cancer is 20%, indicating that the possibility of malfunctioning gene causing cancer is not high. Alternatively, the 75–85% of all chronic illnesses and diseases cannot be explained by genetic disorder but can be related to lifestyle (Locatelli, Lichtenstein et al. 2004; Wong, Gottesman et al. 2005). More than 250 population-based studies indicate that people who eat about five servings of fruit and vegetables a day have approximately half the risk of developing cancer of digestive and respiratory tract, comparing with those who eat fewer than two servings (Surh 2003). Since fruits and vegetables are major sources of antioxidants among other factors, we can hypothesize that these antioxidants are due to their cancer-preventive effects of fruits and vegetables. The data of epidemiological study and large amount of *in vitro* and *in vivo* experiments show antioxidants reduce the risk of pro-inflammatory reaction and cancer (Chu, Sun et al. 2002; Sun, Chu et al. 2002). The antioxidant effects of fruits and vegetables are caused by polyphenolic compounds (Kahkonen, Heinamaki et al. 2003). There are extensively investigated polyphenolic antioxidants exhibiting significant radical scavenging activity: the catechins in green tea, theaflavins in black tea, curcumin in turmeric, resveratrol in red wine, quercitin in apples and the anthocyanins in berries (Kahkonen, Heinamaki et al. 2003; Surh 2003).

Phytochemicals are non-nutritive components in the plant-based diet ('phyto' is from the Greek word meaning plant) that possess significant anticarcinogenic and antimutagenic properties. The active dietary phytochemicals are curcumin, genistein, resveratrol, diallyl sulfide, *S*-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, catechins, eugenol, isoeugenol, dithiolthiones, isothiocyanates, indole-3-carbinol, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, Vitamin C, d-limonene, lutein, folic acid, β-carotene, selenium, Vitamin E, flavonoids, and dietary fiber **(Fig.1).** These dietary agents has been studied that they suppress the inflammatory processes leading transformation, hyperproliferation, and initiation of carcinogenesis. Their inhibitory effects may eventually suppress the final steps of carcinogenesis: angiogenesis and metastasis (Aggarwal and Shishodia 2006).

Large amounts of clinical trials for novel dietary supplement and modified diets to prevent cancer are in progress. However, we should elucidate which component of these dietary agents plays the role in the anti-cancer effects and which mechanism they are related to suppress cancer before they are tested in human intervention trials or before applied to dietary supplements. Because of the great structural diversity of these phytochemicals from fruits and vegetables, it is impossible to predict structure–related health beneficial activity of these compounds. A better approach is to apply each compound to its target signal-transduction pathways. It will be long and slow route to examine the biological effects of tremendous compounds. However, this long and slow route may be the shortcut to bring us to cancer free planet.

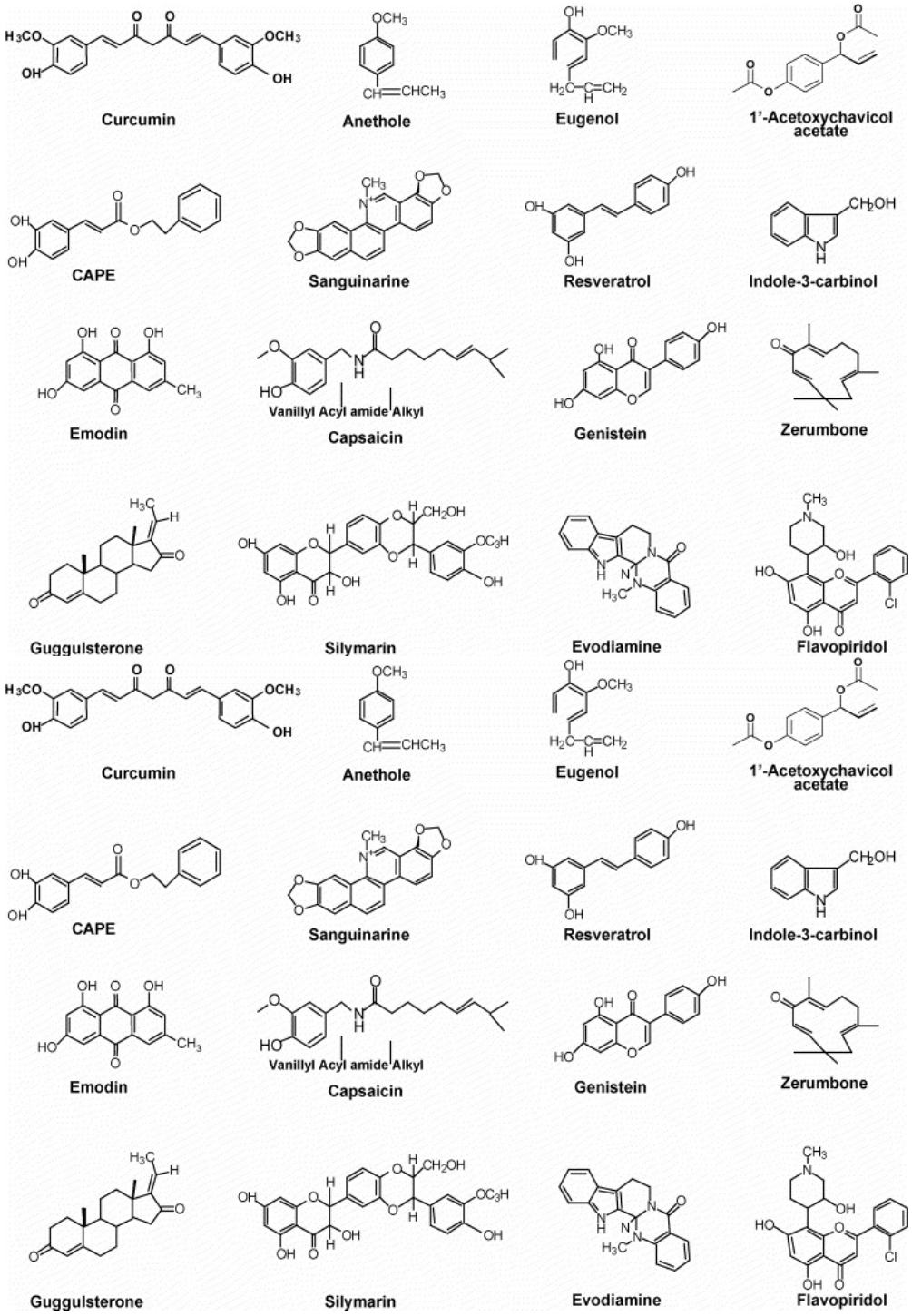



Fig. 1. Chemical structures of dietary phytochemicals (*Biochemical Pharmacology, 71*(10), 1397-1421)

### 1.1.2 Rosemary

Rosemary (*Rosemarinus officinalis* L.) commonly called rosemary, is a woody perennial herb and has evergreen needle-like leaves that are often used in cooking. Rosemary is used as an aromatic culinary spice adding distinctive flavor to chicken, breads, and many other foods. It is native to the mediterranean region and thrives in somewhat dry, light, and warm environment. Both the fresh and dried leaves are pungent. The leaves and parts of the flowers contain volatile oil. In history rosemary has been used as a remedy for various diseases and symptoms. It has been known to good for memory and headaches. Rosemary also has good effects on liver, circulation, and digestion. Its extracts have been incorporated into drugs and cosmetics, and used for flavors and fragrance in foods. Their large number of polyphenols such as carnosic acid, carnosol, rosemarinic acid, and ursolic acid are main constituents of these various bioactivities. Among the antioxidant compounds in rosemary leaves, almost 90% of the antioxidant activity can be attributed to carnosol and carnosic acid. Carnosic acid is the most potent antioxidant constituent, but it is unstable at high-temperature, easily oxidized, and weak against light.

Rosmaric acid was widely studied for its antimicrobial properties (Bult, Herman et al. 1985). Carnosol reduces the generation of peroxynitrite radicals and nitrite. Carnosol inhibits lipopolysaccharide (LPS) and interferon-γ (IFN-γ) induced nitrite production by mouse peritoneal cells (Chan, Ho et al. 1995). Rosemary extract inhibits peroxidation of phospholipid liposomes. Carnosol and carnosic acid show antioxidant activity (Aruoma, Spencer et al. 1996). Richheimer et al analyzed several commercial rosmary extract and isolated the major phenolic diterpenes: carnosic acid and carnosol.

They examined their relative antioxidant activities in soybean oil (Richheimer, Bernart et al. 1996). In both supplementation of diets and intraperitoneal injection of rosemary extract and carnosol inhibit the in vivo formation of mammary 7,12-dimethylbenz[a]anthracene (DMBA)-DNA adducts and initiation of DMBA-induced mammary tumorigenesis in female rats (Singletary, MacDonald et al. 1996). Carnosol, rosmanol, and epirosmanol had an inhibitory activity to lipid peroxidation and oxidized apo B formation in human bloods low-density lipoprotein (LDL) (Zeng, Tu et al. 2001). Carnosol suppresses the NO production and iNOS gene expression by reducing nuclear factor-κB (NF-κB) subunits translocation and NF-κB DNA binding activity in activated macrophage and down-regulate the inhibitor κB (IκB) kinase (IKK). Carnosol also inhibited LPS-induced p38 and p44/42 mitogen-activated protein kinase (MAPK) (Lo, Liang et al. 2002). Carnosol can limit the invasive ability of B16/F10 mouse melanoma cells by reducing matrix metalloprotemase-9 (MMP-9) expression and activity through suppressing ERK 1/2, AKT, p38, and JNK signaling pathway and inhibition of NF-κB and AP-1 binding activity (Huang, Ho et al. 2005). Carnosol prevents Apc-associated intestinal tumorigenesis, potentially via its ability to enhance E-cadherin-mediated adhesion and suppress beta-catenin tyrosine phosphorylation (Moran, Carothers et al. 2005). Crude extracts of rosemary is studied its anti-proliferative property on several human cancer cell lines and its antioxidant and anti-inflammatory properties in vitro in a mouse RAW 264.7 macrophage/monocyte cell line. This study shows that crude ethanolic rosemary extract has differential anti-proliferative effects on human leukemia and breast carcinoma cells (Cheung and Tai 2007). Carnosic acid inhibits MMP-9 activity and expression in human aortic smooth muscle cells. The production of reactive

oxygen species and the nuclear translocation of NF-κ B p50 and p65 induced by tumor necrosis factor-α (TNF-α) were dose-dependently suppressed by carnosic acid pre-treament (Yu, Lin et al. 2008). These results give us insight to the detailed mechanism involved in anti-inflammatory activity of rosemary constituents. During the cooking, long term storage, or processing, carnosol can be transformed to one of its oxidized form, rosmanol. The amount of rosmanol in natural rosemary leaves is relatively low. However, the quantity of rosmanol is increased after cooking or processing. Thus, the bioactivity of rosmanol is very interesting and important to study.

### 1.1.3 Inflammation

Inflammation is a complex response of our body in response to traumatic, infectious, post-ischaemic, toxic or autoimmune injury. It is a set of interactions among soluble factors and cells that can be happened in any tissue of our body. This process normally contributes to recovery from infection and to heal the wound. Inflammation can be classified two categories: acute inflammation and chronic inflammation. Acute inflammation is the initial response of the immune system against pathogens, injured tissue, and small particles and a rapid self-limiting process. Eicosanoids and vasoactive amines which increase the movement of plasma and leukocytes into infected site mediate the acute inflammation. A cascade of biochemical reaction propagates the immune response, achieving the resolution. However, during the acute inflammation, if phagocytes fails to eliminate pathogen, or immune system cannot repair injured tissue, the inflammation can be developed to chronic inflammation. In chronic inflammation, various cytokines and growth factors are released, resulting recruitment of higher order

immune cells such as leukocytes, lymphocytes and fibroblasts. The inflammation can lead to persistent tissue damages by these cells. In addition, many research groups report evidence that chronic inflammation lies at the basis of many diseases of advanced age such as heart attacks, Alzheimer's diseases, and cancer (Coussens and Werb 2002; Mueller 2006). To preserve healthy body, two sets of mechanisms must be matched: the ability to increase a rapid inflammatory response to injurious pathogens and the ability to keep this response from prolonged. Since the inflammatory process consists of sets of signal cascades, each signal or molecule is responsible for mediating the upper or down-levels of inflammatory response. Thus, inflammation can be considered in terms of its check points. By blocking, trapping each signal molecule, or triggering stop signal, the inflammatory process can be inhibited or suppressed (Nathan 2002). There are many chemical reagents are studied showing these blocking activities. Chemical agents that have anti-inflammatory activities may reduce the incidence of various disease derived from inflammation.

### 1.1.4 Process of Acute Inflammation

Acute inflammation is a short-term response of our body, usually appearing within a few minute or hours after exposure to the injurious stimulus and stopped when this stimulus is eliminated.

The acute inflammation is initiated by resident immune cells which are already present in all tissues. The immune cells such as macrophages, dendritic cells, and mast cells are activated by an infection, burning, or other injuries and release inflammatory mediators which can cause the clinical signs of inflammation. There are five principal

signs of acute inflammation:

- Redness
- Elevated heat
- Swelling
- Pain
- Loss of function

Once the tissue is infected, blood vessels are expanded and blood flow is increased to transport large amount of immune cells to infected sites. The redness and elevated heat are caused by the combination of the vasodilation and its resulting increased blood flow. Swelling is an accumulated leakage of plasma and fluid into the tissue from highly permeable the blood vessels. Some of the released mediators increase the sensitivity to pain. The mediator molecules also alter the blood vessels to allow the migration of leukocytes from the blood vessels into the tissue. The leukocytes, mostly neutrophils, move to the injurious site along with the chemically attractive environment created by the local cell. The loss of function may be the result of a neurological reflex action in response to pain. If the stimulation is removed, the acute inflammatory response will not be maintained. Inflammatory mediators having short life cycle are quickly degraded in the tissue. Thus, inflammation ceases once the stimulus has been removed (Robbins, Kumar et al. 2005).

### 1.1.5 Inducible Nitric Oxide Synthase (iNOS)

Nitric oxide (NO) is a gaseous free radical which is used to signal to the surrounding smooth muscle cells to relax the endothelium of blood vessels. This

relaxation results vasodilation and increases blood flow. Because nitric oxide is highly reactive, it can diffuse freely across membranes, which makes nitric oxide ideal transient signaling molecule to communicate between adjacent cells and with a single cell (Stryer and Lubert 1995). Once the human immune system is activated, nitric oxide is also generated by phagocytes such as monocytes, macrophages, and neutrophils. As an immune response NO couples with superoxide ($O_2$-) to form peroxinitrite (ONOO-) in a diffusion-dependant manner which is extremely reactive, comparing with NO or $O_2$- alone. NO has also been known as a chemically potential molecule causing DNA damage by nitration and oxidation (Szabo and Ohshima 1997). NO is synthesized by the enzymatic reaction converting L-arginine into L-citrulline by Nitric oxide synthase (NOS). NOSs are classified into three isomers by the location of expression in the body and the manner of expression, constitutive or inducible. Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutive NOS which are expressed in neuronal tissues and vascular endothelial cells, while inducible NOS (iNOS) is expressed in a variety of cell types in response to various stimuli such as endotoxin and endogenous pro-inflammatory mediators. With these infectious and pro-inflammatory stimuli, iNOS is highly induced to produce NO (Knowles and Moncada 1994; Knowles 1996). eNOS regulates blood vessel tone and nNOS provides neurotransmitter and neuromodulator functions. A constitutive expression of iNOS is detected in the lung epithelium and the small intestine. Furthermore, sustained iNOS expression occurs in virus-infected lymphocytes. Once it is activated, iNOS produces large amounts of NO until substrate is damaged. Particularly, because of its critical functions in inflammation-related disease, iNOS has been drawn considerable attention (Hickey, Granger et al.

2001). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) are the most prominent cytokines which stimuli iNOS. In addition, lipopolysaccharide (LPS) has been extensively studied as an endotoxin. LPS is one of the activator which triggers the important intracellular signal pathway, NF-κB. iNOS gene expression and subsequent mRNA translation is controlled by NF-κB pathway (Kleinert, Wallerath et al. 1998).

### 1.1.6 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (prostaglandin G/H synthase, COX) donates 2 oxygen molecules to arachidonic acid to form $PGG_2$ by peroxidation, which in turn is reduced to $PGH_2$. This leads to the formation of $PEG_2$, a bioactive prostanoid, via concerted activation of PGE synthase (PGES). There are three isoforms of COX. First, COX-1, is constitutively expressed in most normal tissues at relatively low levels and believed to have some housekeeping functions such as forming prostanoids in the stomach and platelets (Simmons, Levy et al. 1989; Smith, Garavito et al. 1996). On the other hand, COX-2 is regulated by mitogens, tumor promoters, cytokines, and growth factors and LPS insult. It has been known to function in the inflammatory release of PGs. COX-2 is over-expressed in practically every premalignant and malignant condition involving the colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck, and esophagus (Mardini and Fitzgerald 2001). Finally, COX-3, a splice variant of COX-1 is expressed in brain. However, the protein expression and functional relevance of COX-3 in human has not been elucidated (Schwab, Schluesener et al. 2003). COX-2-derived bioactive lipids, including prostaglandin $E_2$, are potent inflammatory mediators that promote tumor

growth and metastasis by stimulating cell proliferation, invasion, and angiogenesis. Therefore, high levels of prostaglandins may promote the development of malignancy (Mann, Backlund et al. 2005; Yoshimura, Matsuyama et al. 2005). **Fig. 2** shows the regulation of COX-2 in cancer cells. Various stimulations such as growth factors [epidermal growth factor (EGF)], tumor promoters (phorbol esters and bile acids), chemotherapy (taxanes), and oncogenes (HER-2/neu) induce COX-2. Stimulating either protein kinase C (PKC) or RAS activates mitogen-activated protein kinase (MAPK), which, in turn, activates transcription factors of *COX-2* such as activator protein 1 (AP-1) and nuclear factor κB (NF-κB). These transcription factors mediate the expression of *COX-2* (Zhang, Subbaramaiah et al. 1998; Smith, DeWitt et al. 2000; Subbaramaiah, Hart et al. 2000; Subbaramaiah and Dannenberg 2003). By contrast, Transcription of COX-2 is regulated by wild-type p53. COX-2 is also regulated by post-transcriptional mechanisms. The 3′-untranslated region (UTR) of COX-2 mRNA contains a series of Shaw-Kamen sequences [AUUUA, also known as AU-enriched elements (AREs)] that possess instability message (Cok and Morrison 2001). Increased binding of HuR to Shaw-Kamen sequences of *COX-2* causes, at least in part, the increase in stability of COX-2 mRNA in colon cancer (Dixon, Tolley et al. 2001). Oncogenes, cytokines, growth factors and tumor promoters induce COX-2 by enhancing mRNA stability in addition to stimulating transcription (Sheng, Shao et al. 2000). In addition, prostaglandin $E_2$ ($PGE_2$) induces COX-2 by activating tyrosine kinase activity of the EGF receptor, but it is not known whether this positive feedback mechanism is relevant in human tumors (Subbaramaiah and Dannenberg 2003).

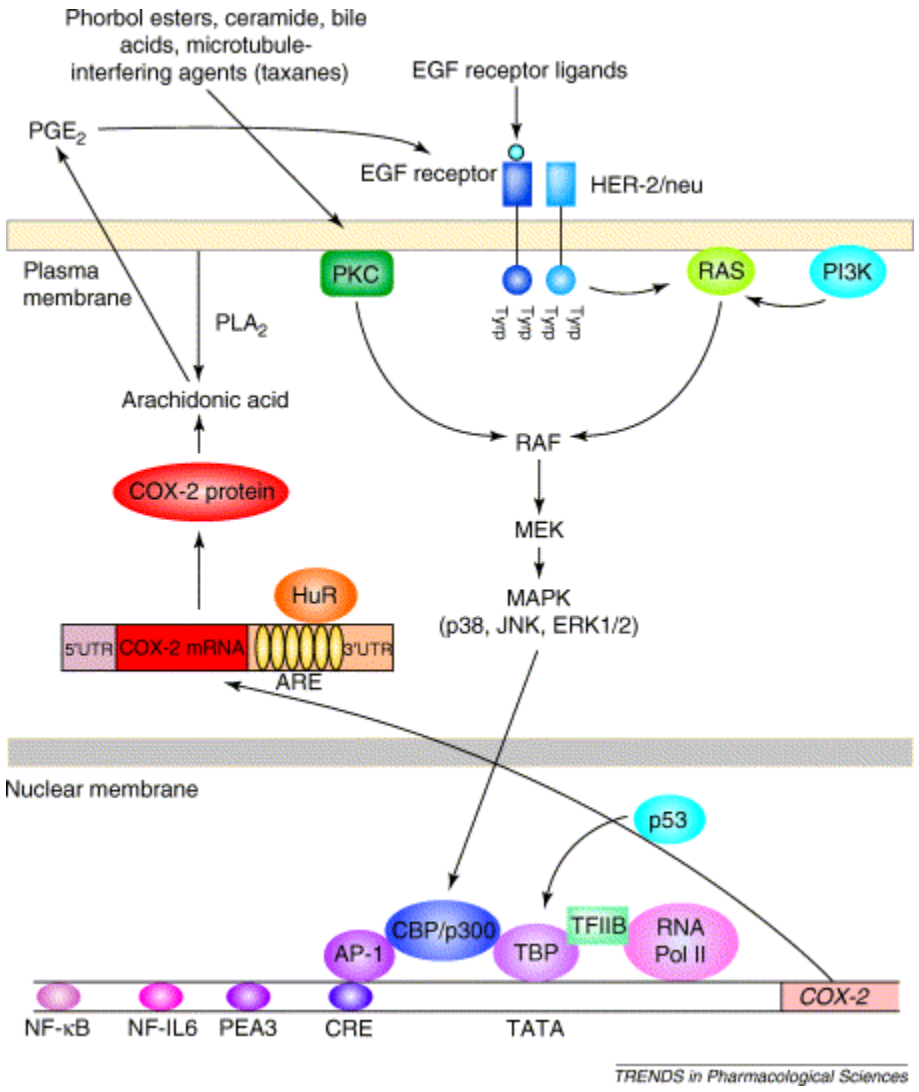



Fig. 2. Regulation of cyclooxygenase-2 in cancer cells (*Trends in Pharmacological Sciences, 24*(2), 96-102)

### 1.1.7 Nuclear factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is one of the most important regulators of pro-inflammatory gene expression. NF-κB plays a pivotal role in the synthesis of cytokines such as TNF-α, IL-1β, IL-6, and IL-8 that are inhibitors or enhancers of the inflammatory process (Brenmer and Heinrich 2002; Huang, Ghai et al. 2004). Inactive NF-κB is present in the cytoplasm of all cells. By the outer stimuli it is activated and translocated into the cell nucleus generating the usual sequence of events. NF-κB has been known to consist of a family of Rel-domain containing proteins; e.g., Rel A (also called p65), Rel B, c-Rel, p50 (also called NF-κB1), and p52 (also called NF-κB2). By phosphrylation p100 is cleaved producing p52, whereas p105 is cleaved to form p50. Similarly, a family of anchorin-domain containing proteins such as IκBα, IκBβ, IκBγ, IκBα ε, bcl-3, p105, and p100 keep NF-κB in its inactive state within the cytoplasm. NF-κB consists of a heterotrimer of p50, p65, and IκBα in cell cytoplasm. p65 and p50 also contain C-terminal transactivation domains. The p65 transactivation domain was activated by phosphorylation. The phosphorylation, ubiquitination, and degradation of IκBα release the p50-p65 heterodimer. The liberated heterodimer translocates to cell nucleus and binds its specific 10 base pair consensus site GGGPuNNPyPyCC. IκBα kinase (IKK) complex consists of IKKα, IKKβ, and IKKγ (also called NEMO) and phosphorylates IκBα at serine residues 32 and 34. By the stimuli of TNF and most other agents IKKβ play an key role in NF-κB activation, which is indicated in gene deletion studies (Aggarwal 2004).

In LPS induced macrophage expression of iNOS and COX-2 is mediated by NF-κB (Pan, Lai et al. 2006). NF-κB in LPS induced macrophage is initiated by a cascade of

events leading to the activation of inhibitor κB (IκB) kinase (IKK). The p65 transactivation domain at serine 536 is phosphorylated by LPS in monocytes and macrophage. In mouse embryonic fibroblasts lacking either IKKα or IKKβ, IKKβ is needed to phosphorylate p65 at serine 536, while IKK is partially required for p65 phosphorylation. The LPS-induced p65 phosphorylation on serine 536 was independent of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Furthermore, the phosphorylation on serine 536 increased the p65 transcription activity (Yang, Tang et al. 2003). Many studies imply cytokine in the induction of transcription activity of NF-κB through extracellular signal-regulated mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/AKT pathways (Chandrasekar, Marelli- Berg et al. 2004; Je, Lee et al. 2004; Agarwal, Das et al. 2005). Members of the toll-like receptor (TLR) family, particularly TLR4, are now recognized as central receptors for LPS (Chow, Young et al. 1999). Activated TLR4 stimulates various protein kinases, including PI3K and phosphoinositide-dependent kinase (PDK). The phosphorylated PI3K activates AKT, which in turn activates IKK. In addition, mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated kinase (ERK), p38 and c-Jun n-terminal kinase (JNK), and PI3K/AKT pathways have been reported that these kinases also induce the NF-κB (Guha and Mackman 2001). **Fig. 3** shows schematic diagram of NF-κB pathway in LPS induced macrophage.

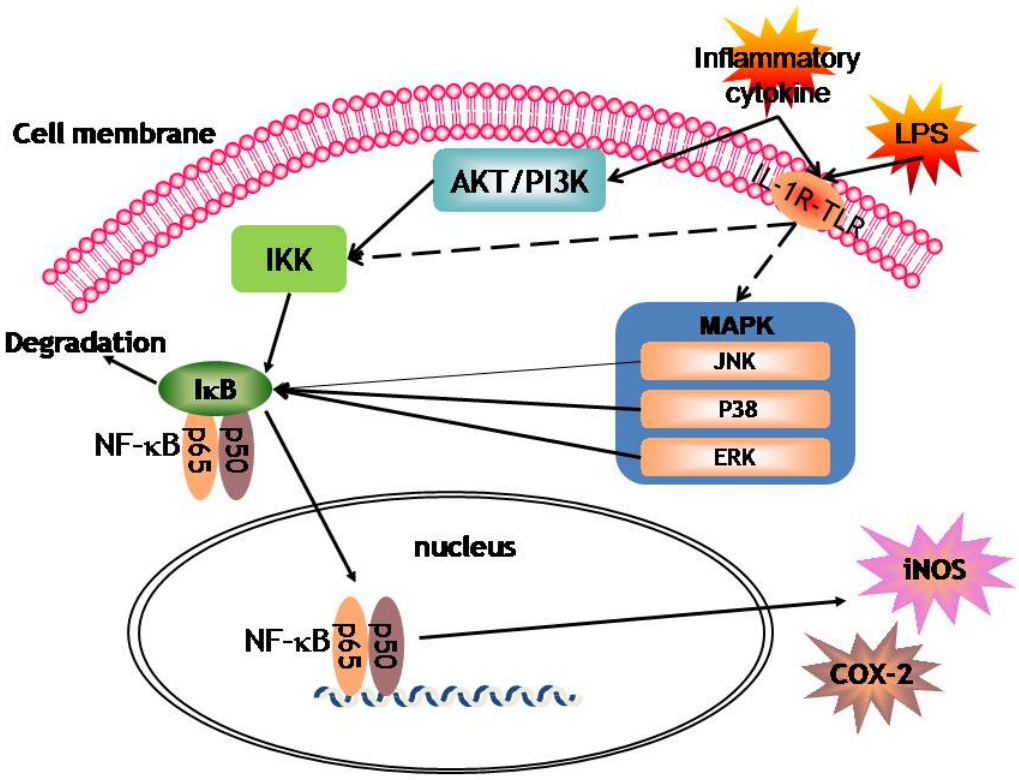



Fig. 3. NF-κB pathway in LPS induced RAW 246.7 macrophage

### 1.1.8 MAP Kinase

There has been growing attention in MAPK pathway as a target molecule for cancer prevention and therapy. The MAPK cascades consist of extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 kinases. Growth-inducing tumor promoters, including 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), activate MAPKs through activating ERKs and JNKs. Although the signaling cascade from growth factor receptors to ERKs is relatively well understood, the pathway leading to JNK activation is not clear. (Cowley, Paterson et al. 1994). Whereas, stress-related tumor promoters, such as ultraviolet (UV) irradiation and arsenic, potently activate JNKs/SAPKs and p38 kinases (Bode and Dong 2000). **Fig. 4** presents the MAP Kinase signal cascades. The MAPK pathway consists of a sequential cascade. TPA-induced or ultraviolet-induced activation of various GTP proteins activates MAP3Ks. The activated MAP3Ks activate MAP2Ks that activate MAPKs (ERK, JNK, and p38), activating transcription factors such as NF-κB and AP-1 which result cell growth, proliferation, and apoptosis. (Seger and Krebs 1995; Bode and Dong 2000).

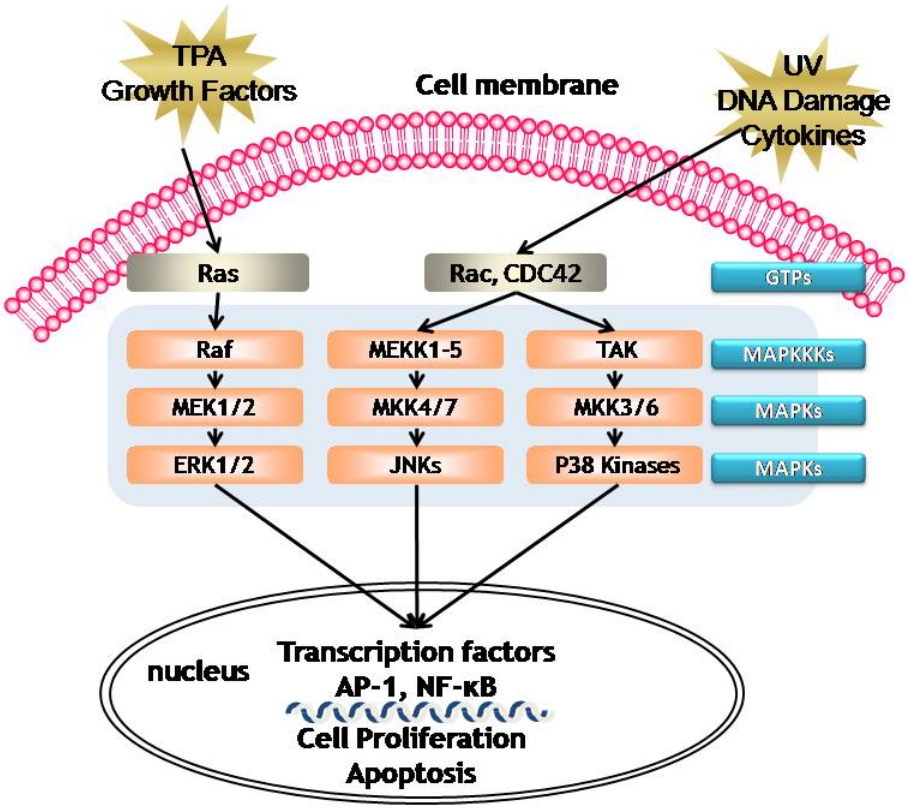



Fig. 4. MAP kinase cascades leading to AP-1 and/or NF-κB activation

### 1.1.9 PI3K/Akt

Protein kinase B or Akt (PKB/Akt) is a serine/threonine kinase, which in mammals comprises three highly homologous members known as PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). When cytokine specific receptors are activated, PI3K can be activated. Recently, it is shown that the PI3K regulatory subunit, p85, contains two regulatory domains: a GTPase-responsive domain (GRD) and its inhibitory domain. GRD can mediate PI3K activation through binding small GTPases (Ras, Rac1) and an inhibitory domain that can block these binding events (Chan, Rodeck et al. 2002). Ras molecules are also believed to activate Raf/MEK/ERK cascades (Yan, Roy et al. 1998). Activated PI3K converts phosphatidylinositol (4,5)-phosphate [$PIP_2$] into phosphatidylinositol (3,4,5)-phosphate [$PIP_3$], which results in membrane localization of phosphotidylinositol-dependent kinase-1 (PDK1) via its pleckstrin homology (PH) domain. Inactive Akt is obtained to the lipid-rich plasma membrane by its PH domain and is phosphorylated at residues T308 and S473 by PDK1 and possibly other kinases (Chang, Lee et al. 2003). Akt primarily mediates a large amount of downstream substrates may participate in malignat transformation. Some of these substrates are Bad, procaspase-9, I-κB kinase (IKK), CREB, the forkhead family of transcription factors (FKHR/AFX/FOX), glycogen synthase kinase-3 (GSK-3), $p21^{Cip1}$, and Raf (Romashkova and Makarov 1999; Nicholson and Anderson 2002).

### 1.1.10 STAT and C/EBP

JAK/STAT pathway is discovered while the mechanism of cytokine signaling is investigated. After the binding of cytokines to their related receptor, members of janus

activated kinase (JAK) family of tyrosine kinases activate signal transducers and activators of transcription (STATs). Signaling through the JAK/STAT pathway is initiated when a cytokine binds to its specific receptor. Cognate receptor associated members of the JAK family of kinases are activated by the conformational changes in the cytoplasmic portion of receptor. The JAKs then sequentially activate each other by phosphorylating specific receptor tyrosine motifs, which serve as docking sites for STATs and other signaling molecules. STATs and other signaling molecules are recruited to the receptor and are then phosphrylated by a JAK at tyrosine residue. Activated STATs dissociate from the receptor, dimerize, translocate to the nucleus and bind to members of the γ-activated sites (GAS) family of enhancers. There are four members of the JAK family in mammals, Jak1, Jak2, Jak3 and Tyk2. They are over 1000 amino acids in length, ranging in molecular weight from 120 to 130 kDa. The seven STAT proteins identified in mammals range in size from 750 and 850 amino acids. The recruitment of unique sets of STATs to each receptor subfamily constitutes a critical step in defining the specificity of the subsequent biological response.

STAT1 and STAT2 knockout mice show defects in their biological response to both types of IFNs. Characterization of these mice has provided important insight into the role the IFNs play in regulating both innate and acquired immunity (Meraz, White et al. 1996; Park, Li et al. 2000). STAT3 is an important transcription factor participating the production of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-10 which plays a major role in inflammatory disease (Schindler 2002). STAT4 is activated by IL-12 and STAT4 is activated by Il-13, which play a critical role in the development of the Th1 subset of T helper cells. These cells are known to play an important role in regulating

immune response (Kaplan, Schindler et al. 1996). STAT5 is found to be encoded by two linked genes: STAT5a and STAT5b, which are 96% identical. STAT5a-null mice are defective in prolactin-dependent mammary development, while STAT5b knockout mice fail to respond effectively to growth hormone (Teglund, McKay et al. 1998).

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that all contain a highly conserved basic-leucine zipper domain at the C-terminus. This domain is involved in dimerization, translocation, and DNA binding of C/EBPs. The activity and/or expression level of three C/EBP members (α, β and δ) is regulated by a number of inflammatory agents, including LPS and a range of cytokines (Ramji and Foka 2002). C/EBPs have also been shown to play an important role in the transcriptional activation of iNOS and COX-2 promoters. Among the members of C/EBP transcription factors, C/EBPβ has been implicated in inducing the expression of COX-2 stimulated by endotoxin and cytokines (Wadleigh, Reddy et al. 2000). Furthermore, both C/EBPβ and C/EBPδ are also involved in induction of glial iNOS (Saha and Pahan 2006) as well as the inflammatory mediator produced in LPS induced RAW264.7 macrophage (Liu, Tseng et al. 2003). **Fig.5** illustrates the signal pathway of STATs and C/EBPs.

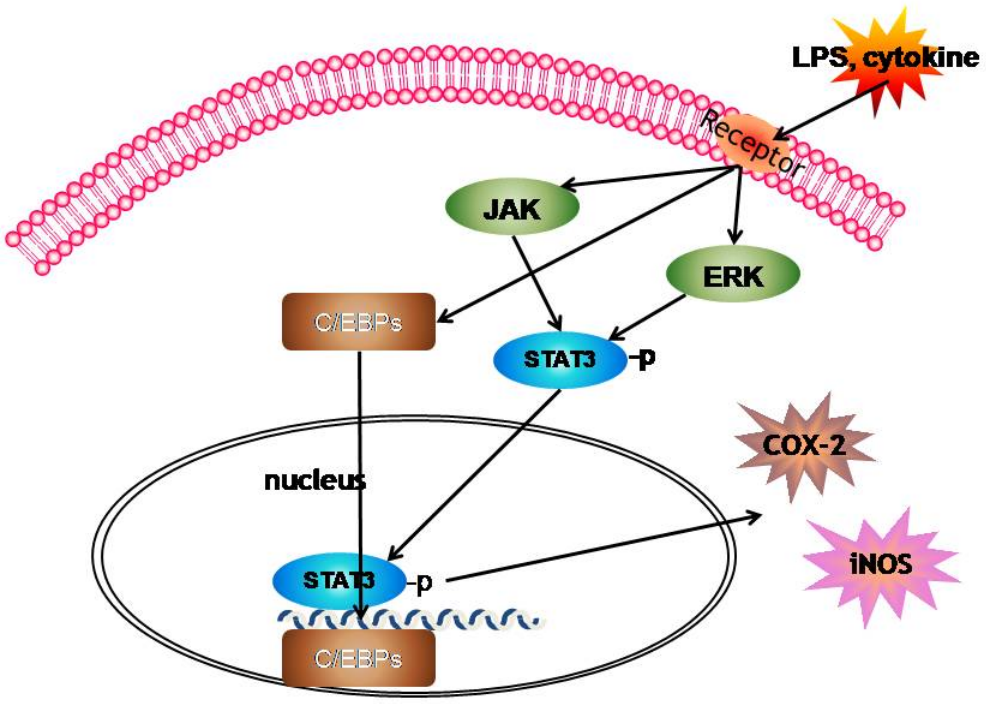



Fig. 5. Signal pathway of Jak/STAT3 and C/EBPs in LPS induced RAW 264.7

## 1.2. Hypothesis

From the previous research and preliminary studies, rosemary extracts have various antioxidant activities. Carnosic acid and carnosol are the most abundant and powerful compounds in rosemary extracts. Current studies have shown these compounds have anti-inflammatory and anti-cancer activities. As a food ingredient, rosemary is easily exposed to heat and oxygen abundant environment during food processing and cooking. Especially, carnosol is apt to be converted into rosmanol via oxidation in food processing. The objectives of these studies were to determine the anti-inflammatory effects of rosmanol transformed from carnosol in rosemary extract. Carnosol has known as an anti-inflammatory compound in rosemary extract. Rosmanol is transformed from carnosol via oxidation and also expected to have an anti-inflammatory property mediated through multiple mechanisms including NF-κB pathway. This hypothesis will be tested using LPS induced Raw 264.7 macrophage model and TPA induced ear edema on mouse model.

## 1.3. Objectives

- To determine the anti-inflammatory effect of rosmanol transformed from carnosol in rosemary extract.
- To determine inhibitiory activity on iNOS and COX-2 expression in LPS induced Raw 264.7 macrophage.

We treated rosmanol dose dependently in LPS induced macrophage and measured the expression of iNOS and COX-2. To elucidate detailed mechanism, well-known up-stream pathway on expression of iNOS and COX-2, NF-κB pathway, was studied.

Akt/PI3K pathway known to trigger the onset of NF-κB pathway and MAPK also related to NF-κB pathway are studied. These experiments are described in Chapter II.

- To determine Inhibition Effects of Rosmanol and Carnosol on TPA (12-O-Tetradecanoylphorbol-13-Acetate) - induced inflammation in Mouse Ears.

We tested the anti-inflammatory effect of rosmanol and carnosol on skin using TPA induced mouse ear model. Rosmanol and carnosol were treated 10 mins after the treatment of TPA on mouse ear for 4 days. We examined the migration of immune cells and alterations of inflamed tissue by histology and immunohistochemical staining. Chapter III deals with this experiment.

# 2. Chapter II. Inhibitory Activities of Rosmanol on Lipopolysaccharide (LPS)-Induced iNOS and COX-2 Expression in RAW 264.7 Murine Macrophage

## 2.1. Background and Rationale

From nineteenth century link between inflammation and cancer has been the first page of scientific issue (Balkwill and Mantovani 2001; Coussens and Werb 2002). Many researchers have found the correlation between inflammation and several human diseases including heart attack and Alzhemer's disease (McGeer and McGeer 2002; Woodward, Lowe et al. 2005). Macrophages play an important role in various inflammatory responses by up-regulating the expression of pro-inflammatory cytokines and growth factors such as tumor necrosis factor-α (TNF- α), interleukin-1 family, transforming growth factor-β (TGF- β) and platelet-derived growth factor (PDGF). Macrophage also represents the main source of inflammatory enzyme, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Mantovani, Allavena et al. 2008). LPS is a component of the cell walls of Gram-negative bacteria, and activates monocytes and macrophages to involve in inflammatory response (Sica, Allavena et al. 2008).

NO has been identified as a neurotransmitter in central nervous system and a potent vasorelaxant regulating the blood pressure in vascular system, and antimicrobial reagent against bacterial pathogen. However, excess amount of NO produced by inducible nitric oxide synthase (iNOS) and its derivatives, such as nitrogen dioxide and peroxynitrite, is implicated in pathogenesis of septic shock, inflammation and carcinogenesis (Nicholas, Batra et al. 2007). iNOS is widely expressed in various cells, including vascular smooth muscle cells, hepatocytes and kupffer cells, and is highly

expressed in LPS-activated macrophages (Nathan and Xie 1994). Prostaglandin (PG) produced at inflammatory sites by inducible cyclooxygenase-2 (COX-2), is a precursor of a wide group of biological active mediators such as prostaglandin E2 (PGE2), prostacyclin, and thromboxane A2. COX-2 is another inducible enzyme that catalyzes biosynthesis of PGs by conversion of arachidonic acid to prostaglandin $H_2$ ($PGH_2$). Particularly $PGE_2$ contributes to pathogenesis of various inflammatory diseases, edema, angiogenesis, invasion and growth of tumor. Over-expression of COX-2 occurs in certain epithelial cancer tissue. COX-2 is also markedly expressed in inflammatory cells stimulated by LPS, pro-inflammatory cytokines and tumor promoters (Ohshima and Bartsch 1994).

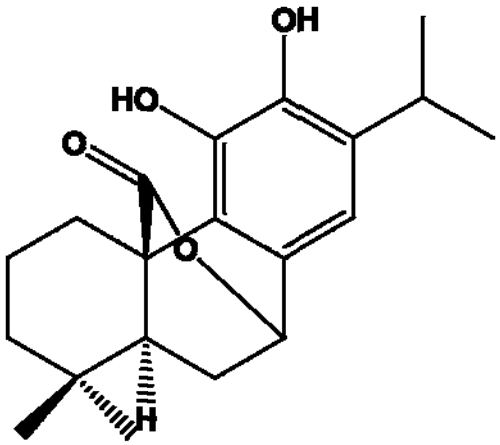
Nuclear factor-κB (NF-κB) is a pivotal transcription factor regulating gene expression involved in inflammation, immunity, cancer development, and its progression (Rockey and Chung 1998). Among the transcription regulators in the promoter regions of iNOS and COX-2, NF-κB seems to work as the most essential transcription factor for expression of these inflammatory enzymes in LPS induced macrophage (Winston, Krein et al. 1999). LPS stimulates toll-like receptors (TLRs) in inflammatory cells and downstream inhibitor κB kinases (IKKs), which in turn phosphorylates the inhibitor κB (IκB), degrades and leads NF-κB to translocate into the cell nucleus. In addition, mitogen-activated protein kinases (MAPKs) such as extracellular signal regulated kinase (ERK), p38 and c-Jun NH2-terminal kinase (JNK), and PI3K/AKT pathways have been reported that these kinases also induce the NF-κB (Guha and Mackman 2001). Another important transcription factor involved in inflammatory gene expression is signal

transducer and activator of transcription-3 (STAT-3). In response to cytokines and growth factors, members of STAT family are phosphorylated by the receptor associated kinases, and form homo/hetero–dimer, and then translocate into the cell nucleus where they play their role as transcription factor (Schindler 2002). The promoter region of iNOS gene in murine macrophage contains the STAT-binding γ-activated sites (GAS) and most of the translocated STAT dimmers recognize and bind to members of GAS which is necessary for expression of iNOS in IFN-γ and LPS-induced RAW 264.7 macrophages (Gao, Morrison et al. 1997; Marrero, Venema et al. 1998).

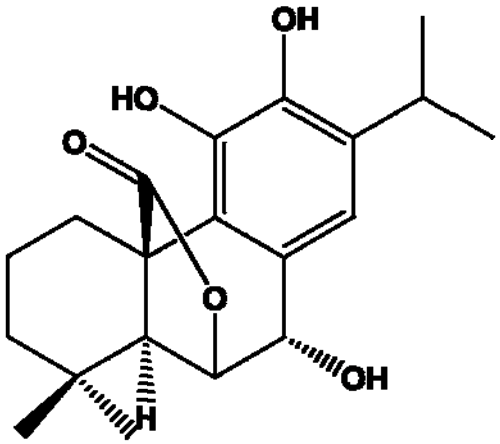
Rosemary (Rosmarinus officinals L.) leaves are widely used for seasoning to enhance food flavoring (Wei and Ho 2006). Previous studies reported that rosemary possesses pharmacological and physiological properties including antioxidant, anti-inflammatory, anti-cancer and anti-proliferate activities (Singletary, MacDonald et al. 1996; Cheung and Tai 2007). Rosemary extract contains volatile oil, phenolic acids, flavonoids and diterpenes. Among the antioxidant substances in rosemary, several phenolic diterpenes such as carnosol and carnosic acid have been shown to exhibit strong antioxidant activity (del Bano, Lorente et al. 2003). Carnosic acid has been demonstrated to be unstable during processing and storage. In the presence of oxygen, carnosic acid is easily transformed into other diterpenes such as carnosol and rosmanol (Wei and Ho 2006). It has been studied that rosmanol is a potent antioxidant having inhibition effect of superoxide anion production, as well as lipid peroxidation and free radical scavenging activities (Haraguchi, Saito et al. 1995; Zeng, Tu et al. 2001; Escuder, Torres et al. 2002).

Lo et al performed extensive anti-inflammatory molecular signaling study with carnosol (Lo, Liang et al. 2002). Rosmanol easily converted from carnosol by processing

such as cooking and long term storage has similar structure with carnosol (**Fig. 6**). Thus, we may expect rosmanol also has anti-inflammatory effect. The aim of this study was to determine the anti-inflammatory effects of rosmanol. In this study, we examined the anti-inflammatory effects of rosmanol using LPS-stimulated murine macrophage RAW 264.7. The results showed that rosmanol suppressed LPS-induced NO and PGE2 production by inhibiting NF-κB, STAT3 and C/EBP, as well as the upstream, p38, ERK1/2, and PI3K/Akt signaling pathways.




Carnosol




Rosmanol

**Fig. 6. Chemical structure of carnosol and rosmanol**

## 2.2. Material and Method

### 2.2.1 Reagents

LPS (Escherichia coli 0127: E8), sufanilamide, naphthylethylenediamine dihydrochloride, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Rosmanol was semi-synthesized from carnosol according to the method of Marrero et al. Briefly, carnosol in acetone was treated with aqueous sodium bicarbonate (5%) and the mixture was stirred at room temperature for 6.5 h, after which the acetone was evaporated under reduced pressure. The reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate, washed with salt solution, and dried over anhydrous sodium sulfate. The residue was purified by silica gel column chromatography eluted with *n*-hexane/acetone (4:1) to yield rosmanol with 99% purity, as determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

### 2.2.2 Liquid Chromatography-Electron Spray Ionization Mass Spectrometry (LC-ESI-MS)

An HPLC-MS system was composed of an auto-sampler injector (Leap Technologies, Switzerland), an HP1090 system controller, with a variable UV wavelength (190-500 nm) detector, an Evaporizing Laser Scattered Deposition (ELSD) detector and an ESI-MS detector from Micromass VG Platform II mass analyzer (Micromass, Beverly, MA). ESI-MS conditions were as following: acquisition mode, ESI-positive; mass scan range, 100-800 amu; scan rate, 0.4 sec; cone voltage, 25 volts; source temperature: 150 °C; probe temperature: 550 °C. Analytical HPLC conditions on

HPLC-MS: column: Chromeabond WR C18, (Macherey-Nagel, Bethlehem, PA; 3 μm, 120 Å; length and OD: 30 x 3.2 mm); injection volume, 15 μL; flow rate: 2 mL/min; run time: 3 min. Mobile phase consisted of acetonitrile and H2O with 0.05% TFA, typical gradient of 10 - 90 % acetonitrile and the gradient varied.

### 2.2.3 Cell Culture

RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin. When the cells reached a density of $2\text{-}3\times10^6$ cells/mL they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Rosmanol dissolved in dimethylsulfoxide (DMSO, as final concentration of 0.05%) and LPS were added. Cells were treated with 0.05 % DMSO as vehicle control.

### 2.2.4 Nitrite Assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. After centrifugation at 1000 g for 20 min, 100 μL of each supernatant medium was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was measured with an enzyme-linked immunosorbent assay plate reader (Dynatech MR-7000; Dynatech Labs,

Chantilly, VA).

### 2.2.5 Determination of $PGE_2$

The culture medium of control and treated cells was collected, centrifuged and stored at –80 °C until tested. The level of $PGE_2$ released into culture medium was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Assay Designs, Inc., Ann Arbor, MI).

### 2.2.6 Western Blot Analysis

The stimulated murine macrophage cell RAW 264.7 were washed with PBS and lysed in an ice-cold lysis buffer (10 % glycerol, 1% Triton X-100, 1 mM $Na_3VO_4$, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 20 mM Tris buffer (pH 7.9), 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN)) on ice for 1 h, followed by centrifugation at 175,00 g for 30 min at 4 $^{o}$C. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Inc. Hercules, CA).

An equal amount of total cellular protein (50 μg) was resolved by SDS-polyacrylamide minigels and transferred onto immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membrane was then blocked with blocking solution (20 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide) at room temperature for 1 h and then, incubated with the primary antibody at 4 $^{o}$C overnight. The membrane was then washed with 0.2 % TPBS (0.2 % Tween-20/PBS) and subsequently probed with anti-mouse, anti-rabbit, or

anti-goat IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) and visualized using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) (ECL). Primary antibodies of specific protein were purchased from various locations as listed: for anti-COX-2 and anti-iNOS, Transduction Laboratories (Lexington, KY), for anti-C/EBPβ and C/EBPδ, Santa Cruz Biotechnology (Santa Cruz, CA), for anti-IκBα, anti-p65, anti-phospho (Ser 32)-specific IκBα, New England Biolabs (Ipwich, MA), for anti-β-actin monoclonal antibody, Oncogene (Oncogene Science Inc., Uniondale, NJ), for anti-phospho-STAT3 (Tyr705 and Ser727), anti-phospho-Akt (Ser473), anti-phospho-p65 (Ser536), anti-phospho-p38 (Thr180/Tyr182), antiphospho-ERK1/2 (Thr202/Tyr204), STAT3, ERK, p38, and Akt antibodies, Cell Signaling Technology (Beverly, MA) were used to determine the level of phosphorylated proteins.

### 2.2.7 Semiquantitative RT-PCR

Total RNA was isolated from mouse macrophage RAW264.7 cell using Trizol® Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Changes in the steady-state concentration of mRNA in iNOS, COX-2 and β-actin were assessed by reverse-transcription polymerase chain reaction (RT-PCR). A total of 2 μg RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Renfrewshire, U.K.) to a final volume of 20 μL. RT reactions were performed at 50 $^{o}$C for 50 min and 70 $^{o}$C for 15 min in Gene Cycler thermal cycler (Bio-Rad). The thermal cycle conditions were initiated at 95 $^{o}$C for 1 min, and 30 cycles of amplification (94 $^{o}$C for 30 s, 58 $^{o}$C for 25 s, and 72 $^{o}$C for 1 min), followed by extension at 72 $^{o}$C for 3 min.

The PCR products were separated by electrophoresis on 2 % agarose gel and visualized by ethidium bromide staining. Amplification of β-actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primer: iNOS, forward primer 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' (2944-2968), reverse primer 5'-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3' (3416-3440); COX-2, forward primer 5'-GGAGAGACTATCAAGATAGTGATC-3' (1094-1117), reverse primer 5'-ATGGTCAGTAGACTTTTACAGCTC-3' (1931-1954); G3PDH, forward primer 5'- TGAAGGTAGGTGTGAACGGATTTGGC -3', reverse primer 5'- CATGTAGGCCATGAGGTCCACCAC -3'. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

### 2.2.8 Transient Transfection and Luciferase Assay

The luciferase assay was performed as described by George et al (George, Bungay et al. 1997) with some modifications. RAW 264.7 cells were seeded in a 60-mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco BRL). The cells were then transfected with a p-NF-κB-Luc plasmid reporter gene (Stratagene, Jalla, CA), murine iNOS promoter plasmid (piNOS/GL3) and COX-2 promoter plasmid (pCOX2/GL3) using LipofectAMINE$^{TM}$ reagent (Gibco BRL, Life Technologies, Inc.). After 24 h of incubation, the medium was replaced with complete medium. After another 24 h, the cells were trypsinized and equal numbers of cells were plated in 24-well tissue culture plates for 5 h. The cells were then incubated with 100 ng/mL LPS and either rosmanol or pyrrolidine dithiocarbamate (PDTC) for different periods of time. Luciferase activity was assayed by means of the britelite$^{TM}$ plus

luciferase reporter gene kit (PerkinElmer Life And Analytical Sciences, Inc, Boston, MA). Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single photon counting mode for 0.1 min/well.

### 2.2.9 Extraction of Nucleus and Cytosolic Protein

Nuclear and cytoplasmic extracts were prepared as previous reported with slight modification (Saha and Pahan 2006). The cells were suspended in hypotonic buffer (10 mM $NaH_2PO_4$, 10 mM NaF, 5.0 mM EDTA, 1.0 phenylmethanesulphonylfluoride (PMSF), 1.0 % NP-40, and 5.0 mM $MgCl_2$) for 10 min, on ice, followed by centrifugation at 4,000 × g for 15 min. The supernatants containing cytosolic proteins were collected. The pellet containing nuclei was resuspended in hypertonic buffer (10 mM $NaH_2PO_4$, 10 mM NaF, 5.0 mM EDTA, 1.0 PMSF, 1.0 % NP-40, 5.0 mM $MgCl_2$, and 2 % NaCl) for 3 h, on ice, followed by centrifugation at 175,00 g for 30 min. The supernatants containing nucleus proteins were collected for further western blot analysis.

### 2.2.10 Statistical Analysis

Data are presented as means ± SE for the indicated number of independently performed experiments. One way Student's *t*-test was used to assess the statistical significance between the LPS- and rosmanol plus LPS-treated cells. A $P$-value $< 0.05$ was considered statistically significant.

## 2.3. Results

### 2.3.1 Effect of Rosmanol on the Production of NO and $PGE_2$ in RAW 264.7 cells

We measured the level of nitrite and prostaglandin $E_2$ to investigate the effects of rosmanol on NO and $PGE_2$ production in LPS induced RAW 264.7 cells. The level of nitrite and prostaglandin in the culture media of RAW 264.7 cells were determined at 24 h after co-treatment with 100 ng/mL LPS and various concentrations of rosmanol. As shown in **Fig. 7**, rosmanol inhibited LPS-induced nitrite production in a dose-dependent manner, but did not interfere with the reaction between nitrite and Griess reagents (data not shown). Treatment with rosmanol also significantly decreased LPS-induced $PGE_2$ production (**Fig. 8**). We also performed an indirect nitrite assay to examine the possibility of rosmanol that inhibits the intrinsic activity of NOS enzyme and as a result of NO production. Cells were stimulated with LPS for 12 h and washed with PBS to remove LPS. The cells were then treated with different doses of rosmanol and incubated another 12 h. The nitrite in the media was determined after treatment of different doses of rosmanol. There was no difference between rosmanol treated group and LPS-induced control (**Fig. 9).** The western blotting analysis also exhibited that the treatment of rosmanol after LPS induction did not affect the level of iNOS and COX-2 in LPS-stimulated cells (**Fig. 10**). Taken together, rosmanol did not affect the production of nitrite in these cells whose a certain amount of iNOS was already expressed. This result was indicating that rosmanol did not influence the activity of NOS enzyme.

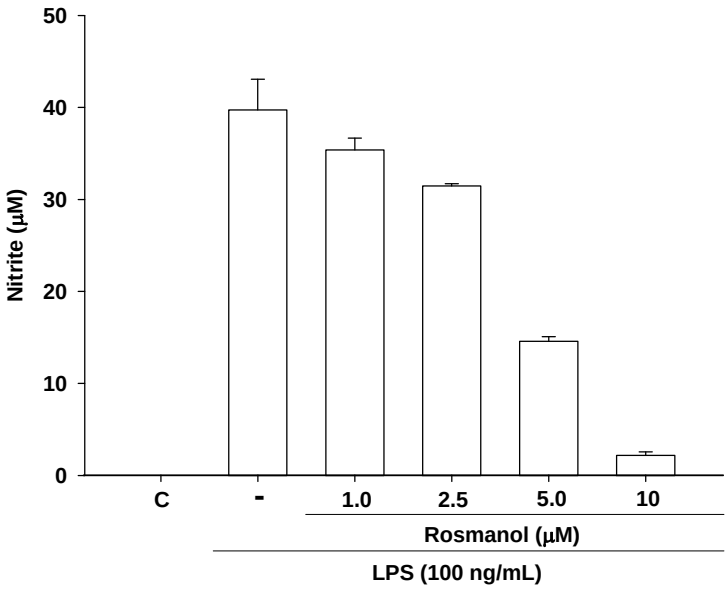



Fig. 7. Effect of LPS-induced NO production in RAW 264.7, The cells were treated with 100 ng/mL LPS only or with different concentrations of rosmanol. After incubation for 24 h, 100 μL of culture media were collected for nitrite assay.

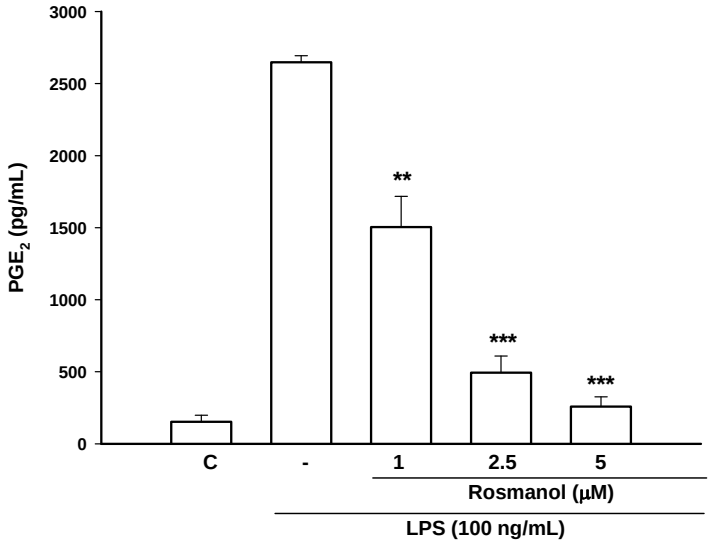



Fig. 8. Effect of rosmanol on LPS-induced PGE2 production, The cells were treated with 100 ng/mL LPS only or with different concentrations of rosmanol. After incubation for 24 h, 100 μL of culture media were collected for $PGE_2$ assay. The values are expressed as means ± SE of triplicate tests. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate statistically significant differences from the LPS-treated group

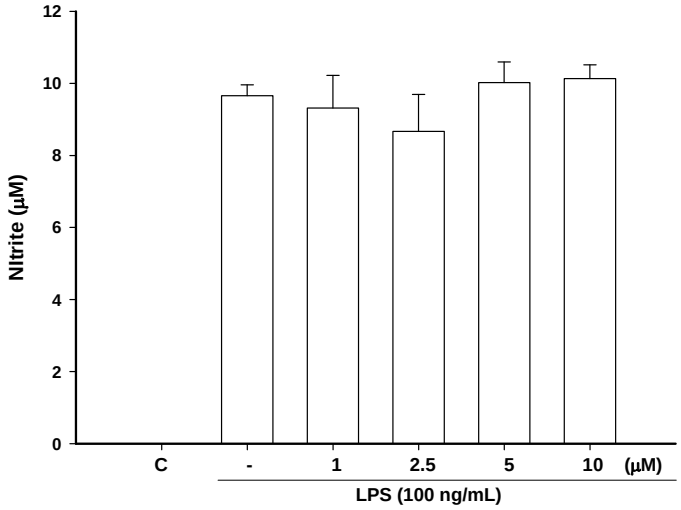



Fig. 9. NO assay of iNOS activity test on LPS induced RAW 264.7, The cells were stimulated with 100 ng/mL LPS for 12 h, and were washed with PBS to remove LPS before treated with different concentration of rosmanol for another 12 h. The culture media and cell lysates were then collected for nitrite assay

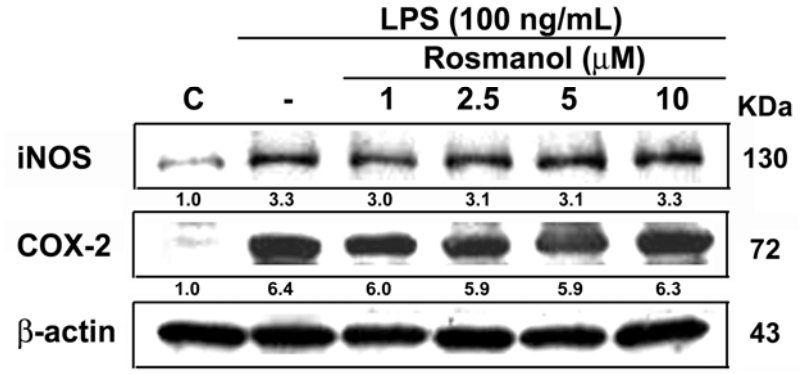



Fig. 10. Western blot analysis of iNOS activity test on LPS induced RAW 264.7, The cells were stimulated with 100 ng/mL LPS for 12 h, and were washed with PBS to remove LPS before treated with different concentration of rosmanol for another 12 h. The culture media and cell lysates were then collected for Western blot analysis

### 2.3.2 Rosmanol Inhibits LPS-induced iNOS and COX-2 Gene and Protein Expressions

It has been reported that iNOS and COX-2 are the key enzymes for the production of nitrite and $PGE_2$, respectively (Nathan and Xie 1994; Winston, Krein et al. 1999). We examined the effects of rosmanol on LPS-induced iNOS and COX-2 expression by western blotting analysis. As shown in **Fig. 11**, the protein levels of iNOS and COX-2 were undetectable in RAW264.7 cells without LPS-stimulation. Treatment with LPS alone markedly increased iNOS and COX-2 protein levels, whereas co-treatment with rosmanol dose-dependently reduced the expression of iNOS and COX-2 proteins. The reduced expression of iNOS and COX-2 protein was correlated with reduced production of total nitrite and $PGE_2$ in culture media (**Fig. 8**). In addition, RT-PCR was done to investigate whether rosmanol suppressed LPS-mediated induction of iNOS and COX-2 through pretranslational mechanism. The results from RT-PCR analysis showed that LPS treated alone resulted in gene expression of iNOS and COX-2 (**Fig. 12**). As compared to the LPS alone group, co-treatment with rosmanol markedly decreased the levels of iNOS and COX-2 mRNA in a dose-dependent manner, but did not affect the expression of the housekeeping gene G3PDH. We also investigated the effect of rosmanol on LPS-induced iNOS and COX-2 protein level. RAW264.7 cells were stimulated with LPS for 12 h, then protein synthesis was stopped by treatment with cycloheximide (CHX, 10 μM) for 2 h, and then rosmanol was added into culture media for a further 2 h. The result, as shown in **Fig. 13**, exhibits that the addition of rosmanol to LPS-stimulated cells after CHX treatment had no significant effect on iNOS and COX-2 protein level, which suggests rosmanol did not degrade iNOS and COX-2 or alter their stability. This result indicates

that rosmanol inhibit the expression of iNOS and COX-2 at the transcription level.

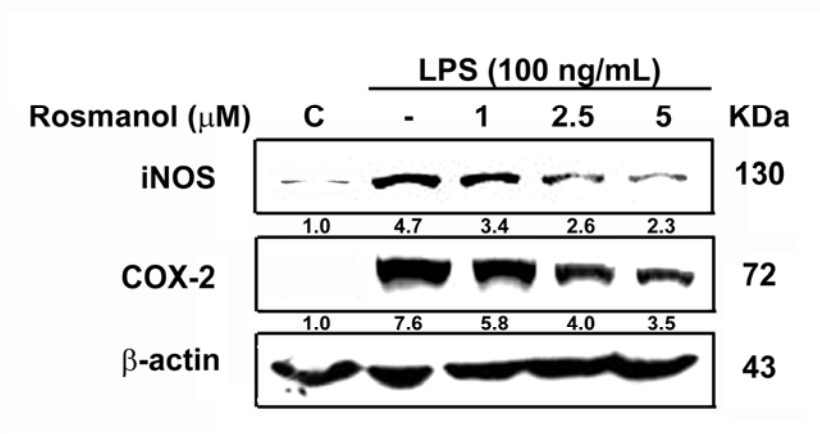



Fig. 11. Effect of rosmanol on LPS-induced iNOS and COX-2 expression in RAW 264.7, Cells were treated with 100 ng/mL LPS only or with rosmanol for 24 h. The levels of iNOS or COX-2 in cell lysates were analyzed by western blotting. β-actin was used as a loading control. This experiment was repeated three times with similar results

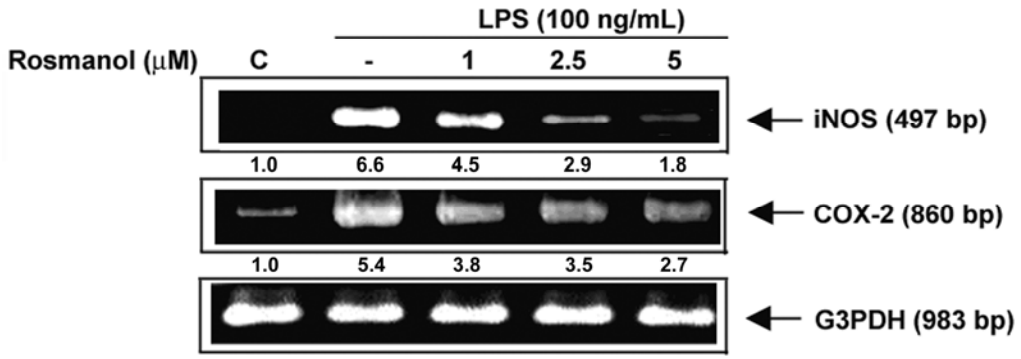



Fig. 12. Effect of rosmanol on LPS-induced iNOS and COX-2 gene expression in RAW 264.7, Cells were treated with 100 ng/mL LPS only or with rosmanol as described previously, after incubation for 5 h, total RNA were isolated and the mRNA expressions of iNOS and COX-2 were determined by semiquantitative RT-PCR. This experiment was repeated three times with similar results

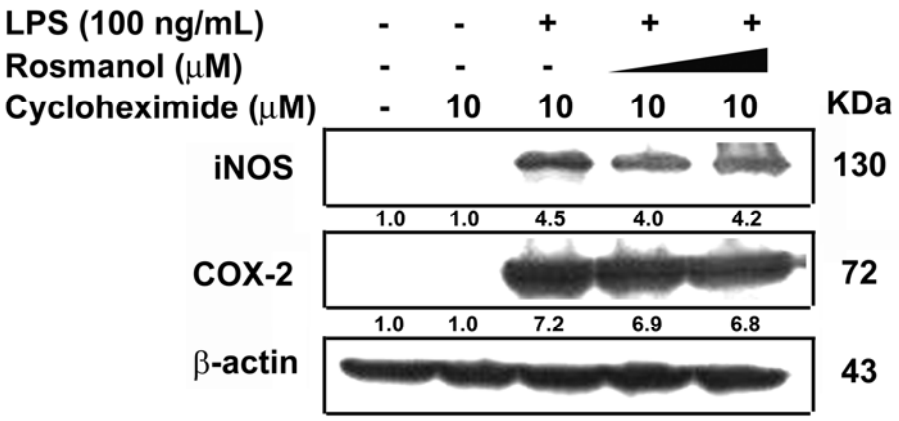



Fig. 13. Effect of rosmanol on LPS-induced iNOS and COX-2 expression after treatment of cycloheximide, Cells were stimulated with 100 ng/mL LPS for 12 h and after treatment with CHX (10 μg/ml) for 2 h to stop subsequently protein synthesis, then rosmanol (2.5 and 5μM) were added into culture media for further 2 h. The levels of iNOS or COX-2 in cell lysates were analyzed by western blotting. β-actin was used as a loading control. This experiment was repeated three times with similar results.

### 2.3.3 Rosmanol Inhibits NF-κB Nuclear Translocation and Activation in LPS-Stimulated Macrophages

Previous research demonstrated that NF-κB activation played a pivotal role in expression of both iNOS and COX-2 in LPS or other inflammatory cytokines induced cell (Baeuerle and Henkel 1994). Because amounts of iNOS and COX-2 mRNA were reduced by rosmanol, we then examined the effects of rosmanol on the activation of NF-κB. We separated the cell lysate into nucleic and cytosolic fractions and then which were subjected to Western Blot analysis, because the activated NF-κB was translocated into cell nucleus. Compared to non treated control group, LPS treated positive control showed translocation of the NF-κB subunit, p50 and p65, to nucleus after 60 mins of stimulation with LPS (**Fig. 14**). Rosmanol significantly reduced the amount of p50 and p65 in cell nucleus which is translocated from cytosol after LPS-stimuli. PARP, a nuclear house-keeping protein, and β-actin, a cytosolic house-keeping protein, were used to confirm whether the same amount of protein in each fraction was subjected and the addition of any contaminant during extraction of each fraction. The phosphorylation of p65 at serine 536 was also suppressed by rosmanol in does dependant manner. In an additional study, RAW 264.7 cells were transiently transfected with a NF-κB-dependent luciferase reporter plasmid and then treated with LPS alone or with various concentrations of rosmanol. As shown in **Fig. 15**, LPS-induced transcription activity of NF-κB was strongly reduced by rosmanol. To determine the effects of rosmanol on iNOS and COX-2 promoter activities, piNOS/GL3 and pCOX2/GL3 were also transfected into RAW264.7 cells. **Fig. 16** and **17** show that transfected cells treated with LPS for 15 h markedly

increased the iNOS and COX-2 promoter activities by 3.3 and 10.1-fold compared with non-treated control group. Rosmanol strongly inhibited the promoter activities both of iNOS and COX-2. Rosmanol showed stronger activity than PDTC, known as a NF-κB inhibitor, in COX-2 promoter activity test.

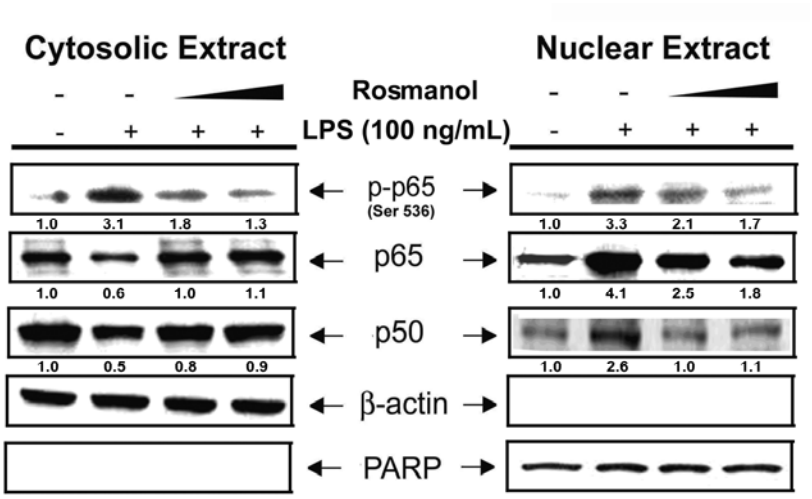



Fig. 14. Effect of rosmanol on translocation of NF-kB in RAW 264.7, The cells were treated with 100 ng/mL LPS alone or with rosmanol (2.5 and 5 μM) for 1 h. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting.

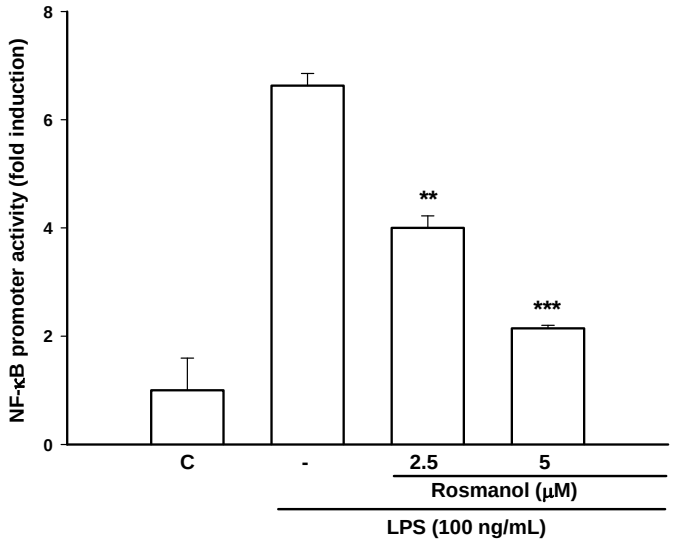



Fig. 15. Effect of rosmanol on pNF-kB promotor activity in RAW 264.7, pNF-κB-Luc reporter plasmid were transiently transfected into RAW264.7 cells. Cells were then treated with 100 ng/mL LPS in the presence or absence of rosmanol (2.5 and 5 μmol) or PDTC (20μmol) for 6 h. After incubation, cells were harvested and luciferase activities were determined as described in materials and methods. The results are expressed as means ± SE of triplicate tests. *P < 0.05, **P < 0.01 and ***P < 0.001 indicates statistically differences from the LPS-treated group.

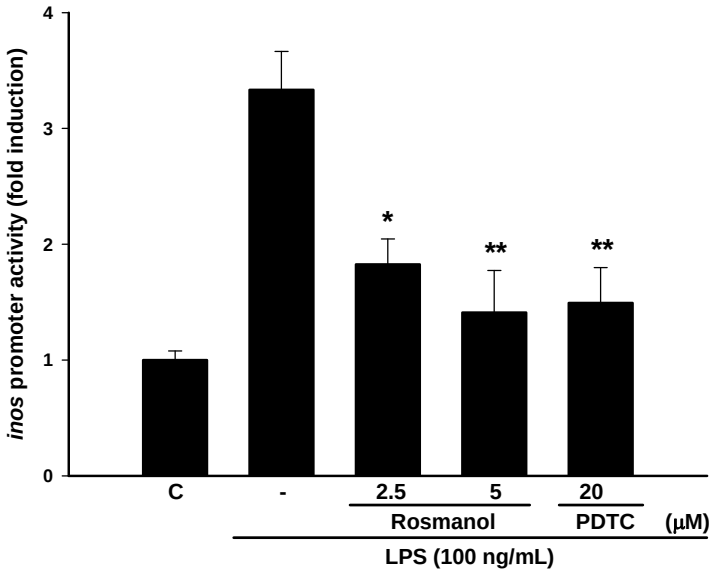



Fig. 16. Effect of rosmanol on piNOS promotor activity in RAW 264.7, Murine iNOS promoter plasmid (piNOS/GL3) were transiently transfected into RAW264.7 cells. Cells were then treated with 100 ng/mL LPS in the presence or absence of rosmanol (2.5 and 5 μmol) or PDTC (20μmol) for 18h. After incubation, cells were harvested and luciferase activities were determined as described in materials and methods. The results are expressed as means ± SE of triplicate tests. *P < 0.05, **P < 0.01 and ***P < 0.001 indicates statistically differences from the LPS-treated group.

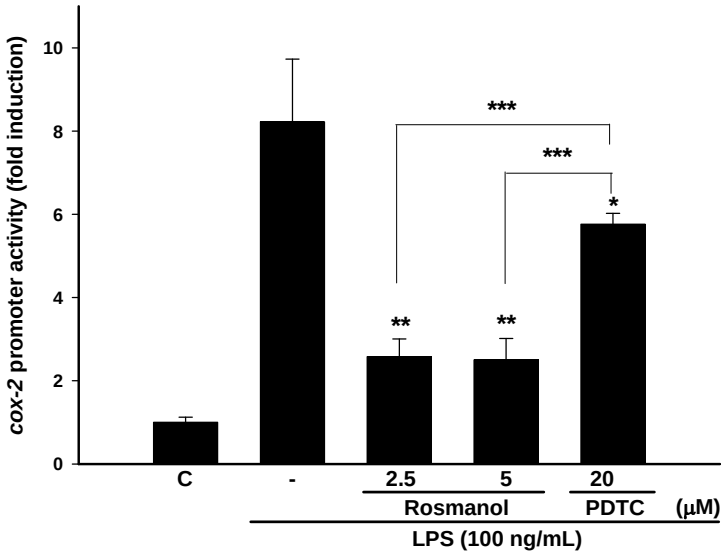



Fig. 17. Effect of rosmanol on pCOX-2 promotor activity in RAW 264.7, Murine COX-2 promoter plasmid (pCOX2/GL3) were transiently transfected into RAW264.7 cells. Cells were then treated with 100 ng/mL LPS in the presence or absence of rosmanol (2.5 and 5 μmol) or PDTC (20μmol) for 15h. After incubation, cells were harvested and luciferase activities were determined as described in materials and methods. The results are expressed as means ± SE of triplicate tests. *P < 0.05, **P < 0.01 and ***P < 0.001 indicates statistically differences from the LPS-treated group.

### 2.3.4 Inhibitory Effects of Rosmanol on LPS-Induced Phosphorylation and Degradation of IκB

The expression of IκB is transient, because IκB is degraded by proteasomal process in LPS-mediated activation of NF-κB. To see the alteration in the level of IκB we measured the level of IκB in time-course. Therefore, we further investigated the effects of rosmanol on LPS-induced phosphorylation and degradation of IκB. First, we treated LPS on RAW 264.7 cells and measured the level of IκB and phosphorylated IκB at 0, 15, 30, 45, 60, and 120min. Second, to examine the effect of rosmanol LPS and rosmanol were co-treated and then measured the level of IκBα and phosphorylated IκBα at same time course with previous one. Treatment with LPS caused the phosphorylation of IκBα protein. From 15min certain amount of phosphorylated IκBα was expressed and at 30min maximum amount of IκBα was degraded. From 45min the levels of IκBα was recovered gradually (**Fig. 18**). Rosmanol effectively attenuated the increased phosphorylation of IκBα and sustained the IκBα protein content (**Fig. 19**). These results suggest that rosmanol inhibit activation and translocation of the NF-κB through inhibiting phorphorylation and the degradation of IκBα, the bounding protein of this dimer.

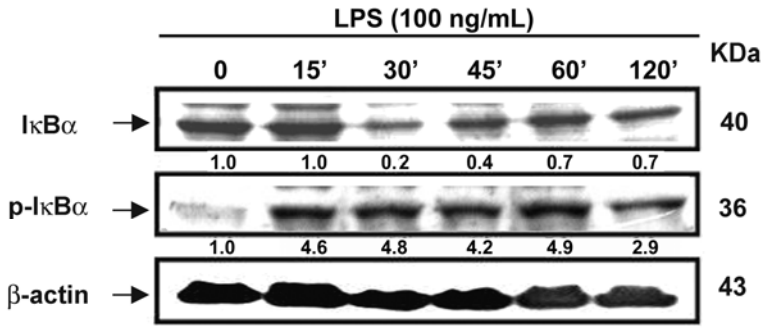



Fig. 18. LPS-induced phosphorylation and degradation of IκB, The cells were treated with 100 ng/mL LPS alone for different times. Total cell lysates were prepared for Western blot analysis. These experiments were repeated three times with similar results.

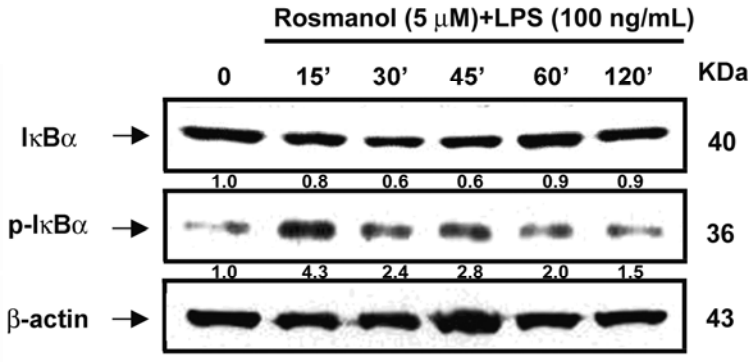



Fig. 19. Effect of rosmanol on LPS-induced phosphorylation and degradation of IκB, The cells were co-treated with LPS and rosmanol for different times. Total cell lysates were prepared for Western blot analysis. These experiments were repeated three times with similar results.

### 2.3.5 Rosmanol Inhibits Phosphorylation and Nuclear Translocation of STAT3 and C/EBP Expression in LPS-Stimulated Macrophages

JAK/STAT3 is another key signaling pathway involved in expression of cytokines such as Il-1β and Il-6, and iNOS in LPS-induced RAW 264.7 macrophage (Marrero, Venema et al. 1998; Samavati, Rastogi et al. 2009). We next explored the effects of rosmanol on the activation of STAT3 by measuring the levels of p-STAT3 (Ser727 and Tyr705) in nuclear extracts. As shown in **Fig. 20**, LPS caused nuclear translocation and phosphorylation of STAT3 (Ser727 and Tyr705) in RAW264.7 cells. Compared with LPS treatment group, rosmanol markedly reduced the nuclear translocation and phosphorylation of STAT3 (Ser727 and Tyr705) induced by LPS but did not affect the total levels of STAT3 protein.

It is well known that transcription factor CCAAT/enhancer-binding protein (C/EBP) is also involved in regulating iNOS and COX-2 gene expression (Lee, Sung et al. 2003). To determine whether C/EBP is inhibited by rosmanol in LPS-treated macrophage, we measured the level of C/EBPβ and C/EBPδ after 2 hours of treatment of LPS in RAW 264.7 cells. The increased expression of both C/EBPβ and C/EBPδ in LPS treated group was observed, whereas both of them were greatly decreased in rosmanol treated group **(Fig 21)**. These results suggest that rosmanol not only inhibit NF-κB activation, but also inhibit the STAT3 and C/EBP signaling pathways while it inhibits inflammatory gene express in LPS-induced macrophage.

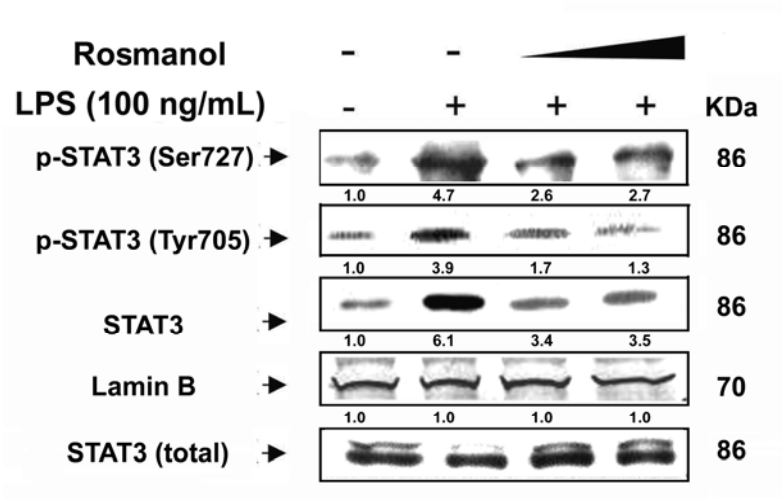



Fig. 20. Effect of rosmanol on LPS-induced phosphorylation of STAT3 in RAW 264.7, The cells were treated with 100 ng/mL LPS with or without rosmanol (2.5 or 5 μmol) for 1 h. Nuclear fractions were assayed for p-STAT3 (Ser727 and Tyr705) and STAT3 by Western blotting analysis. Lamin B was used as an internal control for nuclear fraction.

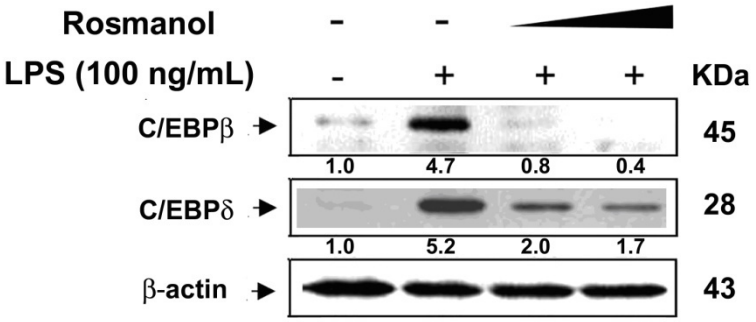



Fig. 21. Effect of rosmanol on LPS-induced translocation of C/EBPs in RAW 264.7, The cells were treated with 100 ng/mL LPS with or without rosmanol (2.5 or 5 μmol) for 1 h. Total cell lysates were determined for C/EBPβ and C/EBPγ by Western blotting analysis.

### 2.3.6 Rosmanol Inhibits Phosphorylation of PI3K/Akt, p38, and ERK1/2 MAPK Kinase in LPS-Stimulated Macrophages

MAPKs pathways are the major signaling pathway involved in various cellular responses. Previous research showed that p38 and p44/42 MAPK play an important role in LPS-induced iNOS and COX-2 expression in murine macrophages Activation of MAPK requires phosphrylation at threonine and tyrosine residues. (Guha and Mackman 2001). In addition, cytokine-medicated activation of the PI3K/Akt pathway is also involved to the activation of NF-κB pathway and its downstream inflammatory gene expression (Sizemore, Leung et al. 1999). To determine whether rosmanol can regulate these upstream signaling pathways, we investigated the level of phosphorlyated of p38, p44/42 MAPK and PI3K/Akt with/without rosmanol in LPS-stimulated macrophages. In only LPS treated group large amount of phosphrylated ERK1/2 and p38 MAPK were observed after 30 min of treatment. However, in LPS and rosmanol co-treated group phosphorylation of ERK1/2 and p38 MAPK was decreased compared to LPS treated control. At 5μM of rosmanol treated group phosphrylated ERK was dramatically decreased (**Fig. 22**). The phosphorylation of PI3K and Akt was also observed after 30 min of co-treatment with rosmanol and LPS. The phosphorylation of Akt and PI3K decreased compared to LPS treated control (**Fig. 23**). Specific inhibitors of these kinases were used to confirm the role of these upstream kinases involved in LPS-induced iNOS and COX-2 expression.

As shown in **Fig. 24**, pretreatment with SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and LY294002 (PI3K/Akt inhibitor) strongly inhibited LPS-induced NO production as well as the protein levels of iNOS and COX-2. However, treatment with

ERK inhibitor, PD98059, decreased only the expression of COX-2, but had almost no effect on NO production and iNOS expression. In addition, rosmanol reduced not only NO production, but also iNOS and COX-2 expression. These western blot analyses suggest that rosmanol might block LPS-induced NF-κB activation by inhibiting ERK1/2, p38 MAPK and PI3K/Akt /IKK pathways which is related to the degradation of IκBα and their downstream-gene expresseion such as iNOS and COX-2.

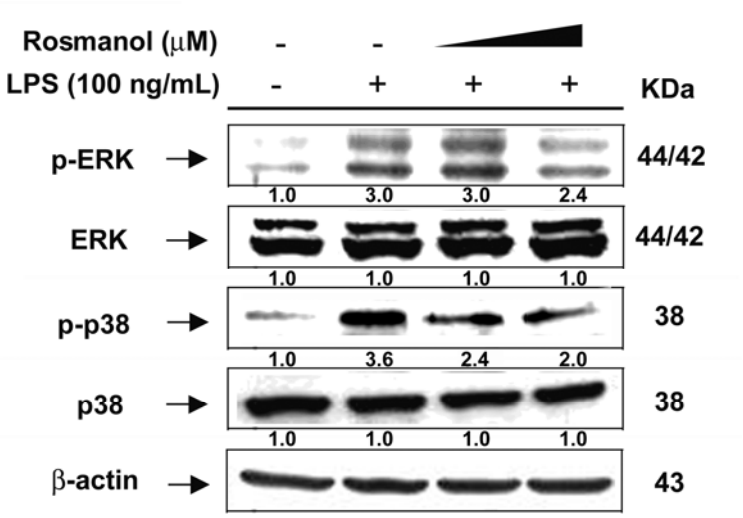



Fig. 22. Effects of rosmanol on phosphorylation of MAPK in RAW 264.7, The cells were treated with 100 ng/mL LPS with or without rosmanol (2.5 or 5 μmol) for 30 min. Total cell lysates were then prepared for determination of p-ERK, p-p38, ERK, and P38 by Western blotting analysis.

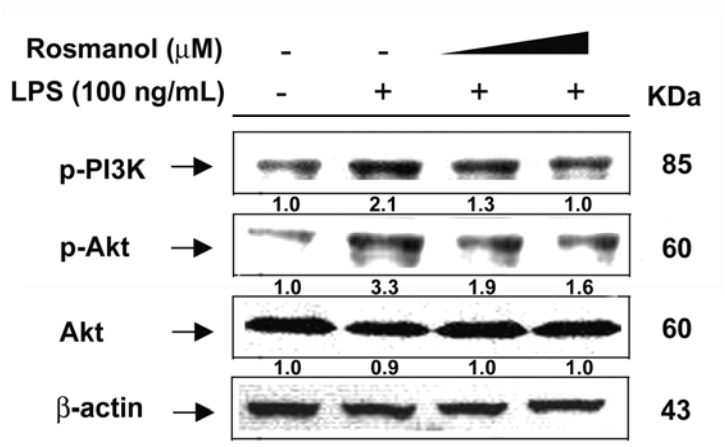



Fig. 23. Effects of rosmanol on phosphorylation of PI3K/Akt in RAW 264.7, The cells were treated with 100 ng/mL LPS with or without rosmanol (2.5 or 5 μmol) for 30 min. Total cell lysates were then prepared for determination of p-Akt, p-PI3K and p-Akt by Western blot analysis.

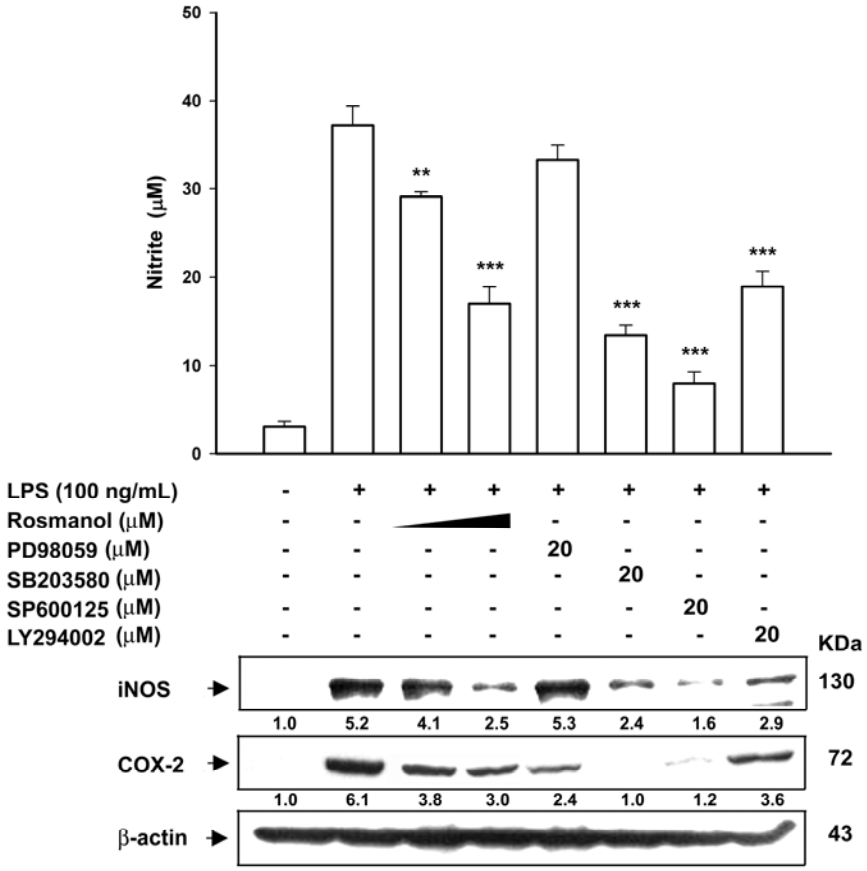



Fig. 24. Effects of rosmanol and kinase inhibitors on iNOS and COX-2 expression in RW 264.7, Cells were pre-treated with rosmanol (2.5 or 5 μmol) or different specific kinase inhibitors for 30 min, and then treated with LPS (100 ng/ mL) for another 12 h. The culture media were collected for nitrite assay and total cell lysates were prepared for Western blot analysis

## 2.4. Discussion

In this study we examined the effects of rosmanol on LPS-induced expression of iNOS and COX-2 in murine macrophage and also provided molecular evidence for these effects of rosmanol serving as potential anti-inflammatory reagent for the first time. Over-production of NO by iNOS occurred in various cell types after stimulation with cytokine and endotoxin, and causes various inflammatory diseases and tumorigenesis (Nathan and Xie 1994; Ohshima and Bartsch 1994). High level of $PGE_2$ synthesized by COX-2 was also occurred in various cancer tissues and implicated in proliferation of cancer cell, angiogenesis, and tumor growth (Claria 2003; Meric, Rottey et al. 2006). Because there is a causal relationship between inflammation and cancer, iNOS and COX-2 are considered as potential molecular targets for chemoprevention (Mantovani, Allavena et al. 2008; Pan and Ho 2008). In our present study, we found rosmanol dose-dependently inhibited LPS-induced NO and $PGE_2$ production by reducing gene expression of iNOS and COX-2. While rosmanol was reducing the production of NO, it just reduced NO by inhibiting gene expression of iNOS, not by affecting the enzyme activity of iNOS. Additional experiments were carried out to test the effect of rosmanol on iNOS and COX-2 protein stability, and neither of these proteins was degraded or altered after rosmanol treatment. These results indicate that rosmanol might inhibit NO and $PGE_2$ production through regulating the transcription levels of iNOS and COX-2, and its upstream molecules in LPS induced RAW 264.7.

There is a large number of binding sites for transcription factors in the region of iNOS and COX-2 promoters, including NF-κB, activator protein-1 (AP-1), interferon regulatory factor-1 (IRF-1), STATs and CCAAT/enhancer-binding protein (C/EBP),

according to cell types and stimulation (Lee, Sung et al. 2003). Among the promoter regions of iNOS and COX-2, the transcription factor NF-κB is mainly responsible for their transcription by LPS stimulation (Xie, Kashiwabara et al. 1994). We also showed that rosmanol markedly inhibited nuclear translocation and transcriptional activity of NF-κB in LPS-induced RAW264.7 cells. Inhibition of NF-κB activation by rosmanol might be through suppressing the phosphorylation and degradation of IκB. In experiments of iNOS and COX-2 promoter analysis, rosmanol and NF-κB inhibitor, PDTC, also reduced the LPS-induced transcriptional activity of both iNOS and COX-2. In comparison with PDTC, rosmanol showed much greater inhibition in COX-2 promoter activity. Because not only NF-κB, but also other transcription factors are involved in regulating the COX-2 gene, greater activity of rosmanol indicates that rosmanol can regulate more than one more transcription factors except NF-κB.

STAT3 is an important transcription factor participating the production of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-10 which plays a major role in inflammatory disease (Schindler 2002). In previous studies, it was shown that the promoter region of iNOS gene in murine macrophage contains the STAT-binding γ-activated sites (GAS) (Marrero, Venema et al. 1998). Activation of STAT3 in response to Il-6 is involved in both phosporylation of Tyr705 and Ser727. Phosphorylation of STAT3 at tyrosine 705 is required for dimerization, nuclear translocation of this transcription factor, and DNA binding. In addition, phosphorylation at Ser 727 is also crucial for nuclear translocation and DNA binding of STAT3 (Schuringa, Schepers et al. 2001). In our study, we showed that treatment with LPS alone markedly increased the

phosphorylation of STAT3 at Tyr 705 and Ser 727 in nuclear levels, whereas rosmanol strongly reduced the phosphorylation of STAT3 both at Tyr 705 and Ser 727, as well as inhibited nuclear translocation. It has been reported that phosphorylated of STAT3 at Tyr 705 is mediated mainly by receptor associated kinases (JAKs) and phosphorylation at Ser 727 is regulated by ERK1/2 and mTOR (Galdiero, Vitiello et al. 2006) . Based on these reports we can assume that rosmanol may inhibit phosphorylation of STAT3 at Tyr 705 and Ser 727 through multiple signaling pathways. However, the detailed mechanism remained to be elucidated.

Recently, C/EBPs have also been shown to play an important role in the transcriptional activation of iNOS and COX-2 promoters. Among the members of C/EBP transcription factors, C/EBPβ has been implicated in inducing the expression of COX-2 stimulated by endotoxin and cytokines (Wadleigh, Reddy et al. 2000). Furthermore, both C/EBPβ and C/EBPδ are also involved in induction of glial iNOS (Saha and Pahan 2006) as well as the inflammatory mediator produced in LPS induced RAW264.7 macrophage (Liu, Tseng et al. 2003). We investigated that rosmanol reduced the levels of C/EBPβ and C/EBPδ stimulated by LPS, which suggested another possible mechanism of rosmanol for inhibition of iNOS and COX-2 gene transcription. We exhibited that stronger effect of rosmanol on reducing iNOS and COX-2 promoter activities compared to PDCT, known as a NF-κB inhibiter (**Fig. 17**). The inhibition effect of rosmanol on inhibiting C/EBPβ and C/EBPδ may support this stronger effect of rosmanol on inhibiting COX-2 promoter activity.

LPS stimulation of human monocytes triggers onset of several intracellular

signaling pathways including IKK-NF-κB pathway and three MAPK pathways such as ERK 1/2, JNK, and p38. These signaling pathways in turn activate a variety of transcription factors that include NF-κB (p50/ p65) and AP-1 (c-Fos/c-Jun), which coordinate the induction of many genes encoding inflammatory mediators (Guha and Mackman 2001). LPS or cytokine stimulation of vascular smooth muscle cells leads to activation of the PI3K-Akt pathway, which then activates the NF-kappaB pathway. Thus, the PI3K-Akt pathway controls the expression of iNOS in LPS and/or cytokine induced vascular smooth muscle cells (Hattori, Hattori et al. 2003). We also explored the effects of these upstream kinases on LPS-indcued iNOS and COX-2 expression in RAW264.7. We found blocking p38, JNK and PI3K with their specific inhibitors resulted in suppressing both iNOS and COX-2 expression, as well as suppression of NO production, whereas inhibition of ERK only suppressed COX-2 protein levels (Fig. 24). Treatment with rosmanol significantly reduced the activation of PI3K/Akt, ERK1/2, and p38 MAPK (**Fig. 22,23**) and subsequent decreased levels of iNOS and COX-2 (**Fig. 24**) were observed. As mentioned above, LPS can activate the PI3K/Akt, NF-κB, STATs and MAPKs pathways and these pathways are related to expression of iNOS and COX-2 directly or in directly. Taken together our results and previous reports, rosmanol suppressed iNOS and COX-2 expression by regulating these kinases. In addition, PI3K has been suggested as a key upstream regulator of the NF-κB, STATs and MAPKs pathways (Marrero, Venema et al. 1998). We can suggest that “loss of function” of PI3K by rosmanol may play a key role in suppression of LPS induced effects, including the disruption of activating transcription factors on iNOS and COX-2 promoter in RAW 264.7 cells. Considering these results, rosmanol might suppress LPS-induced NF-κB

translocation through inhibiting the activation of these intracellular signaling cascades and subsequently suppressing the gene transcription of iNOS and COX-2.

## 2.5. Conclusions

In this study we observed the anti-inflammatory effects of rosmanol on LPS-induced iNOS and COX-2 expression in murine macrophage. Rosmanol reduced production of NO and $PGE_2$ by regulating their gene expression of iNOS and COX-2, not by affecting enzyme activity or altering enzyme stability. This action was carried out by inhibiting signal pathways of NF-κB, the major transcription factor responsible for the iNOS and COX2 expression, reducing the degradation and phosphorylation of IκBα.

MAPK such as ERK1/2 and p38 and PI3K/Akt are particularly affecting the degradation and phosphorylation of IκBα resulting the phosphorylation and translocation of NF-κB. Rosmanol also reduced these kinases. In addition, nuclear translocation of STAT3 and the expression of C/EBPβ and C/EBPδ protein subsequently are related to the expression of iNOS and COX-2. Rosmanol also inhibits these proteins. This study exhibited that rosmanol suppressed the gene expression of iNOS and COX-2 not only by inhibiting major NF-κB pathway, but also by inhibiting its alternate pathways such as nuclear translocation of STAT3 and the expression of C/EBPβ and C/EBPδ. This results showed that anti-inflammatory activity of rosmanol is multi-potent, because if one inhibition pathway may be blocked, the alternate pathway of rosmanol still can work. Based on this study, we suggest rosmanol should have great potential as a novel chemopreventive agent for various inflammatory diseases.

# 3. Chapter III. Inhibitory Effects of Rosmanol and Carnosol on 12-O-Tetradecanoylphorbol-13-Acetate-Induced Inflammation in Mouse Ears

## 3.1. Backgrounds and Rationale

The general role of acute inflammation is successful resolution and repair of tissue damage and infection. Acute inflammation is a rapid self-limiting process. Once it accomplishes its role, it is programmed to be stopped. However, due to presence of persistent initiating factors or the failure to remove inflammatory stimuli such as pathogens, outer contaminants, and dead cells, it will continue to persistent inflammation. The persistent inflammation seems to be developed to chronic inflammation, which can lead to scarring and loss of organ function. Moreover, it has been known that this persistent and/or chronic inflammation is closely linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation of cells. Acute inflammation is often characterized by rapid influx of blood granulocytes and neutrophils. Typically, neutrophils are the first recruited effectors of the acute inflammation response, followed prompt differentiation of monocytes into inflammatory macrophages that subsequently proliferate and affect the functions of resident tissue macrophages. When granulocytes are removed and the population of macrophages and lymphocytes are back to normal pre-inflammatory state, resolution of inflammation can be occurred (Coussens and Werb 2002; Serhan and Savill 2005; Mueller 2006).

When our skin gets injury, activated platelets participate in formation of blood clot which is composed of cross-linked fibrin and extracellular matrix proteins. Importantly, platelets release platelet derived growth factor (PDGF), platelet-activating

factor, platelet factor–4 (PF-4), and transforming growth factor–β (TGF-β). These growth factors play a role as a chemotactic attractant to recruit circulating inflammatory cells to wound site, as well as initiator of tissue re-epithelialization, contraction, and stimulant of wound angiogenesis. The variety of chemotactic signals attracts neutrophils and monocytes. Not only growth factors released from activated platelet, but also cleaved peptides from bacterial proteins are categorized into chemotactic signals and attract these cells. Neutrophils normally arrive to wounded site within a few minutes after injury. Their role has been considered to remove the contaminating bacteria, but recent researches have shown that they are also source of pro-inflammatory cytokines and chemokines that are necessary for cell recruitment, activation, and differentiation (Brigati, Noonan et al. 2002; Coussens and Werb 2002). If the infection is not severe, the infiltration of neutrophils is stopped in few days, and the excess amounts of them are phagocytosed by macrophage. Monocytes and macrophages are recruited to inflamed site, being attracted by chemokines and cytokines such as interleukin-1β (Il-1β), interleukin-6 (Il-6), and tumor necrosis factor-α (TNF-α). One mission of activated macrophages is phagocytosis of remaining pathogens, dead cells, and matrix debris. Second one of them is amplifying the immune signals which are initially released by platelets and neutrophils through releasing large numbers of growth factors such as TGF-α, PDGF, IGF-I/II, TNF-α, Il-1, and Il-6. The others are remodeling of extracellular matrix (ECM), angiogenesis by releasing proteases such as matrix metallopoteases (MMPs) and urokinase-type plasminigen inhibitor (uPA) and ECM-components such as thrombospondin-1 (Dipietro 1995). Another cell type mediates innate immune response is mast cell. Once activated, mast cells rapidly release its characteristic granules and various hormonal mediators such

as MMPs, heparin, heparanase, and vascular endothelial growth factor (VEGF) (Artuc, Hermes et al. 1999). Then, the later phase of wound healing is regulated by migration of fibroblasts. They are stimulated by IL-1-α/β and TGF-β-1, -β-2, -β-3 to synthesize ECM components and to be differentiated into myofibroblasts inducing wound contraction. The final stage of wound healing starts with the proliferation and migration of keratinocytes at the wound edge. Keratinocyte-derived uPA and MMPs mediate the dissolution of the fibrin clot and a degradation of collagen. Not only the essential contribution of cell–cell and cell–matrix interactions to healing process, but also the inflammatory response associated with this repair process have to participate together to organize successful tissue repair (Mueller 2006).

Transcription factor, nuclear factor-κB (NF-κB)/Rel family, plays a pivotal role in inflammatory and immune responses. Bacterial and viral infections, as well as pro-inflammatory cytokines, all of them trigger the activation of IKK complex. Activated IKK cause the translocation of NF-κB to cell nucleus through degrading IκB, bounding protein to sustain NF-B complex in cell cytosol. IKK complex is composed of two catalytic subunits and one regulatory subunit. Two catalytic subunits are IKK-α (also known as IKK1) and IKK-β (also known as IKK2), and IKK-γ (also known as NEMO) is the regulatory subunit. IKK complex phosphorylates IκB and liberates NF-κB dimmers through proteasomal degradation. Liberated NF-κB dimmers move into the nucleus and mediate the transcription of target genes. Most of this pathway is depend on the catalytic subunit IKK-β, performing the phosphorylation of IκB (Barnes and Karin 1997). Cyclooxygenase (COX) is the key enzyme in the subsequent transformation of prostaglandins (PGs) and thrombozane (TX) from arachidonic acid. The COX pathway

has clinical importance because it is the major target for non-steroidal anti-inflammatory drugs, which are commonly used for relieving inflammation, pain and fever. In 1991, it was discovered that COX exists in two distinct isozymes (COX-1 and COX-2), one of which, COX-2, is primarily responsible for inflammation but apparently not for gastrointestinal integrity or platelet aggregation. Protective PGs, which especially preserve the integrity of the stomach lining and prevent platelet aggregation, maintain the homeostasis of our body and are synthesized by COX-1. In addition, this constitutively expressed enzyme is present in the brain and spinal cord, where it may be involved in nerve transmission, particularly that for pain and fever. Meanwhile, PGs made by COX-2 are also important in ovulation and in the birth process. The discovery of COX-2 enables us to design "smart drug" which retain anti-inflammatory activity but minimize the risk of gastrointestinal toxicity and bleeding caused inhibition of protective PGs synthesized by COX-1. Highly selective COX-2 inhibitors may not only be anti-inflammatory but may also be active in colon cancer and Alzheimer's disease. Especially, prostaglandin $E_2$ ($PGE_2$) is synthesized by COX-2 and contributed to pathogenesis of various inflammatory diseases, edema, angiogenesis, and tumor growth (Vane, Bakhle et al. 1998; Claria 2003).

Since the transcription factor, NF- κB is known to regulate COX-2 induction, inhibition of NF- κB attenuates the expression of COX-2. (Williams, Tsujii et al. 2000).

Topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA) to the skin has been known to induce inflammatory responses by attracting inflammatory cells which release pro-inflammatory cytokines such as tumor necrosis factors (TNFs), interleukine (Il) -1, 6, and various chemokines (Lewis and Adams 1987). These responses also include

the development of edema, hyperplasia, enhanced release of reactive oxygen species, and over-expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) proteins (Seo, Park et al. 2002; Chung, Park et al. 2007).

Rosemary (Rosmarinus officinalis L.) leaves, widely used food ingredient for seasoning and flavoring, has been known as a traditional medicine for its health beneficial properties such as antimicrobial, antialgesic, and antirheumatic effects. Its antioxidant (Aruoma, Spencer et al. 1996; Ramirez, Garcia-Risco et al. 2006), anti-inflammatory (Altinier, Sosa et al. 2007), and anti-cancer (Singletary, MacDonald et al. 1996) activities are reported by previous research groups. Most of antioxidant properties of rosemary extract contribute to carnosol and carnosic acid. These diterpenes show strong antioxidant activity (del Bano, Lorente et al. 2003). Rosmanol is originating from oxidative transformation of carnosic acid and carnosol (Wei and Ho 2006). In food processing carnosol is easily converted to rosmanol by exposure to oxygen. Thus, as a food ingredient, the property of rosmanol in rosemary is important. Research has proved that rosmanol has a potent antioxidant activity inhibiting superoxide anion production, lipid peroxidation, and free radical scavenging activity (Haraguchi, Saito et al. 1995; Zeng, Tu et al. 2001; Escuder, Torres et al. 2002). There has been no in vivo anti-inflammatory activity study of rosmanol yet.

Our group examined the anti-inflammatory effects of rosmanol using LPS-stimulated murine macrophage RAW 264.7. The results showed that rosmanol suppressed LPS-induced NO and $PGE_2$ production by inhibiting NF-κB, STAT3 and C/EBP, as well as the upstream, p38, ERK1/2, and PI3K/Akt signaling pathways.

Based on in vitro effecs of rosmanol, we may expect the anti-inflammatory effects

of carnosol and rosmanol in vivo. To assess the anti-inflammatory activity of carnosol and rosmanol on skin inflammation induced by TPA in mouse ear, we observed tissue autopies obtained from TPA treated mouse ear. We used histological and immunohistochemical study to examine the difference in skin edema, keratinocytes ulceration and granulocytes infiltration. To further elucidate the molecular mechanisms undergoing anti-inflammatory activity of carnosol and rosmanol, its effect on the TPA-induced activation of COX-2 and the upstream signaling molecules, NF-κB were also explored in this mouse model.

## 3.2. Material and Methods

### 3.2.1 Animals and Reagents

CD-1 strain mice were used for the mouse ear anti-inflammatory studies. Female CD-1 (4-5 weeks old) mice were purchased from Charles River Breeding Laboratories (Kingston, NY, USA). The mice were kept in a controlled room temperature (22±2°C) and humidity (60-80%) under a 12h light/dark cycle for at least 1 week before use. All animal studies were done in accordance with an institutionally approved protocol.

Il-1β and Il-6 ELISA kits were purchased from R&D system (Mineapolis, MN, USA). $PGE_2$ ELISA kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). COX-2, p65, and β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IKKα, IKKβ, and phosphrylated-p65 antibody were purchased from Cell Signaling Technology Inc (Danvers, MA, USA). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

### 3.2.2 Preparation of Test Compounds

40% carnosic acid powder was provided by Dr. Bai from Naturex (South Hackensack, NJ. USA). 5g of extract powder was dissolved in acetone (50 mL) and bubbled with molecular oxygen (0.1 bar) at room temperature overnight. This solution was subjected to chromatography filled with Sephadex LH-20. The solution was eluted by a mixture of *n*-hexane/dichloromethane/methanol (2:1:1). After TLC analysis of the collections of 24 fractions, fractions 11-15 were purified by crystallization with a mixture of dichloromethane and acetone, yielding carnosol (800 mg). Fractions 20-24 were purified by silica gel column chromatography using mixtures of *n*-hexane/acetone (4:1) to yield the rosmanol (200 mg).

Carnosol (100 mg) in acetone (10 mL) was treated with aqueous sodium bicarbonate (5%, 6 mL), and the mixture was stirred at room temperature for 6.5 h. After the acetone was evaporated using rotary evaporator. The reaction mixture was acidified with dilute hydrochloric acid and extracted with ethyl acetate, washed with saturated sodium chloride, and removed the moisture with anhydrous sodium sulfate. The residue was purified by silica gel column chromatography eluting with *n*-hexane/acetone (4:1), yielding rosmanol (70 mg).

### 3.2.3 TPA-Induced Inflammation on Mouse Ear

The skin inflammation was induced in both ears of female CD-1 mice by topical treatment of 10µl of acetone (vehicle) or various concentrations of test compounds in acetone 10 min prior to each application of acetone or 0.4 nmol TPA in acetone. The treatment was done once a day for 4 days. The mice were sacrificed 4h after the last TPA

treatment. Ear samples from each group were pooled and homogenized.

### 3.2.4 Enzyme Immunoassay

Ear tissues were homogenized in PBS containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1mM PMSF, 0.1mM bensethonium, 10mM EDTA, and 20 U aprotinin per each mL. The homogenates were centrifuged at 12,000 × g for 60 min at 4°C. The supernatants were used to assay for the levels of Il-1β and Il-6. The capture antibody, diluted with PBS, was used to coat a 96-well plate overnight at room temperature. The plate was then washed, blocked (1% BSA, 5% sucrose in PBS with 0.05% $NaN_3$), and washed again. The standards were added to the plate leaving at least one zero concentration well and one blank well. The diluted samples (1:10–1:20) were then added to the plate. After incubating for 2h, the plates were washed and the detection antibody was added. After incubating for another 2h the plates were washed and Streptavidin-HRP was added. After 20min incubation, the plates were washed, and substrate ($H_2O_2$) and tetramethylbenzidine were added. After another 20 min incubation, the stop solution (2N of $H_2SO_2$) was added and then, plates were read with a microplate reader at a wavelength of 450 nm.

For $PGE_2$ assay the mouse ear homogenates (100µl) were acidified with 15 µl of 0.1 N HCl, vortexed for 1 min after adding 1 ml of ethyl acetate, and then centrifuged at 3,000 *g* for 5 min (Sorvall RT6000B; Thermo Scientific). The organic layer was collected and evaporated under N2. The dried sample was dissolved in 1 ml enzyme immunoassay (EIA) buffer. The levels of PGE2 were measured using an EIA kit (Cayman Chemical, Ann Arbor, MI). The well plates are pre-coated with goat polyclonal anti-mouse IgG and

blocking proteins. The standards and samples are added to the wells and incubated for an hour with tracer and antiserum. After washed with washing buffer, Ellman's Reagent is added for color development. After incubating in the dark, the plate is read by microplate reader at a wavelength of 420 nm.

### 3.2.5 Western Blot Analysis

The Western blot analyses were carried out in using biopsy samples obtained from the mice ear. Tissues were homogenized in ice-cold RIPA lysis buffer (10 mM Tris-HCL (pH 7.4), 5mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 50 mM $Na_3VO_4$, 1 % (v/v) leupeptin, 1 % (v/v) phenylmethylsulfonyl fluoride, and 0.1 % (v/v) aprotinin) using a Dounce homogenizer (Weaton, Millville, NJ, USA). The homogenates were chilled on ice under vigorous shaking for 15 min and then centrifuged at 14,000 × g for 60 min at 4°C. The supernatant was aliquoted and stored at -70°C. The protein concentration was determined using the protein assay kit according to the manufacturer's instructions (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Equal amounts of lysates (20 μg) were separated on a 10% SDS-PAGE mini gel, and then transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, Bedford, MA, USA) that was blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) containing 0.1% (v/v) Tween 20 for 1 hour. Membranes were incubated overnight at 4 °C with an anti-COX-2 (1:500), anti-phosphorylated P65 (1:500), anti-P65 (1:1000), anti-IKKα (1:500), and anti-IKKβ (1:500) antibody, and then washed before incubation with species-appropriate fluorescently conjugated secondary antibodies for 1 h at room temperature. Membranes were analyzed

using an Odyssey Infrared Imaging System (LI-COR, Millennium Science, Surrey Hills, Australia) and where relevant signal intensity determined using LI-COR imaging software and exported to Microsoft Excel for graphical representation as mean ± S.E.

### 3.2.6 Histopathological and Immunohistochemical Studies

Ear samples from each group were harvested at autopsy and fixed in 10% formalin for 24 h at room temperature. They were dehydrated by graded ethanol, sectioned, and embedded in paraffin. For histological evaluation, tissue sections (5μm) were deparaffinized with zylene and stained with hematoxylin and eosin. All of the individual ears were evaluated histopathologicaly in three H&E-stained sections per each ear treated with/without TPA and samples. In three H&E stained sections of the ear, the inflammation index was determined. The inflammation index was evaluated with modification of previous studies (Suzuki, Kohno et al. 2004; Ju, Hao et al. 2009). It was the summation of scores of four individual inflammatory parameters: degree of infiltrated cell, ulceration, thickness of ear, and hyperplasia of keratinocytes layer. The degree of infiltrated cell, which accounts for 0, 1-25, 26-50, 51-75, or 76-100% of the area examined, was scored as 0, 1, 2, 3, or 4, respectively. The ulceration was scored as 0 (absent), 1 (present). The score of ulceration was the summation of counted ulcerations. Ulceration was defined as an area of keratinocytes lining was missing. Thickness of ear which was total thickness increased in mouse ear was scored as 0 (normal), 1 (1-2 times thicker than normal), 2 (2-4 times thicker than normal), 3 (more than 4 times thicker). Hyperplasia of keratinocytes was also scored as 0 (normal), 1 (mild, 1-2 times thicker than normal), 2 (moderate, 2-4 times thicker than normal), 3 (severe, more than 4 times

thicker).

Immunohistochemistry was carried out with harvested ear from sample treated mouse model. The ear specimen was paraffin - embedded and microtomed into 5 μm-thick tissue sections. The microtomed slides were incubated overnight at room temperature with COX-2, and phosphorylated (p)-P65. The slides were incubated with biotinylated secondary antibody, and then with avidin/biotinylated peroxidase complex for 30 min at room temperature (Vector Labs, Burlingame, CA, USA) and were then incubated with DAB (3'-diaminobenzamine) substrate for exact amount of time. The sections were then counterstained with Modified Harris Hematoxylin. The images were taken randomly using a Zeiss AxioCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope.

### 3.2.7 Statistical Analysis

Data are presented as means ± SE for the indicated number of independently performed experiments. One way Student's *t*-test was used to assess the statistical significance between the TPA- and rosmanol plus TPA-treated ear tissues. The accepted level of statistical significance for the test was $P$-value $< 0.05$.

## 3.3. Results

### 3.3.1 Effect of Rosmanol and Carnosol on TPA-Induced Up-expression of Pro-inflammatory Cytokine Il-1β and Il-6 Protein Levels in Mouse Ear

To examine the effects of carnosol and rosmanol on TPA induced protein levels of Il-1β in mouse ear specimen were measured using ELISA. Both the ears of mice were

treated topically with 0.75 or 1.5 μmol of carnosol and rosmanol 10 mins prior to 0.4 nmol of TPA once a day for 4 days. The mice were sacrificed 4 hours after the last TPA treatment. As shown in **Fig. 25**, topical application of 0.75 μmol and 1.5 μmol of rosmanol inhibited TPA-induced expression of Il-1β in ear by 58, 60%, respectively. In addition, topical application of 0.75 μmol and 1.5 μmol of carnosol before treatment of TPA also greatly reduced expression of Il-6 in TPA-induced mouse ear by 46, 84%, respectively **(Fig. 26)**. Especially, for Il-6 there is a problem in acetone treated negative control group, this group should have lowest production level of Il-6. However, the Il-6 kit detected relatively higher level. So, I used non-treat ear for comparison in this graph and checked this group with the western blotting. In blotting there was no significant band on Il-6 reign around 21-28 kDa (data not shown). This result indicates that both carnosol and rosmanol reduced the expression of Il-1β and Il-6 in TPA induced mouse ear. In low dose the effect of rosmanol is stronger, and in high dose carnosol has stronger effect.

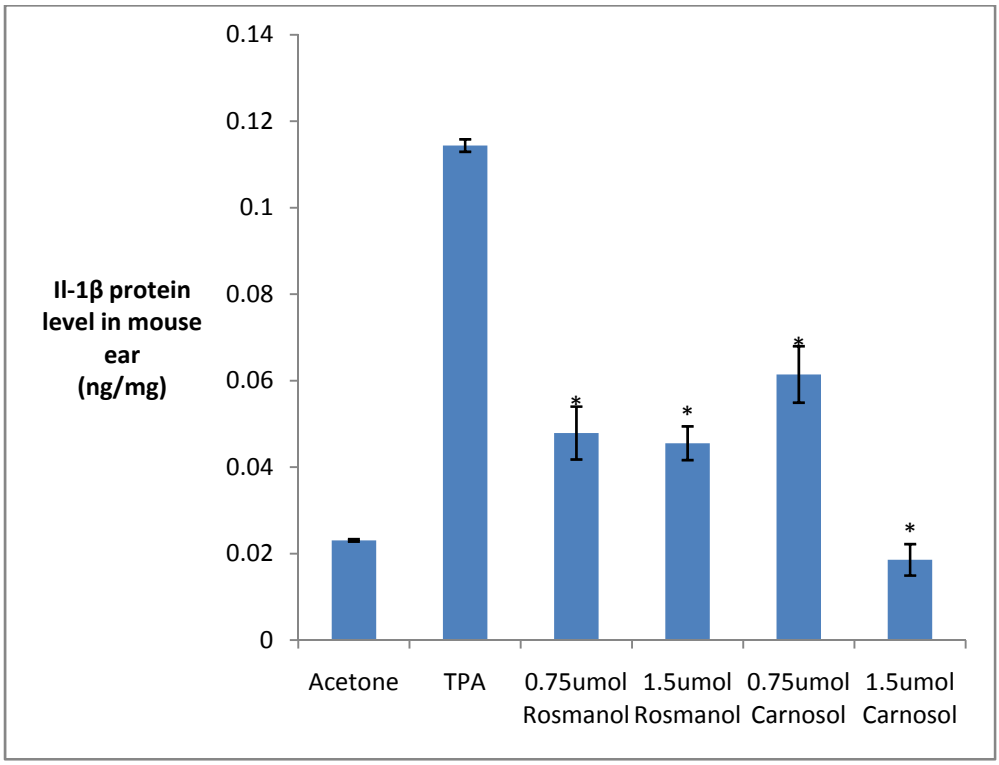



Fig. 25. Effects of rosmanol and carnosol on TPA induced up-expression of Il-1β protein level in mouse ear, Mouse ears were pretreated with 0.75 and 1.5 µmol of rosmanol and carnosol 10 mins before 0.4nmol of TPA topical treatment once a day for four days. The values were expressed as means ± SE of triplicate tests. * indicate statistically significant differences from the TPA-treated group ($P < 0.05$)

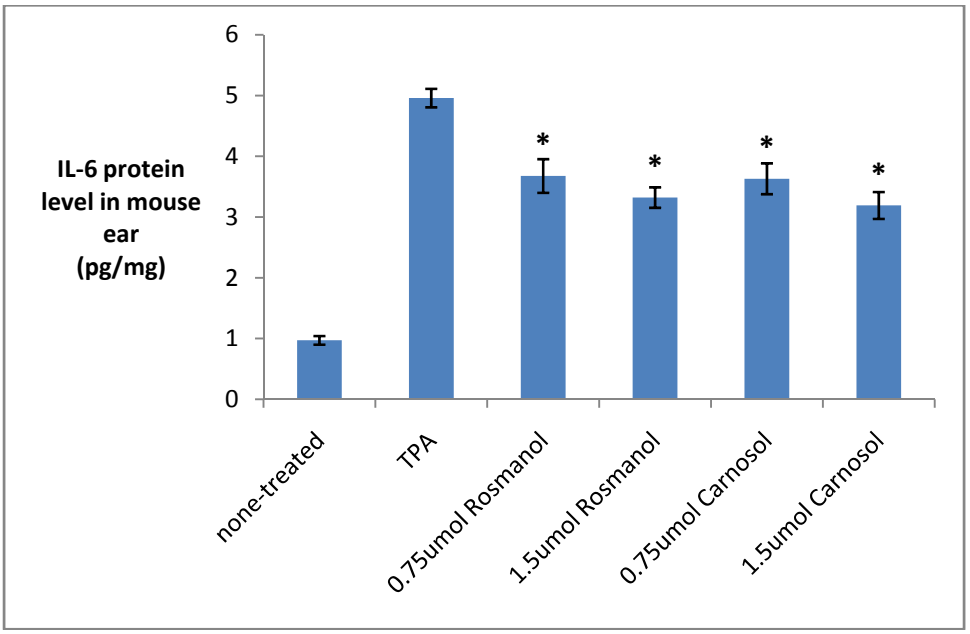



Fig. 26. Effects of rosmanol and carnosol on TPA induced up-expression of Il-6 protein level in mouse ear, Mouse ears were pretreated with 0.75 and 1.5 µmol of rosmanol and carnosol 10 mins before 0.4 nmol of TPA topical treatment once a day for four days. The values were expressed as means ± SE of triplicate tests. * indicate statistically significant differences from the TPA-treated group ($P < 0.05$)

### 3.3.2 Inhibitory Effect of Rosmanol and Carnosol on TPA-Induced Formation of $PGE_2$ in Mouse Ear

$PGE_2$ is known to regulate blood flow and change leakage permeability of blood vessel at early stage of inflammation. COX-2 catalyzes the conversion of arachidonic acid to prostaglandin and related eicosanoids. The level of $PGE_2$ in TPA induced mouse ear was measured by ELISA. The COX-2 expression was determined by western blot analysis. In **fig. 27** the level of $PGE_2$ was decreased in 0.75 and 1.5 µmol of rosmanol treated group by 28% and 33% compared to TPA treated control. 0.75µmol and 1.5 µmol of carnosol also reduced the production of $PGE_2$ by 8% and 23%, respectably. **Fig. 28** showed that the expression of COX-2 protein in rosmanol treated group was decreased significantly in dose-dependants manner. Compared to TPA treated group carnosol also reduced the expression of COX-2. This western blotting data of rosmanol and carnosol was correlated with inhibition effect of them on $PGE_2$ production. In addition, to determine localization of COX-2 in mouse ear, we did immunohistochemical analysis on mouse ear sections using the anti-COX-2 antibody **(Fig. 29)**. In immunohistochemical studies inflammatory cells which released COX-2 were stained in brown color. In TPA induced group large amount of brown stained cells were presented and large amount of infiltrated immune cells which is stained by Hematoxylin and presented as blue were observed. In rosmanol and carnosol treated group not only brown stained cells releasing COX-2, but also blue stained immune cells not releasing COX-2 were decreased dramatically.

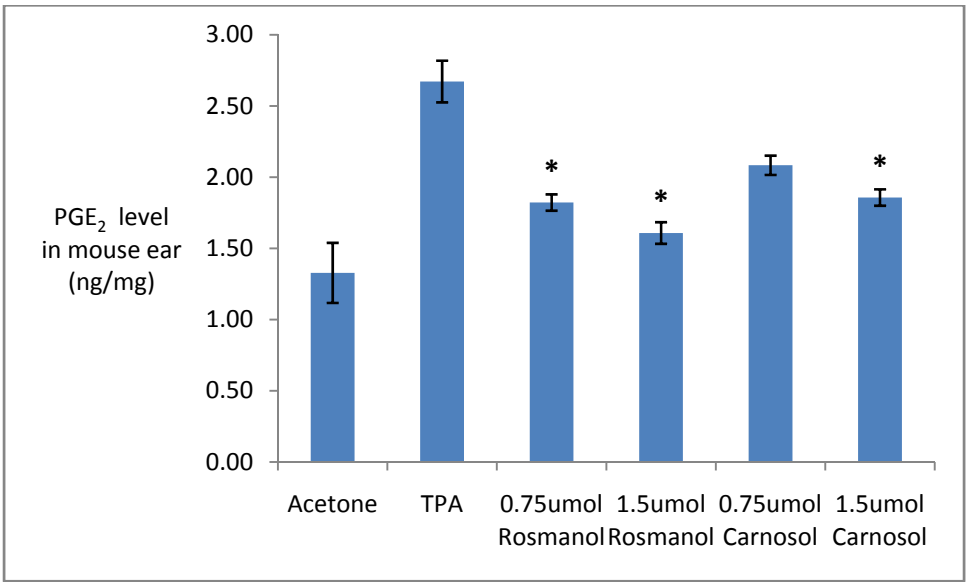



Fig. 27. Effect of rosmanol and carnosol on TPA-induced $PGE_2$ production in mouse ear, Samples were pretreated with 0.75 and 1.5 μmol of rosmanol and carnosol 10 mins before 0.4 nmol of TPA topical treatment on mouse ear. The values were expressed as means ± SE of triplicate tests. * indicate statistically significant differences from the TPA-treated group ($P < 0.05$)

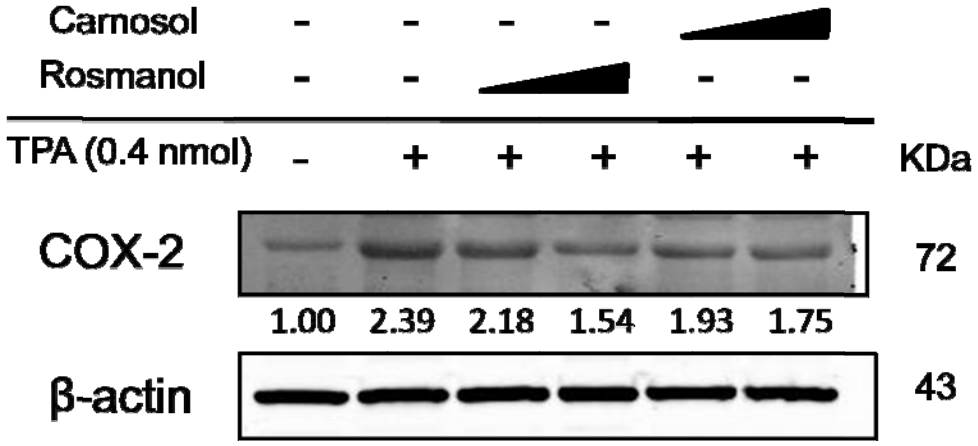



Fig. 28. The expression of COX-2 protein levels in tissue lysates analyzed by Western blotting analysis, Samples were pretreated with 0.75 and 1.5 μmol of rosmanol and carnosol 10 mins before 0.4nmol of TPA topical treatment on mouse ear. β-actin was used as a loading control.

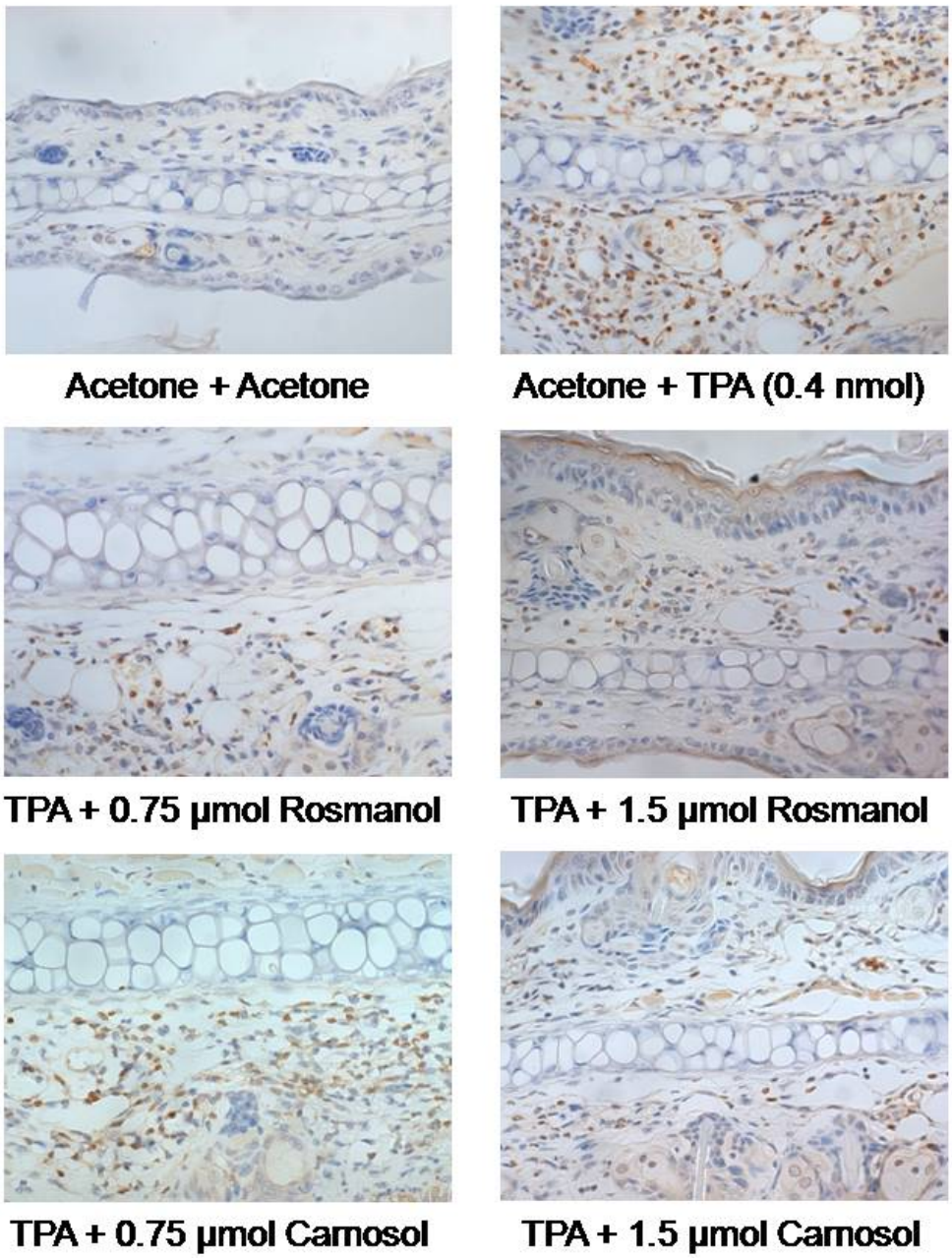



Fig. 29. Immunostained setions of mouse ear treated with rosmanol and carnosol, A section of mouse ear tissue was immunostained with anti-COX-2 antibody and counterstained with hematoxylin. Immunostained COX-2-expressed cells were presented as brown color. Magnification was X400.

### 3.3.3 Inhibitory Effect of Rosmanol and Carnosol on TPA-Induced Persistent Inflammation in Mouse Ear

In H&E stained histology of mouse ear, severe vasodilation, ulceration of keratinocytes, and infiltration of inflammatory cells was detected in TPA treated group **(Fig. 30)**. The rosmanol treatment resulted in lower inflammation index dose-dependently in TPA induced mouse ear **(Tab. 1)**. Among individual inflammatory parameters, the decrease in the degree of ulceration was most prominent ($p < 0.05$). However, not only ulceration, but also thickness of tissue, hyperplasia of keratinocytes, and infiltration of cells were decreased in both rosmanol and carnosol treated group. Assuming based on inflammation index, rosmanol seemed to posses stronger anti-inflammatory effects. Among each treatment, 1.5 μmol rosmanol showed significant decrease in all parameters. Especially, inflammation index of this group was 19% of control. No ulceration was observed and ear tissue thickness was barely changed. In addition, dose-dependent prevention of these histological indicators of persistent inflammation was shown in both rosmanol and carnosol treated group.

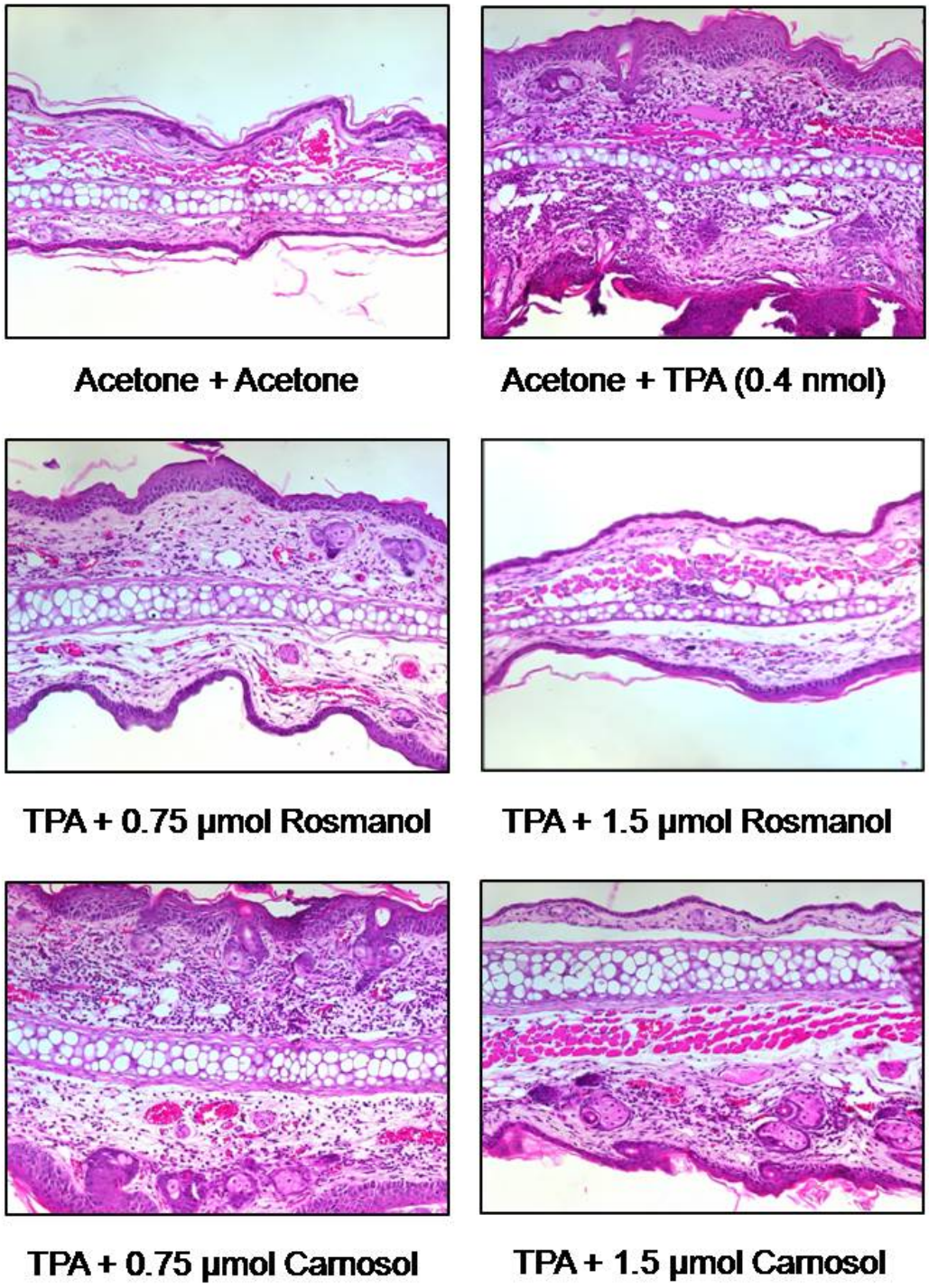



Fig. 30. Histology of TPA-induced inflammtion and hyperplasia in mouse ear, Samples were pretreated with 0.75 and 1.5 µmol of rosmanol and carnosol 10 mins before 0.4 nmol of TPA topical treatment on mouse ear. Sectioned ear tissues were stained with hematoxylin and eosin. Magnification was X200

| Group | Tickness of Tissue | Hyperplasia of Keratinocytes | Infiltration of Cells | Ulceration | Inflammation Index |
|---|---|---|---|---|---|
| Acetone | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| TPA | 3.00 ± 0.00 | 3.00 ± 0.00 | 3.67 ± 0.25 | 4.67 ± 0.48 | 14.33 ± 0.41 |
| 0.75µmol Rosmanol | 1.33 ± 0.00 | 1.67 ± 0.25 | 2.00 ± 0.33 | 1.33 ± 0.25 | 6.33 ± 0.25 |
| 1.5µmol Rosmanol | 0.67 ± 0.25 | 0.67 ± 0.25 | 1.00 ± 0.00 | 0.00 ± 0.00 | 2.33 ± 0.25 |
| 0.75µmol Carnosol | 2.67 ± 0.25 | 2.00 ± 0.00 | 2.67 ± 0.25 | 2.33 ± 0.25 | 9.67 ± 0.25 |
| 1.5µmol Carnosol | 2.00 ± 0.00 | 1.00 ± 0.00 | 1.67 ± 0.25 | 0.33 ± 0.25 | 5.00 ± 0.33 |

Tab. 1. Effects of rosmanol and carnosol on TPA induced persistenat inflammation in mouse ear, Effect of rosmanol and carnosol on TPA induced persistent inflammation in mouse ear. Samples were pretreated with 0.75 and 1.5 µmol of rosmanol and carnosol 10 mins before 0.4 nmol of TPA topical treatment on mouse ear. All the analyzed tissues were sectioned and H&E stained, and three sections per ear tissue were analyzed. The inflammation index was the sum of the individual scores for degree of infiltrated cells, thickness of ear tissue, ulceration, and hyperplasia of keratinocytes. * indicate statistically significant differences from the TPA-treated group (by student's t-test, $P < 0.05$)

### 3.3.4 Rosmanol and Carnosol Inhibit the Phosphorylation of NF-κB-p65 through Inhibiting IKKβ

When NF-κB is inactive and bound to IκB, it is present in cell cytosol. Bacterial or viral infections, as well as pro-inflammatory cytokines such as TNF, Il-1, and Il-6, all of which activates the IKK complex. The activated IKK, especially IKK-β, phosphorylates NF-κB-bound IκB, resulting phosphorylation and subsequent proteasomal degradation of this inhibitory subunit and liberating NF-κB. Released NF-κB enters the nucleus and mediates transcription of target gene (Karin and Greten 2005). However, in cytoplasm there are still active NF-κBs which are phosphorylated and released from IκB, and still not translocated into the nucleus (Charalambous, Lightfoot et al. 2009). If we stained tissue slides with anti-p65 antibody, it is very hard to distinguish which part among the cytosol and nucleus was stained. To avoid this confusion we stained ear tissue with anti-phosphorylated NF-κB-p65 protein. Western blotting was also done with same anti-body to skip fractionation. To elucidate up-stream pathway we determined the expression of IKK β. In **Fig. 31**, brown stained phosphrylated-p65-expressed cells were decreased in carnosol and rosmanol treated group compared to TPA treated group. Moreover, the degree of staining of each cell in sample treated group was also decreased. In TPA treated group most of cells were stained, meanwhile some cells whose NF-κB are not phosphrylated still have blue color. In western blotting analysis (**Fig. 32**) the expression of IKK-β and phosphorylated NF-κB-p65 protein was significantly decreased in 1.5μmol of rosmanol treated group. In addition, rosmanol seemed to have stronger inhibition activity of IKK-β, resulting reduction of the phosphorylated NF-κB.

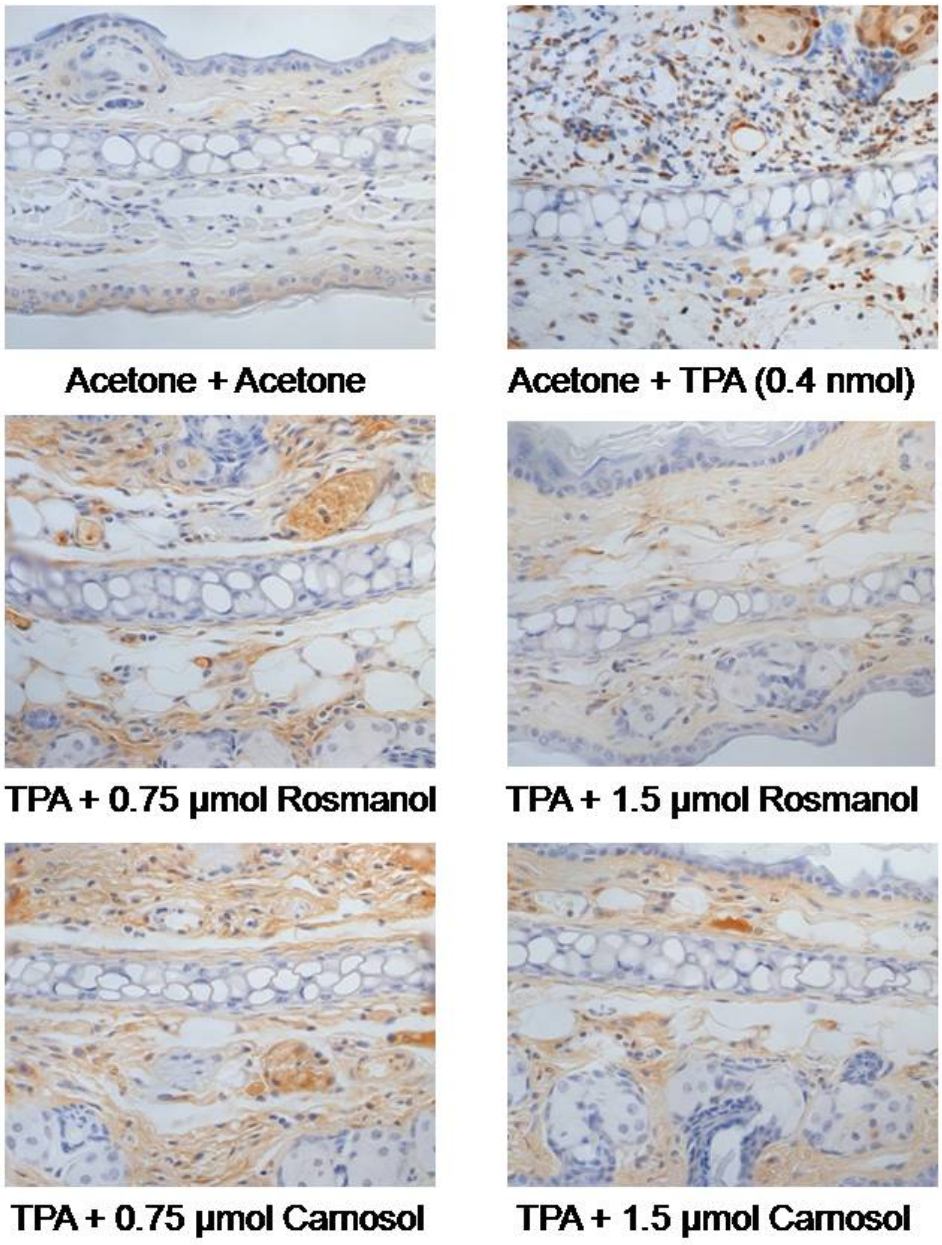



Fig. 31. Immunohistochemistry on rosmanol and carnosol treated ear tissue section against anti-phosphorylated p65-NF-κB, Samples were pretreated with 0.75 and 1.5 µmol of rosmanol and carnosol 10 mins before 0.4nmol of TPA topical treatment on mouse ear. Immunostained COX-2-expressed cells were presented as brown color. Magnification was X400

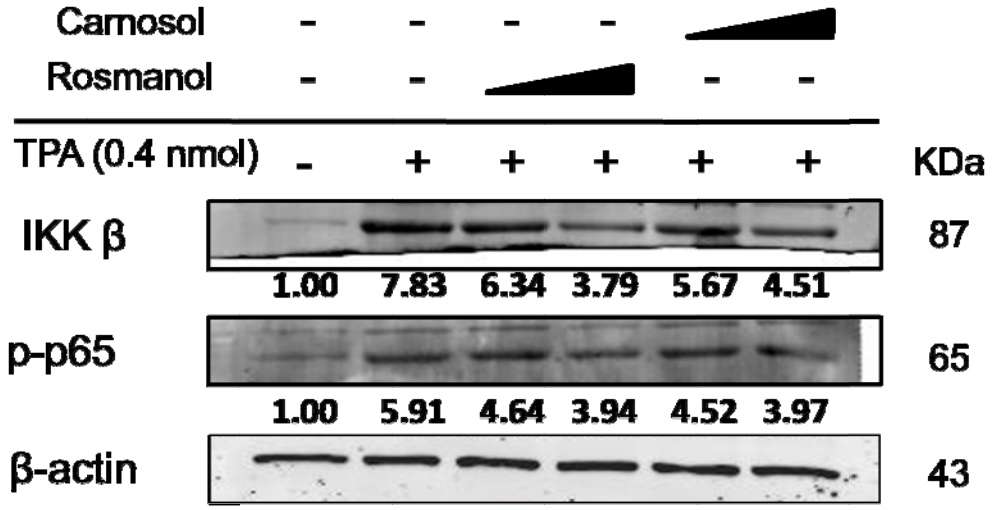



Fig. 32. Western Blotting anaysis for inhibition activity on phosphorylation of p65-NF-κB and expression IKKβ, Samples were pretreated with 0.75 and 1.5 μmol of rosmanol and carnosol 10 mins before 0.4 nmol of TPA topical treatment on mouse ear

### 3.4. Discussion

Polyphenolic antioxidants present in a variety of plants and vegetables, and their biological effects in anti-inflammation and cancer prevention have been investigated over many decades (Surh 2003). One of frequently used food ingredient, rosemary has been studied the antioxidants, anti-inflammation, and anti-cancer benefits of its extract and several potential compounds (Singletary, MacDonald et al. 1996; Lo, Liang et al. 2002; Lai, Lee et al. 2009). However, the potential benefits of rosmanol and carnosol, active compound in rosemary, on in vivo anti-inflammatory model have not been studied.

In the present study, we showed that topical treatment of rosmanol and carnosol inhibits TPA-induced inflammation in mouse ear. We also found that this anti-inflammatory activity of rosmanol and carnosol on TPA-induced inflammation resulted from inhibiting expression of IKK-β using Western blotting analysis against anti-phosphorylated p65 antibody and anti-IKK-β antibody. To our knowledge, this is the first report about the inhibitory activity of rosmanol and carnosol on TPA-induced persistent inflammation in mouse ear.

TPA-induced mouse ear model we used in this study was modified from Huang's model (Huang, Liu et al. 2006). Both Il-1β and Il-6 which are released by granulocytes, mainly neutrophils, participate in early stage of inflammation and play a role as cytokine for cell recruitment, activation, and response. Monocytes and macrophage attracted by these cytokines to the site of injury are activated and become major source of these cytokines (Coussens and Werb 2002). Rosmanol and carnosol reduced the level of Il-1β and Il-6 in dose-dependent manner (**Fig. 25,26**). However, in Il-6 assay, the negative control group which is treated by acetone showed relatively large amount of Il-6

production. To check this phenomenon I applied cell lysates to western blotting against anti-Il-6 antibody. Intereukin families and THF are very difficult to detect in western blotting because of their low weight and small size. So, even though not clear, the blotting showed very low level of Il-6 was detected. Other group in Laboratory of Cancer Research has same problem with Il-6. That group changed the manufacturer of Il-6 detection kit and then they got good result correlated to Il-1β and other inflammatory markers.

For COX-2 and iNOS expression, Chun et al showed that NOS inhibitor aminoguanidine (AG) inhibited the expression of COX-2 protein at the pharmacologically effective dose in TPA induced dermal inflammation model. They concluded that up-regulation of COX-2 by NO may be mediated by activation of NF-κB in mouse skin (Chun, Cha et al. 2004). This critical study on TPA induced dermal inflammation in mouse suggests iNOS may play an important role in expression of COX-2. However, in western blotting analysis against anti-iNOS antibody there was no expressed iNOS. At 75 kDa there detected unknown protein reacted with anti-iNOS antibody, but it was not iNOS because its molecular weight is 130 kDa. We cannot state that there is no iNOS expressed, because the expression of iNOS is transient. We sacrificed mouse 5 hours after the last TPA treatment. During that period expression of iNOS might be already stopped and the expressed iNOS might be degraded. To induce the expression of COX-2 by NO iNOS should be expressed earlier than the expression of COX-2, which supports this assumption. Besides the iNOS expression the decreased level of $PGE_2$ is consequence of the decreased level of COX-2 expression. Pro-inflammatory eicosanoids, $PGE_2$ is implicated in both inflammation and carcinogenesis.

We found that the level of $PGE_2$ in rosmanol - pretreated group was deceased by 28% and 33% from the level of mouse treated with only TPA in dose-dependent manner. Carnosol-pretreated group also showed reducing activity on level of $PGE_2$ dose-dependently (**Fig. 27**). Inhibition activity of rosmanol on COX-2 expression was closely correlated with decreased level of $PGE_2$ (**Fig. 28**).

In H&E staining analysis of ear tissue, we characterized and scored the TPA induced-inflammation of the mouse ear, using the previous description of typical criteria. We determined and choose critical inflammatory parameters considering well-known clinical sign of inflammation. They were ear thickness, degree of infiltrated cells, ulceration, and hyperplasia of keratinocytes.

Lai et al reported anti-inflammatory effects of rosmanol on LPS-induced expression of COX-2 and iNOS in RAW264.7. Among various biomarkers they examined, inhibition of translocation and phosphorylation of NF-κB is the most prominent. Based on their conclusion, rosmanol downregulates inflammatory iNOS and COX-2 gene expression by inhibiting the activation of NF-κB (Lai, Lee et al. 2009), we assumed that inhibition effects of rosmanol and carnosol we investigated were due to inhibiting NF-κB through reducing IKK-β expression. To see the translocation of p65-NF-κB, we have to fractionize the tissue lysates into nucleus and cytosolic extracts. Concept of fractionization is using two different strength of lysis buffer. First, with hypotonic lysis buffer we break the cell membrane, and then nucleus membrane is broken with hypertonic buffer. However, because tissue sample contains various types of cells, degree of lysis will be different depending on cell type. In addition, when p65- NF-κB immunostained slides was observed, cytosolic region of cell may be stained, because not

all activated NF-κBs were not translocated (Charalambous, Lightfoot et al. 2009). To overcome this defect of fractionization, we used anti-phosphorylated p-65 NF-κB protein. Wherever it located, the phosphrylated p65-NF-κB is activated form and ready for transcript. Using immunostaining with anti-phosphorylated p-65 NF-κB protein, we observed phosphrylation of p-65 NF-κB in inflammatory cells in ear tissue. Treatment of rosmanol and carnosol reduced not only number of stained cells, but also degree of staining (**Fig. 31**). Western blotting of IKK-β expression showed that rosmanol reduced the expression of this protein. And the decreases of the expression of phosphorylated p-65 NF-κB is correlated with IKK-β data (**Fig. 32**).

### 3.5. Conclusions

In this study we observed the anti-inflammatory effects of rosmanol and carnosol on TPA-induced iNOS and COX-2 expression in mouse ear. Rosmanol and carnosol reduced production of pro-inflammatory cytokine, Il-1β and Il-6. The level of was reduced in rosmanol and carnosol treated group. The expression of COX-2, the enzyme producing $PGE_2$, was also decreased. This inhibition activity was closely related to inhibiting signal pathways of NF-κB, the major transcription factor responsible for the COX2 expression through reducing the expression of IKK-β. Mostly in overall assay for this study, rosmanol showed stronger activaties than carnosol, whcich seems due to its structure. The polarity of rosmanol was higher than carnosol, and this higher polarity seems to facilitate translocation of cell membrane or play an important role as stronger affinity to interacting molecules.

Based on this study, we suggest rosmanol and carnosol should have great

potential as a novel chemopreventive agent for various inflammatory diseases.

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# Curriculum Vita

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### Educational Backgrounds

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### Publications

1. Kim, H., Lee, J. H., & Suh, H. (2003). Interaction of mesenchymal stem cells and osteoblasts for in vitro osteogenesis. Yonsei Medical Journal, 44(2), 187-197.
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