The Role of Nitric Oxide Synthase and Carnosol in UVB-induced NF-κB Activity and Skin Damage

A dissertation presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

Lingying Tong

December 2014

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This dissertation titled
The Role of Nitric Oxide Synthase and Carnosol in UVB-induced NF-κB Activity and Skin Damage

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ABSTRACT

TONG LINGYING, Ph.D., December 2014, Molecular and Cellular Biology

The Role of Nitric Oxide Synthase and Carnosol in UVB-induced NF-κB Activity and Skin Damage

Director of Dissertation: Shiyong Wu

NF-κB is a transcription factor involved in many signaling pathways, which also plays an important role in UV-induced skin tumorigenesis. UV radiation can activate NF-κB, but the detailed mechanism remains unclear. In this work, evidence is provided to demonstrate that the activation of constitutive nitric oxide synthase(s) (cNOS) plays a role in regulation of IκB reduction and NF-κB activation in human keratinocyte HaCaT cells in early phase (within 6 h) post-UVB. Treating the cells with L-NAME, a selective inhibitor of cNOS, can partially reverse the IκB reduction and inhibit the DNA-binding activity as well as nuclear translocation of NF-κB after UVB radiation. The luciferase reporter assay indicates that UVB-induced NF-κB activation is totally diminished in cNOS null cells. Our data also showed that NF-κB activity is required for maintaining a stable IKKα level since treating the cells with NF-κB or cNOS inhibitors can reduce IKKα level upon UVB radiation. In addition, while NF-κB protects cells from UVB induced death, its pro-survival activity is likely to be neutralized by the pro-death activity of peroxynitrite induced by UVB radiation.

In addition to L-NAME, the effect of a natural compound carnosol is also studied on its protection role in UVB-induced skin carcinogenesis. Carnosol is a natural antioxidant compound extracted from rosemary and sage, which are commonly used in
traditional Mediterranean cuisine. Because of its structure similarity to sex hormones, research has been done on the effect of carnosol mainly on breast and prostate cancers. However, very little is known on the effect of carnosol on UV-induced skin cancer. In this work, the effect of carnosol on UV-induced skin cancer formation and progression is studied. Evidence is provided to reveal that carnosol can partially reduce UVB-induced reactive oxygen species (ROS) elevation and thus protects UV-induced DNA damage in normal skin cells. The data also indicate carnosol can inhibit the UVB-induced NF-κB activation. As a result, the UV-induced normal cell apoptosis can be partially protected. Moreover, it can also prohibit skin cancer cell progression with or without UV radiation. Therefore, there is a reason to believe that carnosol can be a potential therapeutic for chemoprevention and treatment of UVB-induced skin cancers.
This work is dedicated to my dearest father, Jianqiang Tong, who passed away of Amyotrophic Lateral Sclerosis (Lou Gehrig’s disease) in 2013 when I was only a step away from pursuing my Ph.D. degree.
ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Dr. Shiyong Wu, for his unlimited support and continuous guidance for my research during my whole Ph.D. study period. He has always inspired me to dream ambitiously, think creatively and do energetically for the past five years. I have learned dedication, passion, and intelligence to scientific work from him. I am so blessed to have his accompany through all my laughers and tears at every step along the steep academic road.

I also feel heartily indebted to my committee members: Dr. Xiaozhuo Chen, Dr. Robert Colvin, and Dr. Michael Held. It is my great fortune to have their fully support, great enthusiasm, pleasant cooperation and serving on my committee for the past five years. Dr. Chen helped me a great deal when I first arrived the States and assisted me with smooth transition from undergraduate study at CUHK to graduate study at OU. He also gave me a lot of valuable advise and suggestion on my experimental designs, as well as presentation skills and techniques. Dr. Colvin helped me with everything in MCB program, from course curriculum to seminars. Dr. Held spent a lot of time surveying my research proposal and dissertation, and I also enjoyed the pleasant and unforgettable time when I was a TA at his course.

I am particularly grateful to all my former and present colleagues: Dr. Shinhee Lee, Dr. Kimberly Suzanne Parsons, Dr. Lei Wang, Dr. Wei Lu, Dr. Wei Liu, Dr. Luke Carpenter, Dr. Qiong Wu, Dr. Huiwen Cheng, Rachel Morris, Ye Yuan, Huizhi Zhao, Guangyu Fan and Nick Moses, for their great support, kindly cooperation, and lifelong
friendship. Their assistance in my experiments, and companionship in my everyday life made my life in lab so colorful, meaningful and memorable forever.

I would also like to take this opportunity to acknowledge the support from Department of Chemistry and Biochemistry, Molecular and Cellular Biology program, Edison Biotechnology Institute for providing me financial support and wonderful research environment. I would like to specifically acknowledge the warm support from Ms. Eileen Schulz, Ms. Marlene Jenkins, and Ms. Lori Abdella during my graduate study.

Finally, I want to express my deepest appreciation to my parents, Jianqiang Tong and Ping Wang, and my husband Dr. Dan Wang for their unconditional love. They have been taking care of my life in every aspect, and have always been there for me whenever I need. Without their extraordinary patience and great support, the entire work would never been possible.

THANK YOU ALL!
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LIST OF ABBREVIATIONS

ANX5       Annexin V  
ATF        Activating Transcription Factor  
β-TrCP     β-tranducin repeats containing protein  
CHOP       CCAAT-enhancer-binding protein Homologous Protein  
CK2        Casein Kinase 2  
CPD        Cyclobutane Pyrimidine Dimer  
eNOS       Constitutive Nitric Oxide Synthase  
DAPI       4’6-diamidino-2-phenylindole  
eIF2α       Eukaryotic Initiation Factor 2-Alpha  
eIF2AK      eIF2α Kinase/s  
ELISA      Enzyme-linked Immunosorbent Assay  
EMSA       Electrophoretic Mobility Shift Assay  
ER         Endoplasmic Reticulum  
FITC       Fluorescein isothiocyanate  
GCN2       General Control Non-depressible Protein Kinase 2  
HaCaT      Human Keratinocytes  
HRI        Heme-regulated inhibitor kinase  
IFNγ       Interferon Gamma  
IkB        Inhibitor of Nuclear Factor Kappa B  
IKK        IkB Kinase  
IRE1       Inositol-requiring Enzyme-1
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<tr>
<td>L-NAME</td>
<td>L-N\textsuperscript{G} -Nitro-arginine Methyl Ester</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<td>MSK</td>
<td>Mitogen and Stress Activated Protein Kinases</td>
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<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<td>NOS</td>
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<td>NOX</td>
<td>NADPH Oxidase</td>
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<td>NT-HBD</td>
<td>N-terminal Hemin-Binding Domain</td>
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<tr>
<td>O\textsubscript{2}⁻</td>
<td>Superoxide</td>
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<td>ONOO⁻</td>
<td>Peroxynitrite</td>
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<td>PDI</td>
<td>Protein Disulfide Isomerase</td>
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<td>PERK</td>
<td>dsRNA Dependent Protein Kinase-like ER Kinase</td>
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<td>PI</td>
<td>Propidium Iodide</td>
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<td>PKR</td>
<td>dsRNA Dependent Protein Kinase</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>SERCA</td>
<td>Sarcoplasmic/ER Ca\textsuperscript{2+}-ATPases</td>
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<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
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<td>TLR</td>
<td>Toll-like Receptor</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>ZF</td>
<td>Zinc Finger</td>
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CHAPTER 1: INTRODUCTION

Objective: The goal of this study is to determine the role of free radicals and/or nitric oxide synthase in regulating UVB-induced NF-κB activity and its physiological effects on skin keratinocyte cells or on skin cancer cells.

Ultraviolet (UV) light is electromagnet radiation with a wavelength in between the range of visible light and X-rays. It is strongly emitted in sunlight, which we are exposed to everyday. There are three types of UV radiation separately according to their wavelength: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). On earth, we received about 90% of UVA radiation, and about 10% UVB radiation, while UVC is completely absorbed by the ozone layer and atmosphere. Besides many beneficial effect of UV light, such as vitamin D production, over exposure to sunshine could also lead to severe skin problems such as skin aging, skin cancer, immune suppression, etc.

According to the National Toxicology Program Report on carcinogens, UV is considered to be a carcinogen, which contributes to most of the estimated two million skin cancer development each year in the States. UV-damaged skin has an increased chance of developing various forms of skin cancers, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and cutaneous malignant melanoma (1,2). Among these cancers, melanoma is lethal and so far has limited treatment methods, while BCC and SCC are less dangerous but could be hard to treat if metastasis starts (3).

One of the key factors that plays a critical role in UV-induced carcinogenesis is NF-κB activation, which is involved in the regulation of cell transformation, apoptosis, migration, adhesion, etc. NF-κB is a transcript factor firstly identified in mature B cells
in 1986. It was so named as it was found in the nucleus bound to the intronic enhancer region of the κ light chain gene in the B cells (4,5). NF-κB is a transcription factor which regulates the expression of many genes mostly related to immune and inflammatory response, along with the genes determining developmental processes, cellular growth and apoptosis (6).

The mammalian NF-κB family is composed of five members: p65 (RelA), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (7). All five family members have a Rel homology domain (RHD) which is responsible for DNA binding and dimerization between different or identical family members, leading to homomeric or heteromeric binding of the subunits. RelA, RelB, c-Rel share a transcriptional activation domain, while p50 and p52 do not (8,9). Thus, of all the possible NF-κB dimers, some function as transcriptional activators (e.g. RelA/p50 heterodimer), but others (e.g. p50/p50 homodimer) do not unless they recruit specific co-activator proteins, and some dimers do not bind DNA at all (10).

As a transcription factor involved in many signaling pathways, the activity of NF-κB is precisely regulated by both canonical and atypical pathways. For the canonical pathway, the inhibitor of NF-κB kinase (IKK) is activated and phosphorylates inhibitor of NF-κB (IκB) at N-terminal serines (Ser32 and Ser36) (11,12). The phosphorylated IκB is then ubiquitin-targeted and rapidly degraded through the polyubiquitin-dependent proteasomal pathway, and then the freed NF-κB translocates into the nucleus with its exposed nuclear localization signal peptide and activates its target genes (13,14). Therefore, ubiquitination and proteasomal degradation pathways are often critical in
regulating NF-κB activity. On the other hand, some stimuli such as UV radiation, regulates NF-κB through a more complicated pathway by regulating IκB translational levels (15,16). Other atypical NK-κB activation pathways include the IκB-independent activation of p50 and p52. Briefly, upon stimulation, both p105 and p100 are proteasomal targeted and by partial proteolysis, they are processed to p50 and p52, which then translocate to nucleus to be activated (17).

The regulation of NF-κB expression and activation is tightly controlled in normal cells. One of the well-known regulatory mechanisms of NF-κB is it can bind to the promoter region and up-regulate its inhibitor IκB. By doing this, the activated NF-κB can increase IκB level and in turn shut down NF-κB itself. In this way, the NF-κB activation in normal cells is transient and naturally regulated. In many cancer cells, NF-κB has a constitutively high level of activity (18,19). This high level of NF-κB activity is suggested to be correlated with cancer development and progression (20,21). In most cases, the activation of NF-κB leads to anti-apoptosis signaling pathways, stimulating cell proliferation, invasion, metastasis, and angiogenesis in cancer cells. Interestingly, the activation of NF-κB is not usually due to mutagenesis of NF-κB or IκB themselves, but due to deregulation of various signaling pathways, including but not limited to pathways that respond to virus infection, receptor activation, and constitutive activation of kinases (20) (22). Therefore, targeting NF-κB as well as its related signaling pathways has always been considered as a potential therapeutic target for cancer treatments.

Most previous studies on UV-induced NF-κB were using UVC light, and the detailed mechanism of UVC-induced NF-κB activation is discussed in Chapter 2. Since
UVC is completely absorbed by the ozone layer and atmosphere, UVB can reach the earth and therefore is considered to be a more physiological light compared to UVC. Thus, in this study, the UVB radiation was used instead of UVC.

In addition to NF-κB, previous research also indicated that free radicals might also play important roles in carcinogenesis, although the detailed mechanism is still unsolved. UV is known to be one of the stimuli to facilitate the production of intracellular free radicals, and the previous research also demonstrated that these intracellular free radicals could regulate the phosphorylation of eukaryotic initiation factor 2 (eIF2) at the alpha subunit. There are four kinases known to be able to phosphorylate eIF2α: (1) the heme-regulated inhibitor kinase (HRI, EIF2AK1); (2) the double-strand RNA dependent protein kinase (PKR, EIF2AK2); (3) double-strand RNA dependent protein kinase-like ER kinase (PERK, EIF2AK3); and (4) general control non-depressible protein kinase 2 (GCN2, EIF2AK4). The details of how free radicals, especially nitric oxide (NO), regulate these four kinases are discussed in Chapter 3. Based on the previous study, it is known that UVB can activate NF-κB, it is also known that UVB can induce intracellular free radical level. Therefore, in this study, if and how free radicals regulate NF-κB activation upon UVB radiation, and its physiological effects are investigated. Moreover, the signaling pathways and physiological effect of carnosol, an anti-oxidant natural compound, upon UVB radiation has also been studied.
CHAPTER 2 : BACKGROUND

Introduction

The nuclear factor-kappa B (NF-κB) plays important roles in the regulation the expression of genes that are mostly related to the immune and inflammatory response, along with genes critical to developmental processes, cellular growth, and apoptosis (6,18,23,24). The canonical NF-κB activation pathway is that upon stimulation, such as with tumor necrosis factor α (TNFα), the inhibitor of NF-κB (IκB) kinase (IKK) is activated and phosphorylates IκBα at N-terminal serines (Ser32 and Ser36) (11,12). The phosphorylation leads to the dissociation of IκBα from NF-κB. While the phosphorylated IκB is ubiquitin targeted and rapidly degraded through the polyubiquitin-independent proteasomal pathway, the freed NF-κB translocates into the nucleus with its exposed nuclear localization signal (NLS) peptide and activates its target genes (13,14,25-29). UV is known to activate NF-κB, though the detailed mechanism is still under elucidation (30,31). Among the three wavelengths, UVA, B and C, UVC is the most extensively studied in the previous research and best known for its induction of NF-κB activation (30). UVC-induced activation of NF-κB does not always follow the canonical pathway (31,32). Compared to other stimuli such as TNFα, UVC activates NF-κB in a delayed and prolonged manner with differential regulatory mechanisms for early- (within 12 h) or late-phase (within 16-24 h) post-UVC (31-33).
Early-phase Activation of NF-κB after UVC

IKK activation or N-terminal serine phosphorylation of IκBα is not increased during the early-phase activation of NF-κB after UVC-irradiation (32). However, while IKK activation is not detected above the basal level post-UVC, IKK activity is required and the IKK targeted serine sites on IκBα are critical for UVC-induced NF-κB activation (33). Several mechanisms were proposed to elucidate the early-phase activation of NF-κB after UVC-irradiation and discussed in the following sections.

The Role of Translational Inhibition in Regulation of UVC-induced Activation of NF-κB

UVC does not accelerate the degradation of IκBα in the early-phase of irradiation because the half-life of IκBα is not reduced during the period. Instead, IκBα synthesis is reduced in accompany with the inhibition of nascent protein synthesis post-irradiation. Since the half-life of IκB is only 140 minutes for complexed endogenous IκB and 40 minutes for free, overexpressed IκB (32), a reduced expression of IκBα leads to a reduction in the net amount of IκB and sequentially NF-κB activation (34,35). The down regulation of IκBα expression is controlled at the translational level via the phosphorylation of alpha subunit of the eukaryotic initiation factor 2 (eIF2α), which plays a critical role in the regulation of protein synthesis. The phosphorylation of eIF2α at the Ser51 inhibits translational initiation (36,37) and activates NF-κB through inhibition of IκB synthesis (38). Four protein kinases are known to phosphorylate Ser51 of eIF2α in response to different stress stimuli. All four eIF2α kinases (EIF2AK) have
been shown to be directly or indirectly involved in NF-κB activation (39). Among them, PERK (EIF2AK3) and GCN2 (EIF2AK4) are activated and mediate NF-κB activation after UVC radiation (34,35,40,41).

UVC-induced eIF2α phosphorylation is a prolonged process. Due to the delayed activation of eIF2α kinases, UVC was believed not to be an inducer of eIF2α phosphorylation (42). The eIF2α phosphorylation was first detected at 4 h post-UVC and PERK was identified as the mediator for UVC-induced phosphorylation of eIF2α (40). Soon after, GCN2 was also identified as a kinase that phosphorylates eIF2α upon UVC-irradiation (41). Both PERK and GCN2 regulate the early phase activation of NF-κB (34,35). The UVC-induced eIF2α phosphorylation and NF-κB activation were significantly inhibited in PERK or GCN2 knockout mouse embryonic fibroblast (MEF) cell lines (34,35). Analysis of NF-κB activation in a MEF cell line containing an eIF2α mutant in which Ser51 was mutated to alanine (MEF<sup>A/A</sup>) showed similar inhibition of NF-κB (34,35). Based on the fact that IKK activity is not induced but is required for UVC-induced IκBα reduction; PERK and GCN2 mediate UVC-induced eIF2α phosphorylation and translational inhibition of IκB synthesis, a model for UVC-induced early phase activation of NF-κB was proposed. UVC activates PERK and GCN2, which phosphorylate eIF2α and inhibit new IκBα synthesis. The existing IκB amount is reduced upon natural degradation through the polyubiquitine pathway and finally NF-κB is activated due to the lack of IκB (Fig. 2.1).
Figure 2.1. Signaling circuit that regulates UVC-induced early-phase activation of NF-κB.

The Contribution of IKK in Reduction of IκBα upon UVC Radiation

IKK is a 700 kDa protein complex consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit NEMO (NF-κB essential modulator or IKKγ) (27,33,43). Activation of the catalytic subunits takes place by intra- and inter-molecular phosphorylation followed by releasing of their kinase domains, which phosphorylate IκBα at Ser32/Ser36 and promote its degradation (11,12). The reduction of IκB in the
early phase of UVC-irradiation does not apparently involve a detectable IKK activation and IkBα phosphorylation at Ser32/Ser36 (31,32). However, the N-terminus of IkBα is required for IkBα reduction after UVC since a deletion mutant of IkBα lacking 36 amino acids at N-terminal is stable upon UVC-irradiation (32). Interestingly, the UVC-induced NF-κB activation is abolished in a cell line that is stably transfected with IkBα(Ser32/Ser36A) mutant, which has both Ser32/Ser36 replaced with Ala, while transient transfected IkBα(Ser32/Ser36A) mutant is not resistant to UVC-induced reduction. These results suggest that while the UVC-induced reduction of excess amount of free IkBα is independent of IKK activity, the degradation of NF-κB-bound IkBα requires active IKK (44).

Further study indicates that UVC-irradiation slightly increases phosphorylation of Ser32/Ser36 on overexpressed IkBα, which can only be detected after capturing by an overexpressed F-box deletion mutant of ubiquitin ligase β-tranducin repeats containing protein (β-TrCP) as a specific phospho-protein substrate trap after immunoprecipitation. In addition, deletion of 25 amino acids at C-terminal of IKKγ/NEMO, which contains a putative Cys2HisCys zinc finger motif, abolishes the UVC-induced activation of NF-κB (33). Based on these results, it is proposed that while an increased activation of IKK is not detected after UVC-irradiation, IKK activity still plays a critical role in regulating IkBα degradation via phosphorylation of Ser32/Ser36 (Fig. 2.1).
The Roles of Casein Kinase 2 (CK2) in Removal IκBα upon UVC Radiation

In addition to the translational inhibition of IκBα synthesis and IKK-mediated IκBα degradation, CK2 also plays roles in regulating IκB degradation and NF-κB activation upon UVC radiation. In response to UVC, CK2 is activated via p38 MAPK pathway and phosphorylates IκBα at a cluster of C-terminal sites, which leads to IκBα degradation and NF-κB activation (45). Knockdown of CK2 using siRNA eliminates UV-induced IκBα degradation (45) while expression of an IκBα mutant with six CK2 target sites replaced with Alanine attenuates UV-induced NF-κB activity (44). UVC-activated CK2 also phosphorylates β-arrestin, which interacts with IκBα and stabilizes the IκBα-NF-κB complex (46). The phosphorylation of β-arrestin reduces its binding affinity to IκBα and leads to the dissociation of IκBα from the IκBα-NF-κB complex.

Regulation of NF-κB Activation

The UVC-induced early phase activation of NF-κB is independent of nuclear signal generated by DNA damage, but dependent on the activation of Src-Ras-Raf signaling cascade (32,47). Overexpression of dominant negative mutant of v-src, Ha-ras, or raf-1 inhibits UVC-induced early activation of NF-κB. Since both src and Ha-ras resident on plasma membrane, it is suggested that the UVC-induced signaling cascade for activating NF-κB is initiated on or near the plasma membrane (47). In the meantime, while IKK is important in regulation of IκB level as discussed above, IKKγ/NEMO, the regulatory subunit of IKK, appears to play an additional role in recruiting cofactor(s) that
are critical for UVC-induced NF-κB activation (33). The interaction between IKKγ/NEMO and the catalytic subunits (IKKα/β) is required for the activation of NF-κB induced by UVC radiation. Furthermore, overexpression of IKKγ/NEMO restores and robustly increases the inducibility of NF-κB after UVC radiation in the IKKγ/NEMO deficient cell line, which suggests that expression level of IKKγ/NEMO may be the rate-limiting step of the UVC-induced NF-κB signaling pathway.

In spite of the kinase-binding domain which interacts with IKKα and IKKβ, IKKγ/NEMO also contains two coiled-coil domains, a leucine zipper motif (LZ) and a putative zinc finger domain at the C-terminal. IKKγ/NEMO is able to be self-multimerized, either in trimerization or tetramerization form through the leucine zipper and C-terminal coiled-coil domain (48-50). The zinc finger domain itself is not involved in the multimerization since deleting it does not significantly affect the multimerization of IKKγ/NEMO (51). However, the extra ZF that added on to IKKγ/NEMO dimer might assist the LZ to recruit upstream factor(s) to promote NF-κB activation (48). The vital role of the ZF in recruiting activator(s) to IKKγ/NEMO from upstream signaling pathways is also applicable to UVC-induced NF-κB activation since expression of ZF mutant with amino acid substitution abolishes the inducibility of NF-κB upon UVC radiation (33). Since the ZF is able to interact with a variety of different molecules (52), it is still not clear whether the ZF of IKKγ/NEMO recruits upstream cofactor(s) to activate IKK or to directly activate NF-κB (Fig. 2.1)
Late Phase Activation of NF-κB after UVC Radiation

The regulation of the late phase activation of NF-κB after UVC exposure is more complex than the original thought (31,32). During the late period post-UVC, IKK is activated, which phosphorylates the N-terminal Ser32 and Ser36 of IκBα and promotes IκBα degradation (32). Meanwhile, UVC-induced eIF2α phosphorylation down-regulates IκB expression at both transcriptional and translational levels (53). However, the reduction of IκB may not be a direct cause of NF-κB activation. Furthermore, neither ubiquitination nor proteasomal degradation has detectable contribution to late-phase UV-induced IκBα depletion (53). Some contradictory theories and potential signaling circuits in regulation of NF-κB activation in the late phase of UVC are discussed below.

Regulation of IκB Reduction

The late phase activation of NF-κB is suggested to be dependent on a DNA-damage-induced and IKK-mediated IκB degradation pathway (31,32). In the late phase of UVC exposure, DNA-damage and the production of IL-1α and autocrine/paracrine induced by DNA-damage are crucial for NF-κB activation. Moreover, unlike early phase, both IKK activation and phosphorylation IκB at Ser32/Ser36 are required for NF-κB activation at late phase (32). In addition to IκB degradation, the level of IκB is also regulated by eIF2α. The previous data showed that at 24 h after UVC exposure, the mRNA level remained unchanged in MEF^{S/S} cells but significantly increased in MEF^{A/A} cells, as well as decreasing of IκB protein level in MEF^{s/s} cells and the intact protein level
in MEF<sup>A/A</sup> cells (53). These results mean that at late onset of NF-κB activation caused by UVC irradiation, the phosphorylation of eIF2α inhibited both the transcriptional and translational level of IκBα and thus regulated NF-κB activation (Fig. 2.2).

The involvement of IKK in regulation of IκB is challenged by the results showing that a membrane-permeable proteasome inhibitor MG132 and an IκBα ubiquitin ligase inhibitor Ro106 failed to influence IκBα levels in MEF cells at 24 hours post-UVC (53). In addition, the phosphorylation of Ser536 of NF-κB (p65), which is a direct target of activated IKK (54,55), is not increased at the late phase of the irradiation (53). These results suggest that there might be an IKK-dependent or -independent pathway existing in different cell lines that regulates IκB level (Fig. 2.2).

**Figure 2.2.** Signaling circuit that regulates UVC-induced late-phase activation of NF-κB.
IκB-independent Activation of NF-κB

While IκB is reduced in the late phase of the irradiation, NF-κB activation does not appear to be dependent on the reduction. NF-κB can be activated in the presence of normal level of IκB in a non-phosphorylatable eIF2α knock-in mouse fibroblast cell line during the late phase of UVC radiation (34,53). These results indicate that IκB reduction in the late phase might not be a direct cause of NF-κB activation and UVC induces the DNA-binding of NF-κB (34). Further analysis on the critical phosphorylation sites of NF-κB shows that an increased phosphorylation of Ser276 of NF-κB, but not Ser536, is correlated to the activation of the protein. Two kinases, mitogen and stress activated protein kinases (MSK) and protein kinase A (PKA) phosphorylate the Ser276 of NF-κB in the nucleus (56) or in the cytosol (9,57,58) respectively. Since the phosphorylation levels of Ser276 of NF-κB is only increased in the nucleus, but not in cytosol, MSK signaling cascade is more likely to be the mediator of UVC-induced late-phase activation of NF-κB (Fig. 2.2).

Translocation and DNA Binding of NF-κB

During the late-phase of UVC-irradiation, NF-κB is activated with increased DNA-binding activity (34) and Ser276 phosphorylation (53). The phosphorylation at S276 is increased even in the presence of normal level of IκB in the nucleus, but is not detected in the cytosol. Since UVC is able to induce NF-κB translocation without activation in cells which overexpress a dominant negative PERK (34), it is likely that
UVC is able to induce an IκB-independent release and translocation of NF-κB into nucleus, where it is phosphorylated and activated by MSK (Fig. 2.2).

In addition to direct regulation of the activation of transcription factors, such as NF-κB, MSK is also able to remodel chromatin, which further regulates transcriptional activity. MSK has two isoforms - MSK1 and MSK2. Both isoforms of MSK are capable of phosphorylating histone 3 (H3) at Ser10 and Ser28 (59-61). However MSK2 has been observed to play a more crucial role in H3 modification (60). Phosphorylation of either of these sites on H3 results in a conformational change, as well as a change in charge on the molecule; both of which play important roles in chromatin binding (59,61). In this way, UVC-induced activation of MSK might play dual roles in regulation of the transcriptional activity of NF-κB via direct phosphorylation of NF-κB as well as modification of the availability of genes for transcription (Fig. 2.2).

Summary

UVC-induced activation of NF-κB has two distinct phases. In the early phase following radiation, IκBα is reduced due to the combined effects of translational inhibition and IKK/CK2 activities. Meanwhile IκKγ/NEMO also recruits cofactor(s) that further promotes activation of the freed NF-κB in the cytosol. On the other hand, in the late phase, NF-κB is released from IκB with an unknown mechanism and translocates into the nucleus, where it is phosphorylated and activated by MSK. In the meantime, MSK phosphorylates chromatin, which further regulates the transcription of genes that are under control of NF-κB.
CHAPTER 3 : NITRIC OXIDE: A REGULATOR OF EUKARYOTIC INITIATION FACTOR 2 KINASES

Introduction

Nitric oxide (NO\(^{\bullet}\)) plays an important role in the control of physiological functions such as muscle relaxation and immune response (62,63). In addition to its physiological significance, changes in NO\(^{\bullet}\) concentration affect gene expression. One mechanism for NO\(^{\bullet}\)-mediated regulation of gene expression is via activation of multiple serine-threonine kinases that phosphorylate the eukaryotic initiation factor 2 (eIF2). When not phosphorylated, eIF2 initiates translation by forming an eIF2•TP•Met-tRNA\(_{i}\) ternary complex, which promotes the binding of Met-tRNA\(_{i}\) to the 40S ribosome-mRNA complex with the hydrolysis of GTP to GDP. To restart the cycle, the guanine exchange factor eIF2B must refresh the eIF2-GDP to eIF2-GTP (64). Phosphorylation of Ser51 in the \(\alpha\) subunit of eIF2 (eIF2\(\alpha\)) stabilizes the eIF2-GDP-eIF2B complex, thus preventing the GDP-GTP exchange and halting translational initiation (65-67).

The four identified serine–threonine eIF2\(\alpha\) kinases (EIF2AKs) are (1) HRI (EIF2AK1), which responds to heme deprivation (68); (2) PKR (EIF2AK2), which is activated by dsRNA produced during viral infection (69); (3) PERK (EIF2AK3), which responds to the accumulation of unfolded protein response (UPR) in the endoplasmic reticulum (ER) (70,71); and (4) GCN2 (EIF2AK4), which responds to amino acid depletion (72). Whereas each EIF2AK is regulated specifically by its activator(s) and inhibitor(s), generation of NO\(^{\bullet}\) can be either an upstream activator (all four EIF2AKs) or a downstream mediator (PKR) of an EIF2AK-activated signaling pathway. Below, the
mechanisms of the NO•-induced or -mediated EIF2AK signaling pathways and their physiological and pathological impacts will be discussed.

The Molecular Mechanisms of NO•-activated or -mediated EIF2AK Signaling Pathways

Activation of HRI via Formation of a Heme–Fe(II)NO• Complex

HRI, as a hemoprotein, is activated by heme deficiencies through a series of autophosphorylations (73-76). Through sequence and mutagenesis analyses, an N-terminal hemin-binding domain (NT-HBD) containing a heme-binding site His119 and a catalytic domain of HRI were identified (77,78). NO• was first shown to activate HRI through binding with the recombinant NT-HBD (79). It was suggested that the binding of NO• to the NT-HBD results in cleavage of the iron–histidyl bond to form a 5-coordinate ferrous nitrosyl [heme–Fe(II)NO] complex (Fig. 3.1A) (79). However, additional evidence shows that the cleavage of the iron–histidyl bond is neither necessary nor sufficient for the activation of HRI by NO• (80,81). The binding of NO• appears to disrupt the inhibitory interactions between the NT-HBD and the catalytic domain, thus activating HRI (81).

By using recombinant N-terminal-deleted mutant and full-length HRI, two heme-binding sites in HRI, His119/120 in the N-terminal and Cys409 in the catalytic domain, were shown to form a complex with one heme, regardless of whether it was a hemin–Fe(III) or heme–Fe(II) (82,83). Hemin–Fe(III) inhibits HRI by forming a stable 6-coordinate hemin–Fe(III)–HRI complex with Cys409 as one of axial ligands (Fig. 3.1B)
In the presence of NO\textsuperscript{•}, heme–Fe(III) can be reduced to heme–Fe(II) (85), which binds to NO\textsuperscript{•} to form a 5-coordinate heme–Fe(II)NO\textsuperscript{•} complex (Fig. 3.1A) and leads to HRI activation with a conformational change (82,84). This model might potentially be affected by the phosphorylation states of HRI and the allostery effect of eIF2 binding, which can affect the heme-binding affinity and HRI conformation (81,84).

![Diagram of 5-coordinate Heme-Fe(II)NO Complex](image)

![Diagram of 6-coordinate Heme-Fe(III)HRI Complex](image)

**Figure 3.1.** Regulation of HRI activation by NO\textsuperscript{•}.
(A) The 5-coordinate heme–Fe(II)NO\textsuperscript{•} complex in active HRI. (B) The 6-coordinate heme–Fe(III)HRI complex in inactive HRI.

**Activation of PERK by NO\textsuperscript{•} via Two Distinctive Mechanisms**

It is commonly accepted that the elevation of NO\textsuperscript{•} leads to ER stress and results in PERK activation. Treating either differentiated or undifferentiated neuroblastoma cells
with the NO\textsuperscript{•} donor S-nitroso- N-acetylpenicillamine (SNAP) was found to induce ER stress and PERK activation (86). In addition, cytokines, such as IL-1, were found to induce ER stress by up-regulating the expression of inducible nitric oxide synthase (iNOS) and thus increasing the intercellular NO\textsuperscript{•} level in chondrocytes and islets of both rats and humans. In both chondrocytes and β cells, PERK was activated by IL-1β in an iNOS-dependent manner since inhibiting iNOS resulted in a decreased expression of ER-stress-associated genes (87,88).

Two mechanisms have been proposed for NO\textsuperscript{•}-induced PERK activation. The first is that NO\textsuperscript{•} induces PERK activation by disrupting Ca\textsuperscript{2+} homeostasis in the ER. NO\textsuperscript{•} inactivates the sarcoplasmic/ER Ca\textsuperscript{2+}-ATPases (SERCA) family proteins on the ER membrane, which are responsible for transporting cytosolic Ca\textsuperscript{2+} into the ER. Simultaneously, NO\textsuperscript{•} activates the ryanodine receptor of Ca\textsuperscript{2+} release channels (RyR), which facilitates the release of Ca\textsuperscript{2+} from ER into cytosol. The inhibition of SERCA and activation of RyR lead to the depletion of Ca\textsuperscript{2+} in the ER and sequentially disrupt the protein-folding process, which increases ER stress and activates PERK (89,90) (Fig. 3.2).

In addition to disrupting the Ca\textsuperscript{2+} channels, NO\textsuperscript{•} also interrupts the flux of Ca\textsuperscript{2+} between mitochondria and ER (91), which are in close proximity (92-94). The NO\textsuperscript{•}-induced depletion of Ca\textsuperscript{2+} in the ER appears to be coupled to a mitochondrial Ca\textsuperscript{2+} influx (91,93,95). When the Ca\textsuperscript{2+} released from ER is collected in the matrix of mitochondria, the mitochondrial membrane loses its potential. This depolarization disrupts the respiratory chain and increases production of reactive oxygen species (ROS), which
further facilitates Ca\textsuperscript{2+} efflux from ER (96) (Fig. 3.2). In an attempt to alleviate ER stress, an elevation of NO\textsuperscript{*} also stimulates an efflux of Ca\textsuperscript{2+} from mitochondria to ER, which activates the p90ATF6-mediated ER-stress response and protects cells from Ca\textsuperscript{2+} flux-caused damage (91).

**Figure 3.2.** Mechanism of Ca\textsuperscript{2+} depletion-mediated activation of PERK.

The other proposed mechanism of NO\textsuperscript{*}-induced PERK activation is through S-nitrosylation of protein disulfide isomerase (PDI), which facilitates the folding of targeted proteins by either forming a disulfide bond or isomerizing a misfolded disulfide bond in the ER (97-99). When cells were treated with a NO\textsuperscript{*} donor, such as S-nitrosocysteine or O\textsuperscript{2-}[2,4-dinitro-5-(N-methyl-N-4-carboxyphenylamino)phenyl]1-(N,N-dimethylamino) diazen-1-iium-1,2-diolate, PDI was S-nitrosylated and inhibited. Accompanying the formation of S-nitrosylated PDI (SNO–PDI) and the loss of PDI activity, the activation of ER-stress-induced genes such as XBP-1, CCAAT-enhancer-binding protein (C/EBP)-homologous protein (CHOP), and PERK was detected (97,98). It was proposed that
when intracellular levels of NO• rise, NO• interacts with PDI on its thioredoxin domain, which forms one or two S-nitrosothiols. Furthermore, the two active thiols might share the NO• group and form a SNO2 group. After S-nitrosylation, the chaperone activity of PDI decreases, which leads to an accumulation of misfolded protein in the ER. This prolonged ER stress activates UPR and PERK (100) (Fig. 3.3). It is worthwhile to notice that although SNO–PDI formation in NO• donor-treated cell lysate was detected by immunoblot and mass spectroscopy (97,98), the formation of SNO2–PDI is only a prediction based on an in silico study (101).

**Figure 3.3.** Mechanism of S-nitrosylation-mediated activation of PERK.

*Production of NO• Depletes L-Arg and Induces GCN2 Activity*

Unlike NO•-induced HRI or PERK activation, the activity of GCN2 is not directly controlled by NO• but rather regulated by NOS-catalyzed production of NO•. L-Arg is the substrate for all three NOSs, including inducible NOS (iNOS) and two constitutive NOSs (cNOSs), which convert L-Arg to NO• and L-citrulline (102-104). Upon
activation of NOS, cellular L-Arg levels begin to decrease, which eventually leads to L-Arg starvation, GCN2 activation, and eIF2α phosphorylation (Fig. 3.4) (105,106). To alleviate the demand for L-Arg in translation, L-Arg-sensitive GCN2 phosphorylates eIF2α, leading to the inhibition of protein synthesis, which also inhibits the translation of iNOS. The reduced expression of iNOS causes less L-Arg to be used to produce NO•, thus decreasing NO• levels and allowing L-Arg levels to build back up (105). An additional effect of the depletion of L-Arg is the uncoupling of cNOSs and the generation of O•−, which then reacts with NO• to generate ONOO−, resulting in further oxidative and ER stress. Thus, NOS-mediated GCN2 activation is often accompanied by PERK activation (106-108). Most, if not all the NOS-mediated activation of GCN2 was based on the studies of cell culture conditions with limited supply of L-Arg (105,106). These results may not apply to animal models, which have a constant supply of L-Arg. In fact, recent study from our lab indicated that the pattern of UVB-induced NO• release in mouse skin tissue was very different from that of cultured skin cells. The UVB-induced NO• production in cultured keratinocytes over time produces a bell-shaped curve with a sharp increase followed by a decrease to baseline, which may be the result of a lack of L-Arg (107). In mouse skin tissue, however, the UVB-induced NO• release remains a steady increase, indicating that the supply of L-Arg is not limited in the irradiated skin.
Figure 3.4. The regulation of GCN2 activity by NOS under cell culture conditions.

PKR Promotes NO\(^\bullet\) Production through NF-\(\kappa\)B

In addition to being an upstream regulator with an unknown mechanism (109), NO\(^\bullet\) production is regulated downstream by PKR. The dsRNA-dependent activation of PKR results in an increased iNOS expression and therefore NO\(^\bullet\) elevation. The elevation in iNOS occurs through the activation of NF-\(\kappa\)B (110), which is known to up-regulate the expression of iNOS (111). Viral or synthetic dsRNA alone or in conjunction with interferon-\(\gamma\) (IFN-\(\gamma\)) increases the expression and activity of NF-\(\kappa\)B, which induces iNOS expression and increases NO\(^\bullet\) production in a PKR-dependent manner (110,112). In U373 MG astroglial cells, PKR activation was required for dsRNA-induced NF-\(\kappa\)B activation and iNOS expression (112). In airway epithelial cells, PKR knockouts lost the ability to induce NF-\(\kappa\)B activation and iNOS expression by dsRNA (110). Furthermore, PKR knockouts also showed reduced interferon-regulatory factor 1 (IRF-1) activity, which decreased the transcription of iNOS (110). Based on the assumption that PKR is able to activate NF-\(\kappa\)B through direct phosphorylation of the inhibitor of NF-\(\kappa\)B (I\(\kappa\)B)
(113) or activation of IκB kinase (IKK) (114), a model for PKR-mediated induction of iNOS expression and NO\(^\cdot\) production is proposed (Fig. 3.5).

![Diagram](image)

**Figure 3.5.** Models for NO\(^\cdot\) production mediated by PKR through dsRNA-induced and PKR-mediated activation of iNOS.

Although there is evidence showing that PKR mediates dsRNA-induced iNOS, the role of PKR in the regulation of NO\(^\cdot\) production through iNOS is still a subject of debate. Additional data indicate that iNOS induction occurs before PKR activation and is independent of functional PKR in cells infected with encephalomyocarditis virus, an RNA virus known to induce PKR activation (115). Moreover, the dsRNA-induced degradation of IκB and translocation of NF-κB into nucleus are also independent of PKR. In PKR knockout mouse islet cells, dsRNA was still able to induce iNOS expression by activating NF-κB (116). These controversial results could be due to tissue-specific signaling for dsRNA-induced iNOS expression (112). Two different signaling pathways are proposed for mediating the dsRNA-induced expression of iNOS. One is a PKR-
dependent activation of the NF-κB signaling pathway and the other is the p38MAPK-dependent but PKR-independent C/EBPβ signaling pathway (112,115). The proposed model is based on the assumption that if the activities of both PKR and p38MAPK are diminished, iNOS is no longer inducible by dsRNA (112).

The Physiological Impacts of the Coordinative Effects of NO•-EIF2AKs

The Role of NO•-HRI Interaction in Regulation Cell Cytostasis and Hg2+ Toxicity

The NO•-induced activation of HRI increases eIF2α phosphorylation and inhibits protein synthesis in nonerythroid cells. Because eIF2 plays an important role in the regulation of cell growth and death, it has been suggested that HRI may contribute to NO•-regulated cell growth, differentiation, and apoptosis (79). Furthermore, it has been found that HRI plays a role in mediating NO•-induced translation inhibition in breast cancer cells (109). In invasive breast tumors, the activities of both cNOSs and iNOS are higher than in benign or normal breast tissues (117). Exposure of breast cancer cells to 1 mM diethylenetriamine NONOate (DETA-NONOate), which releases NO• to a constant concentration of 0.5 µM, activated HRI and led to a gradual decrease in short-half-life proteins and cell growth arrest (118). Whereas the translational inhibition and cell cytostasis caused by exposure to 0.5 µM NO• were reversible, the effects of higher NO• levels were not. Exposure of breast cancer cells to 2 mM DETA-NONOate resulted in the activation of both HRI and PKR and caused a sharp decrease in both short- and long-half-life proteins (109).
Besides promoting cytostasis, the NO•-HRI interaction has been suggested to play a role in regulating Hg\textsuperscript{2+}-induced cytotoxicity (119). Metal cations, such as Hg\textsuperscript{2+}, Cd\textsuperscript{2+}, Zn\textsuperscript{2+}, and Pb\textsuperscript{2+}, inhibit HRI \textit{in vitro}, with their IC\textsubscript{50} values ranging from 0.6 to 8.5 µM. The inhibition of HRI by Hg\textsuperscript{2+}, but not other heavy metal ions, could be reversed by NO• at the micromolar level (119). A potential mechanism for the metal cations-induced inhibition of HRI is that the cations compete with the heme in binding Cys or His and inhibit HRI by forming an inactive Mt\textsuperscript{2+}-HRI complex (Fig. 3.6). Although all these metal cations can potentially interact with His residues, there is a unique interaction between Hg\textsuperscript{2+} and HRI through the formation of a Hg\textsuperscript{2+}-thiol bond with cysteine residues, including Cys409, one of the axial ligands. It was proposed that NO• reverses Hg\textsuperscript{2+}-inhibited HRI by nitrosylating Cys409, likely to form a thiol adduct, S–NO, in the active site (Fig. 3.6).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mechanism.png}
\caption{Mechanism proposed for Hg\textsuperscript{2+} inhibition of HRI and the reverse reaction by NO•.}
\end{figure}
It has thus been suggested that NO• and Hg\(^{2+}\) competitively control protein synthesis in cells, which may be important for cell survival under mercury contamination (119). However, whereas the binding of Hg\(^{2+}\) to Cys on HRI was detected, the formation of HRI–S–NO was based only on the previous studies indicating that Cys could be S-nitrosylated by NO• (120,121). Another possibility is that NO• restores the activity of Hg\(^{2+}\)-inhibited HRI simply by reducing Hg\(^{2+}\) to Hg\(^{+}\), which is then released from HRI. Hg\(^{2+}\) / Hg\(^{+}\) has a relatively high standard reduction potential compared to other metals and is the only one of these tested metals that can be reduced by NO• (119) (Table 1).

Table 3-1 Standard reduction potentials in aqueous solution at 25 °C.

<table>
<thead>
<tr>
<th>Half-reaction</th>
<th>(E^\circ) (V)</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2\text{Hg}^{2+} + 2e^- \rightarrow \text{Hg}^{2+})</td>
<td>0.92</td>
<td>Acidic</td>
</tr>
<tr>
<td>(2\text{Hg}^{2+} + 2e^- \rightarrow 2\text{Hg})</td>
<td>0.85</td>
<td>Acidic</td>
</tr>
<tr>
<td>(\text{Fe}^{3+} + e^- \rightarrow \text{Fe}^{2+})</td>
<td>0.77</td>
<td>Acidic</td>
</tr>
<tr>
<td>(\text{NO}_2^- + e^- + \text{H}_2\text{O} \rightarrow \text{NO}^\cdot + 2\text{OH}^-)</td>
<td>0.37</td>
<td>Neutral</td>
</tr>
<tr>
<td>(\text{Cu}^{2+} + e^- \rightarrow \text{Cu}^{+})</td>
<td>0.15</td>
<td>Acidic</td>
</tr>
<tr>
<td>(\text{Pb}^{2+} + 2e^- \rightarrow \text{Pb})</td>
<td>-0.13</td>
<td>Acidic</td>
</tr>
<tr>
<td>(\text{Cd}^{2+} + 2e^- \rightarrow \text{Cd})</td>
<td>-0.40</td>
<td>Acidic</td>
</tr>
<tr>
<td>(\text{Zn}^{2+} + 2e^- \rightarrow \text{Zn})</td>
<td>-0.76</td>
<td>Acidic</td>
</tr>
</tbody>
</table>

_The Role of PERK in Mediating NO•-induced Apoptosis_

NO• induces apoptosis by depleting Ca\(^{2+}\) in the ER, which increases ER stress and activates PERK (122,123). The proapoptotic role of PERK comes from its ability to induce expression of the apoptotic protein CHOP via phosphorylation of eIF2α and
activation of activating transcription factor 4 (ATF4) (122-125). PERK, along with the ER-stress-induced proteins inositol-requiring enzyme-1 (IRE1) and ATF6, helps mediate the NO\textsuperscript{•}-induced expression of CHOP through multiple signaling pathways (122-124). The discovery that CHOP knockout β cells are more resistant to NO\textsuperscript{•}-induced apoptosis suggests that ER-stress-induced signaling pathways are responsible for NO\textsuperscript{•}-mediated β cell death (123). The involvement of ER-stress-induced signaling pathways in NO\textsuperscript{•}-induced cell death was also observed in other cell lines, including microglial cells (126,127), macrophages (124), and neuronal cells (128) (Fig. 3.7).

Although NO\textsuperscript{•}-induced and ER-stress-mediated PERK activation increases CHOP expression (124), it is still under debate whether PERK-catalyzed eIF2α phosphorylation is the cause of or just occurs simultaneous with NO\textsuperscript{•}-induced apoptosis. In chondrocytes, the NO\textsuperscript{•} donor SNAP increased CHOP expression and cell apoptosis but the NO\textsuperscript{•} inducer IL-1 did not (87), suggesting that SNAP may induce apoptosis via a NO\textsuperscript{•}-independent

![Figure 3.7. NO\textsuperscript{•}-induced and UPR-mediated cell apoptosis pathways.](image)
mechanism. Because of complex chemical structures, NO\textsuperscript{*} donors can modulate cellular processes in mechanisms beyond NO\textsuperscript{*} donation (129). Additionally, cells overexpressing dominant negative PERK or PERK knockout cells showed no increase in cytokine-induced cell death but were more susceptible to the tunicamycin-induced cell death. This implies that NO\textsuperscript{*} is not necessary for PERK-mediated apoptosis because tunicamycin-induced cell death is ER-stress dependent and NO\textsuperscript{*} independent (88). It has also been suggested that instead of mediating cell death, the NO\textsuperscript{*}-induced UPR may play a protective role in cytokine-induced apoptosis. β cells with a knockout PERK or a knock-in nonphosphorylatable eIF2\textalpha, eIF2αS51A, are more susceptible to ER stress (130,131). Also, during NO\textsuperscript{*}-induced apoptosis, the ER-stress-response protein p90ATF6 is digested into p50ATF6, which activates the CHOP gene in the nucleus (124). In addition to activating the CHOP apoptosis pathway, p50ATF6 activates the ER-stress-induced chaperone protein GRP78, which attenuates CHOP-induced apoptosis and promotes cell recovery (123) (Fig. 3.7).

**Potential Roles of PERK in NO\textsuperscript{*}-mediated Physiological Responses**

NO\textsuperscript{*}-induced activation of PERK helps regulating Ca\textsuperscript{2+} flux and therefore affects cardiac and skeleton muscle contraction. NO\textsuperscript{*} regulates muscle contraction by inducing ER Ca\textsuperscript{2+} efflux via activation of Ca\textsuperscript{2+} channels and by inhibiting ER Ca\textsuperscript{2+} influx via inactivation of Ca\textsuperscript{2+}-ATPases, and the resulting Ca\textsuperscript{2+} depletion in the ER leads to ER stress and PERK activation (89,90,132). Through a currently uncharacterized
mechanism, the activated PERK reduces ER Ca\(^{2+}\) efflux and thus relieves ER stress and maintains the integrity of the ER (90). The NO\(^\cdot\)-induced and PERK-mediated protection of ER integrity may also protect brain tissue from damage caused by ischemia and reperfusion (I/R) (133). Treating brain tissue with the NOS inhibitor L-NAME reduced PERK activation and eIF2\(\alpha\) phosphorylation. These treated cells showed an increase in brain tissue damage from I/R, suggesting that translation inhibition protects brain cells from I/R-induced damage. However, the role of PERK in protecting cells from I/R-induced damage is limited to the brain because L-NAME treatments protected kidney and liver cells from damage without inducing eIF2\(\alpha\) phosphorylation (86) (Fig. 3.7).

The long-acting NO\(^\cdot\) donor DETA-NONOate induces PERK activation and eIF2\(\alpha\) phosphorylation. Additionally it is able to preferentially induce macrophage apoptosis in plaques without affecting normal smooth muscle cells or circulating macrophages/monocytes. The removal of macrophages from a plaque affects its stability and decreases the risk of cardiovascular disease. A proposed explanation for the selectivity of NO\(^\cdot\)-induced apoptosis is that plaque macrophages are more metabolically active and thus more sensitive to protein synthesis inhibition. As a result, DETA-NONOate can potentially be used for treatment of atherosclerotic plaques because it specifically targets plaque macrophages (134) (Fig. 3.7).

*Cell Cycle Regulation Mediated by NO\(^\cdot\)-induced Activation of GCN2/PERK*

NO\(^\cdot\) production without a continuous supply of L-Arg can activate GCN2, which coordinates with PERK to regulate the cell cycle upon UVB irradiation. Activation of
GCN2 and PERK promotes UVB-induced G₁ arrest because a knockout of either GCN2 or PERK allows cells to shift from the G₁ phase to the G₂ phase. Interestingly, elimination of eIF2α phosphorylation by knocking in the nonphosphorylatable eIF2αS51A prevented UVB-induced cell cycle shift, which suggests that PERK and GCN2 regulate the mammalian cell cycle via an eIF2α phosphorylation-independent pathway (135).

Cellular Responses to NO• Production and PKR Activation

PKR activation seems to be critical for an innate immune response to viral infections. Innate immune response cells, such as monocytes and macrophages, respond to pathogens by activating a Toll-like receptor (TLR) signaling cascade. DsRNA or single-stranded DNA (ssDNA) interacts with TLR3 and activates an antiviral pathway by increasing the expression of iNOS and thus the production of NO• (136). However, the TLR3-mediated elevation of NO• in monocytes or macrophages in response to dsRNA or ssDNA is diminished when the cells are treated with a PKR pharmacological inhibitor, which inhibits the ATP-binding site of PKR. This suggests that PKR is indispensable for the virus-induced innate immune response (Fig. 3.8).
Figure 3.8. Model for PKR and iNOS-mediated cellular responses.

PKR also plays a role in lipopolysaccharide (LPS)-induced inflammatory response by mediating the activation of the NF-κB-STAT1-iNOS cascade (137). In both microglial and astrocyte cells, PKR was activated within 5 min and was followed by STAT1 activation at 2 h post LPS treatment. Reducing PKR activity by PKR-specific siRNA or 2-AP reduced NF-κB activation, IFN-β production, STAT1 activation, iNOS expression, and NO• production after LPS treatment. The PKR-mediated inflammatory response seems to be LPS specific because ganglioside-stimulated STAT1 phosphorylation is independent of PKR activation (137) (Fig. 3.8).

In addition to phosphorylating eIF2α and inhibiting protein synthesis, PKR also promotes protein degradation via activation of the iNOS-p38MAPK pathway. TNFα or IFN-γ induces the NO•-mediated activation of p38MAPK, which promotes muscle
protein degradation in myotubes by increasing ROS production. Inhibition of PKR, p38MAPK, or iNOS in myoblasts attenuates ROS production and protects proteins from degradation. This suggests that NO• production regulates protein degradation and is important in decreasing the overall protein concentration in a cell, though the mechanism for the ROS formation is not known (138) (Fig. 3.8).

Whereas activation of PKR increases cytosolic NO• production, a higher level of NO• produced by 2 mM DETA-NONOate can also activate PKR, which along with HRI inhibits protein synthesis as discussed earlier (109). Interestingly, NO•-induced and PKR-mediated translation inhibition appears more effective on cancer cells than on normal cells. PKR autophosphorylation is induced by DETA-NONOate in breast cancer cells but not in normal mammary epithelial cells. It appears that inactive PKR exists as a monomer in cancer cells, but is bound to its inhibitor p58 in normal cells (139,140). NO• is able to interact with and activate only monomeric PKR, not the PKR-p58 heterodimer. Thus, DETA-NONOate has the potential to be used as an anti-cancer drug with limited side effects on healthy cells (109,141) (Fig. 3.8).

Conclusion

Via different mechanisms, NOS-catalyzed NO• production regulates the activation of the four EIF2AKs and thus inhibits protein synthesis. Conversely, activation of EIF2AK2 (PKR) increases iNOS expression and thus NO• production. Coordination between NO• production and EIF2AK activation is well regulated. A shift
of the balance between NO• production and EIF2AK activity could change the responses of cells to environmental stimuli. Therefore alteration of this balance could be a potential target for treating various diseases, such as artery plaque formation and cancers.
CHAPTER 4: THE ROLE OF CONSTITUTIVE NITRIC OXIDE SYNTHASE IN UVB-INDUCED NF-κB ACTIVITY

Introduction

UV radiation can cause skin damage and result in increased chance of developing skin cancer (142,143). Upon UV radiation, NF-κB is one of the transcription factors that can be activated and regulate the expression of genes which regulate immune and inflammatory responses, as well as genes involved in regulation of cellular growth and apoptosis (6,23,24,31,144). Because of its crucial role in cellular responses to environmental stress, the regulation of NF-κB activation has been widely studied. In canonical NF-κB activation, IKKα phosphorylates IκBα at Serine 32 and 36, which leads to its dissociation from NF-κB and being degraded. NF-κB, freed from IκB, then translocates into the nucleus and activates its target genes (11,12). While many NF-κB activators go through this pathway, some stimuli like UV, reactive oxygen species (ROS) and hypoxia activate NF-κB through much more complex cellular pathways which have not been fully elucidated (34,53,145,146).

Our previous studies indicated that UVB radiation quickly induces the activation and uncoupling of constitutive nitric oxide synthase (cNOS), which led to an imbalance of [NO\(^\cdot\)]/[ONOO\(^-\)] (147). The \(\text{NO}^\cdot\) production and [NO\(^\cdot\)]/[ONOO\(^-\)] imbalance can induce the phosphorylation of the eIF2α and inhibits protein synthesis (106). The eIF2α-mediated translation inhibition has been shown to play a critical role in the regulation of NF-κB activation and cell apoptosis post-UVC (34,35,148). However, the roles of cNOS in regulation of NF-κB activation after UVB radiation are still unclear. The relationship
between cNOS and NF-κB in mediating cell survival or death after UVB radiation is also not known. In this study, it is demonstrated that cNOS activity plays important roles in mediating a complex signalling circuit in the regulation of NF-κB activation and cell survival after UVB radiation.

Material and Methods

Cell Culture

Human keratinocyte HaCaT cells (kindly provided by Dr. Nihal Ahmad, University of Wisconsin-Madison), HeLa cells and HEK293 cells were grown in Dulbecco’s Minimal Essential Medium (Cellgro) supplemented with 10% fetal bovine serum and penicillin/streptomycin, at 37°C with 5% CO₂.

UVB Radiation

UVB was generated from a Bench XX-Series UV Lamp (UVP Inc.) equipped with two 15-watt UVB tubes (UVP Inc.). Medium was removed before exposing the cells to UVB. After UVB radiation, fresh medium was added to the culture plates with or without drugs, and the cells were continuously incubated at 37°C with 5% CO₂ until further analysis.

Drug Treatments

L-N⁵-Nitro-arginine methyl ester (L-NAME, Sigma) was added to cells to a final concentration of 1 mM at 1 h before UVB radiation. After irradiation, cells were
continuously incubated with L-NAME (1 mM) for either 1 h and replaced with fresh medium (acute treatment), or for the whole period until further analysis (continuous treatment). S-nitroso-N-acetylpenicillamine (SNAP, Invitrogen) was added to cells to the indicated final concentration at 1 h before UVB-irradiation. After irradiation, the cells were continuously incubated with the same concentration of SNAP until further analysis. BAY11-7085 (5 µM, Sigma), JSH-23 (10 µM, Sigma), MG132 (10 µM, Sigma) or Ro106 (10 µM, Sigma) were added to cells immediately after UVB radiation and kept in the media for the whole period until further analysis.

Western Blot Analysis

Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl pH 8.0, 0.1% SDS) with proteinase inhibitor mixture (Complete™, Roche Applied Science) was used to lyse cells. Cell lysate was incubated on ice for 15 min and then centrifuged at 14,000 rpm at 4 ºC for 15 min. Protein concentration was measured by Protein DC Assay kit (Bio-Rad Laboratories). Equal amounts of protein were subjected on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then blocked in 5% milk in Tris buffered saline plus Tween 20 (TBST) for 45 min and probed with anti-NF-κB p65 (Santa Cruz), anti-IκB (Santa Cruz), anti-phospho (Ser32/Ser36)-IκB (Cell Signaling), anti-eIF2α (Santa Cruz), anti-phospho (Ser52)-eIF2α (Invitrogen), anti-IKKα (Santa Cruz) or anti-β-actin (sigma) at 4 ºC overnight. After washing with TBST, the membrane was incubated with corresponding HRP-conjugated anti-rabbit or anti-mouse antibody for 45 min at room temperature. Membrane was then washed three
times in TBST followed by two times in TBS, and developed in West Pico SuperSignal chemiluminescent substrate (Pierce).

**ELISA for NF-κB Activity**

Cells were harvested with 0.25% trypsin-EDTA, and nuclear extracts were separated from cytoplasmic extracts by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacturer’s protocol. NF-κB activity in the nuclear extract was detect by the enzyme-linked immunosorbent assay (ELISA)-based Transcription Factor Assay Kits for NF-κB p50 and p65 (Thermo Scientific) or electrophoretic mobility shift assay (EMSA). For ELISA, NF-κB binding buffer and Poly(dI·dC) were added into the wells followed by nuclear extracts and incubated for 1 h with mild agitation. After three times wash with washing buffer, antibody against p65 was added and incubated for 1 h without agitation. After three times washing, the secondary antibody was added and incubated for 1 h without agitation. The chemiluminescent substrates were then added to the wells and chemiluminescence was measured by luminometer (Molecular devices Spectra Max M2).

**Electrophoretic Mobility Shift Assay**

A 22-bp synthetic oligonucleotide (5’-AGTTGAGGGGACTTTCCAGGC-3’) containing the specific NF-κB-binding site was annealed and labeled with γ-32P-ATP using T4 polynucleotide kinase. A DNA-binding reaction mixture of total 20 µL containing poly(dI·dC), labeled probe, binding buffer (10 mM pH 8.0 Tris HCl, 150 mM
KCl, 0.5 mM EDTA, 0.1% Triton-X 100, 12.5% Glycerol and 0.2 mM DTT) and 10 µg of cell nuclear extract was incubated at room temperature for 30 min and loaded onto a 5% non-denaturing polyacrylamide gel for electrophoresis. The gel was run in 0.5X Tris borate-EDTA buffer at 120 V, transferred to a double layer of Whatman paper and dried on a gel dryer for 45-60 min at 76 °C. The dried gel was used to expose an autoradiography film (Denville) at -80 °C and the NF-κB bound 32P-labeled DNA was detected, and the band intensity was analyzed by Image J.

**Reporter Transfection and Luciferase Activity Assay**

HeLa and HEK293 cells seeded in 96-well plate were cotransfected with NF-κB luciferase reporter containing 3X binding sites of NF-κB (kindly provided by Dr. Jian Jian Li, University of California, Davis) together with CMV-Renilla plasmid using lipofectamine 2000 (Life Technologies). At 24 h post transfection, cells were exposed to UVB radiation with or without L-NAME treatment. Luciferase activity was measured at 6 h post-UVB using Dual-Glo luciferase assay kit (Promega). The reading of luciferase signal was normalized to the reading of Renilla following manufacture’s instruction.

**Immunofluorescence Staining of NF-κB**

Cells were fixed with 3.6% formaldehyde for 10 min at room temperature, rinsed with PBS three times and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then blocked with blocking buffer (2 mg/mL BSA in PBS) for 1 h before incubating with mouse anti-p65 monoclonal antibody (Santa Cruz) for 1 h. After three times
washing with PBS, cells were incubated with a fluorescein-conjugated horse anti-mouse antibody (Vector Labs) for 1 h, washed with PBS and mounted with ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). The pictures were taken by NIKON Eclipse E600 with an exposure time of 0.1 s and analyzed with NIS-Elements Imaging Software Basic Research 3.2. Three cells of each group were randomly picked for quantification analysis.

\[cNOS\text{ Silencing Using RNA Interference}\]

Lipofectamine RNAiMAX reagent, scrambled siRNA, nNOS (human) siRNA (siRNA ID 117855) and eNOS (human) siRNA (siRNA ID 106158) were all purchased from Life Technologies. 2.5x10^6 cells were seeded in a 6-well tissue culture plate in antibiotic-free medium the day before transfection. 4 µL of Lipofectamine RNAiMAX and 2 µL (10 µM) siRNA were prepared separately in 100 µL DMEM medium (free of FBS and antibiotics) and then mixed and incubated for 5 min at room temperature. The mixture was then added to 1 mL of the medium with cells, the medium volume was added up to 2 mL 8 h after transfection. The cells were then incubated at 37 °C with 5% CO₂ for 16 h before UV radiation.

\[Real-TIme\text{ PCR}\]

Total RNA was extracted by the RNeasy Mini Kit (Qiagen). First strand cDNA was reverse-transcribed from 1 µg RNA using SuperScript III Reverse Transcriptase (Life Technologies). Briefly, in a 20 µL reaction system, 1 µL of 50 µM oligo(dT)₂₀ and
1 µL 10 mM dNTP mix were added, and heated to 65 °C for 5 min followed by incubation on ice for 2 min. Then 5X first-strand buffer and 0.1M DTT and 1µL of Superscript III Reverse Transcriptase were added with 5 min incubation at 25 °C followed by 60 min 50 °C and 15 min 75 °C heating. Quantitative real-time PCR using Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) (Thermo scientific) was then performed. Primers (Life Technologies) of the targeted human genes were as follow:

IKK: 5’-GAGATACAGCGAGCAGATGAC-3’,  
      5’-ATGACACCAACCTCAGCATAG-3’;

GAPDH: 5’-TGCACCACCAACTGCTTAGC-3’,  
       5’-GGCATGGACTGTGGTCATGAG-3’;

β-Actin: 5’-CACTCTTCCAGCCTTCCTCC-3’  
       5’-CGGACTCGTCATACCTCCTGCT-3’;

The real time PCR was performed on Bio-rad iCycler with the following protocol: 95 °C for 10 min; 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s for 40 cycles. Data were analyzed by ΔΔCt method.

Cell Survival Analysis

Total cell death was analyzed by determination of the loss of membrane phospholipid symmetry and membrane integrity using a fluorescein isothiocyanate (FITC) conjugated-annexin V (ANX5)/propidium iodide (PI) apoptosis detection kit (BD Biosciences) following the manufacturer’s protocol. Briefly, the cells were harvested by 0.25% trypsin digestion, combined with the cells floating in the medium and washed
twice with cold PBS. The cells were then suspended in ANX5 binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). The cell suspension was mixed with 5 µL ANX5-FITC and 5 µL PI. The cell mixture was incubated for 15 min in dark at room temperature and the ANX5/PI double-stained cells were analyzed using a FACSort Flow Cytometer (BD Science) equipped with CellQuest software (BD Science). Total cell number of 1x10⁵ was used for each analysis. The number of cells positive for ANX5, PI and both ANX5/PI were counted. Cell survival rate (R) was calculated as:

\[ R = \frac{1 \times 10^5 - \text{number of positive stained cells}}{1 \times 10^5}. \]

**Clonogenic Assay**

Immediate after treatment, cells were harvested with 0.25% trypsin-EDTA and counted, and then 5x10³ cells/well were plated in 6-well plates. After 6 days, cells were fixed with ice-cold methanol for 10 min at -20 °C and then stained by 1% crystal violet in 25% methanol for 10 min. Cells were then rinsed with distilled water and colonies with a size greater than 0.4 mm were counted by Kodak IS in vivo F system equipped with Kodak Molecular Imaging Software (Eastman Kodak).

**Statistics**

Student’s t-test was used for all the statistical data analysis and \( p<0.05 \) is considered as statically significant.
Results

Both cNOS Activation and NO* Elevation Mediate UVB-induced IκB Reduction via Translation Pathway

The phosphorylation of eIF2α and sequentially the inhibition of protein synthesis play an important role in regulation of UVC-induced IκB reduction and NF-κB activation (34,35). Because cNOS mediates UVB-induced eIF2α phosphorylation via activating eIF2A kinases 3 (PERK) and 4 (GCN2) (106), we examined whether cNOS also regulates UVB-induced IκB reduction and NF-κB activation. First, the effect of acute (1 h post UVB) or continuous (6 h post UVB) treatment of L-NAME (1 mM), a selective inhibitor of cNOS (149), on IκB protein expression level was analyzed in UVB-irradiated HaCaT cells. The data indicated that the acute or continuous treatment of L-NAME alone did not alter the IκB level in the cells (Fig. 4.1A, Lanes 2 and 3 vs. 1) without UVB irradiation. While the acute treatment had no statistic significant effect on IκB (Fig. 4.1A, Lane 5 vs. 4), the continuous treatment partially protected the reduction of IκB level at 6 h post-UVB (Fig. 4.1A, Lane 6 vs. 4). Further more, the protective effect of L-NAME on IκB at 6 h post-UVB was dependent on it concentration. The effect started at 0.1 mM and appeared to be saturated at 1 mM (Fig. 4.1B). These results indicated that cNOS activity plays a role in regulation of IκB after UVB radiation.

Activated cNOS mediates UVB-induced eIF2α phosphorylation via two pathways. Immediately after UVB radiation, in the first pathway, the coupled cNOS-catalyzed NO* production depletes L-Arg, which leads to the activation of GCN2; and in the second pathway, the uncoupled cNOS-catalyzed O2•− production, which rapidly reacts with NO*
to form ONOO\textsuperscript{\textendash} and activates PERK (106). To further determine the mechanism of cNOS-mediated NF-κB activation, we examined the effect of SNAP, a NO\textsuperscript{\textendash} donor (150), on the protective effect of L-NAME on UVB-induced IκB reduction. Interestingly, the partial protection of L-NAME on the IκB (Fig. 4.1C, Lane 13 vs. 8) was diminished with a low dose (50 μM, Fig. 4.1C, Lane 9 vs. 13) but not effect by higher doses (100-500 μM, Fig. 4.1C, Lane 10-12 vs. 13) of SNAP treatment, indicating NO\textsuperscript{\textendash} level also plays a role in regulation of IκB reduction after UVB radiation. In addition, UVB did not induce a detectable amount of Ser32/Ser36 phosphorylated IκB (p-IκB) but induced a high level of Ser52 phosphorylated eIF2α (p-eIF2α) (Fig. 4.2, Lane 3). The UVB-induced eIF2α phosphorylation was decreased with L-NAME treatment (Fig. 4.2, Lane 4 vs. 3) suggesting cNOS protects IκB reduction via translation regulation pathway.
Figure 4.1. The effect of cNOS on IκB reduction in the early-phase after UVB radiation. HaCaT cells were exposed to 50 mJ/cm² UVB irradiation with or without drug treatment as indicated. The expression level of indicated proteins were measured by Western blot analysis. (A) Cells were treated with acute (1 h post UVB) or continuous (kept until cell collection) of L-NAME (1 mM) and its statistical analysis. *, p<0.05 versus corresponded control; **, p<0.05 versus UVB alone. (B) Dose-dependent treatment of L-NAME and its effect on IκB. (C) Cells were treated with L-NAME and different dose of SNAP.
Figure 4.2. The effect of cNOS on IκB and eIF2α phosphorylation. HaCaT cells were exposed to 50 mJ/cm² UVB irradiation with or without L-NAME treatment as indicated. The amounts of p-IκB (Ser32/Ser36), p-eIF2α (Ser52), total IκB and total eIF2α protein level were measured by western blot analysis.

Both nNOS and eNOS are Involved in Regulation of UVB-induced NF-κB Activation

To determine whether the cNOS-mediated IκB reduction is correlated to NF-κB activation after UVB radiation, the effect of L-NAME on the DNA-binding activity of NF-κB was studied using an ELISA assay (Fig. 4.3) and EMSA assay (Fig. 4.4). The data indicated that the acute or continuous treatment of L-NAME alone had no statistically significant effect on the NF-κB activity (Figs. 4.3 and 4.4, Lanes 2, 3 vs. 1). For the ELISA assay, the data showed that the DNA-binding activity of NF-κB was increased to about 1.8 fold at 6 h after UVB irradiation (Fig. 4.3, Lanes 4 vs. 1). While the acute treatment of L-NAME did not have a statistic significant effect on NF-κB activity (Fig. 4.3, Lane 5 to 4), the continuous treatment of L-NAME inhibited the UVB-induced NF-κB activity to about 0.8 fold at 6 h post-UVB irradiation (Fig. 4.3, Lane 6 vs.
4. Similar results were observed with EMSA assay, which showed NF-κB activity increased to about 2.7 fold with UVB alone, and continuous treatment of L-NAME reduced the induction to 1.4 fold while acute treatment of L-NAME showed no statistically significant change (Fig. 4.4). To further confirm the role of cNOS in regulation of UVB-induced NF-κB, the NF-κB activity post-UVB in HeLa cells was determined using a NF-κB driven luciferase assay (Fig. 4.5). The data indicated that the activity of NF-κB could be induced by UVB radiation to about 1.4 fold, while with the treatment of L-NAME, the induction could totally be demolished (Fig. 4.5).

**Figure 4.3.** The effect of cNOS on DNA-binding activity of NF-κB at early phase post UVB. HaCaT cells were treated acute or continuous L-NAME (1 mM) with UVB exposure. Nuclear and cytoplasmic proteins of the cells were separated after cell collection at indicated time point. The nuclear proteins were used to study the NF-κB activity of binding to its target DNA sequence by using ELISA assay. Error bar represents standard deviation of three independent experiments. *, p<0.05 versus control group; **, p<0.05 versus UVB group.
Figure 4.4. UVB-induced NF-κB activity reduced by cNOS inhibitor. HaCaT cells were treated acute or continuous L-NAME (1 mM) with UVB exposure. Nuclear and cytoplasmic proteins of the cells were separated after cell collection at 6 h post UVB radiation. The nuclear proteins were used for the EMSA DNA-binding assay. Error bar represents standard deviation of three independent experiments. *, p<0.05 versus control group; **, p<0.05 versus UVB group.
Figure 4.5. The effect of L-NAME on UVB-induced NF-κB activity determined by NF-κB driven Dual-Glo luciferase assay in HeLa cells. HeLa cells were used for NF-κB driven dual-glo luciferase assay. Cells were transiently transfected with NF-κB driven luciferase reporter and CMV-Renilla plasmid. 24 h after transfection, cells were exposed to UVB radiation with or without L-NAME treatment. *, p<0.05 versus UVB alone group; **, p<0.05 versus UVB group.

In addition to DNA-binding activity of NF-κB, the continuous treatment of L-NAME also had a stronger inhibitory effect on nuclear translocation of NF-κB at 6 h post-UVB than the acute treatment of L-NAME (Fig. 4.6). As shown by the semi-quantitative analysis, nuclear NF-κB increased from 31% to 75% with UVB alone and decreased to 48% with continuous treatment of L-NAME (Fig. 4.6, Bottom Panel, Bar 1 vs. 4 vs. 6). Meanwhile the acute treatment of L-NAME did not statistically significantly affect nuclear translocation of NF-κB (Fig. 4.6, Bottom Panel, Bar 5 vs. 4). These results were correlated to the IκB reduction and NF-κB activity with the same treatment (Figs. 4.1 to 4.5).
Figure 4.6. The effect of L-NAME on translocation of NF-κB upon UVB radiation.

Top, HaCaT cells were treated with acute or continuous L-NAME with UVB radiation. At 6 h post UVB, cells were fixed with formaldehyde and stained with p65 antibody and FITC-conjugated secondary antibody, mounted with mounting reagent containing DAPI. Pictures were taken by NIKON Eclipse E600, and analyzed by NIS-Elements Imaging Software Basic Research 3.2, with an exposure time of 1s. Bottom, quantitative analysis of p65 fluorescent signal. Three cells of each group were randomly picked for the quantitative analysis of the signal intensity of p65 both in cytoplasm and nucleus. Error bar represents standard deviation of three randomly chosen cells. *, p<0.05 versus corresponded control; **, p<0.05 versus UVB alone.
**SiRNA Knockdown of nNOS and/or eNOS Knockdown Partially Protected IκB from Reduction and Retained NF-κB in Cytosol post-UVB**

Both nNOS and eNOS are expressed in HaCaT cells as previously showed (147). To determine the contribution of each isoform of cNOSs in regulation of UVB-induced reduction of IκB, the extent of effect of nNOS and/or eNOS knockdown on IκB expression after UVB irradiation was analyzed. The data showed that treating the cells with nNOS and/or eNOS siRNA partially reduced the expression level of both cNOSs (Fig. 4.7). The data also showed that while it did not alter the background level of IκB (Fig. 4.8, Lanes 3-5 vs. 1, 2), the siRNA knockdown of nNOS and/or eNOS could partially protect IκB from UVB-induced reduction (Fig. 4.8, Lanes 8-10 vs. 6, 7). The increased level of IκB correlated to an increased retention of NF-κB in cytosol post-UVB (Fig. 4.9) indicating the activity of both cNOSs contributes to UVB-induced IκB reduction and NF-κB nucleus translocation.

Since the effect of siRNA knockdown is not as significant as L-NAME treatment, HEK293 cells which is known to be null for both nNOS and eNOS (151) were used to confirm the effect of cNOS in UVB-induced NF-κB activation. The data demonstrated that the inducibility of NF-κB by UVB was totally diminished in cNOS null HEK293 cells (Fig. 4.10A), while TNFα treatment could induce the NF-κB activity to about 2 fold compared to control group (Fig. 4.10B). The result confirms that the early activation of NF-κB upon UVB radiation is cNOS dependent.
Figure 4.7. Western blot analysis of cNOS silencing in HaCaT cells. HaCaT cells were transiently transfected with either nNOS or eNOS siRNA or both as cNOS knockdown. Scramble siRNA was also transiently transfected into cells as negative control. Cells were lysed at 24 h post transfection and the protein level of either nNOS or eNOS was determined by western blot analysis. *, $p<0.05$ versus control.
Figure 4.8. The effect of cNOS silencing on IκB protein level. 24 h after siRNA transfection, cells were exposed to UVB radiation. At 6 h post UVB radiation, cells were lysed and the protein level of IκB was determined by western blot analysis with quantitative analysis. *, p<0.05 versus corresponded control; **, p<0.05 versus UVB alone.
Figure 4.9. The effect of cNOS silencing on NF-κB translocation.
24 h after siRNA transfection, cells were exposed to UVB radiation. At 6 h post UVB radiation, cells were fixed with formaldehyde and stained with p65 antibody and FITC-conjugated secondary antibody, with DAPI containing in the mounting reagent. Pictures were taken by NIKON Eclipse E600, and analyzed by NIS-Elements Imaging Software Basic Research 3.2, with an exposure time of 1s. Quantitative analysis was also performed for p65 fluorescent signal. Three cells of each group were randomly picked for the quantitative analysis of the signal intensity of p65 both in cytoplasm and nucleus. Error bar represents standard deviation of three cells. *, $p<0.05$ versus control; **, $p<0.05$ versus UVB.
Figure 4.10. NF-κB activity cannot be induced by UVB radiation in HEK293 cells. (A) HEK293 cells were exposed to UVB radiation and luciferase signal was measured 6 h post UVB radiation. (B) Cells were treated with TNFα for 3 h as positive control. *, p<0.05 versus control.

Cross Regulation among cNOS, IkB, NF-κB and IKKα after UVB Radiation

Previous studies suggested that IkB reduction in the early-phase (within 12 h) post-UVC is dependent on the background activity of IKKα, but independent of induced-activation of IKKα (31,34). Previous studies also suggested that UVC-induced NF-κB activation contributes to IkB synthesis (34). To further determine if UVB-induced cNOS activation is involved in regulation of IkB level via up-stream and/or down-stream signaling pathways, the effect of L-NAME with two commonly used NF-κB inhibitors, BAY11-7085 and JSH-23 on ubiquitin or proteasomal degradation pathway-mediated IkB degradation was compared. BAY11-7085 inhibits IkB phosphorylation and JSH-23 interferes the binding of NF-κB to its target DNA (152,153). The data showed that L-NAME and BAY11-7085, but not JSH-23, had the same effect on partially protecting IkB from UVB-induced reduction (Fig. 4.11. Lanes 5, 11 or 8 vs. 2). Interestingly, in
combined treatments, the effect of L-NAME and JSH-23, but not BAY11-7085, could be added on top of the effect of a proteasome inhibitor MG132 or an ubiquitin ligase inhibitor Ro106 in protecting IκB from UVB-induced reduction (Fig. 4.11. Lanes 6,7; 9,10; 12, 13; vs. 3, 4). These results indicated that cNOS is independent of ubiquitin and proteasome pathway in protecting IκB reduction after UVB radiation.

![Figure 4.11](image-url)

**Figure 4.11.** The ubiquitination and proteasomal effects on UVB-induced IκB reduction. HaCaT cells were treated with L-NAME (1 mM), BAY11-7085 (5 µM), JSH-23 (10 µM), with or without combined treatment of Ro106 (10 µM) and MG132 (10 µM). The amount of total IκB was measured by Western blot analysis. The ratio of IκB/β-actin represents the average of 3-6 sets of data. *, p<0.05 versus control; **, p<0.05 versus UVB alone; #, p<0.05 versus corresponded UVB without MG and Ro treatment (Lane 2, 5, 8 or 11); ^, p<0.05 versus UVB with corresponded MG132 or Ro106 (Lane 3 or 4).
Since the IKKα plays a critical role in regulation of IκB degradation through ubiquitin and proteasome pathway (154-156) and IKK expression is regulated by NF-κB (157), whether UVB-induced cNOS-mediated NF-κB activation would have an effect on IKKα expression was examined. Again, L-NAME, BAY11-7085 and JSH-23 were used in the study. Data showed that the three inhibitors and UVB alone had no effect on IKKα expression by themselves (Fig. 4.12, Lanes 2-5 vs. 1). However, the combinational treatment of UVB with each inhibitor significantly reduced the IKKα expression (Fig. 4.12, Lanes 6-9 vs. 5). Moreover, the continuous treatment was more effective than the acute treatment of L-NAME on the inhibition of IKKα expression (Fig. 4.12, Lane 9 vs. 8). To determine the mechanism for the NF-κB mediated IKKα expression post-UVB, the mRNA level of IKKα under the same treatments was investigated. The results indicated that the mRNA of IKKα decreased to approximately 30% at 6 h post UVB while none of the three inhibitors could rescue the reduction (Fig. 4.13) indicating cNOS-mediated NF-κB activation is critical in preventing IKKα degradation post-UVB.
Figure 4.12. The effect of NF-κB activity on IKKα protein level. HaCaT cells were treated with L-NAME 1 h prior to UVB radiation, BAY11-7085 and JSH-23 were added immediate after UVB. Cells were lysed at 6 h post UVB radiation and the amount of IKKα was measured by Western blot analysis and quantitative analysis. Error bar represents standard deviation of three sets independent experiments. *, p<0.05 versus control group. **, p<0.05 versus UVB alone.
Figure 4.13. The effect of NF-κB activity on IKKα mRNA level. HaCaT cells were treated with L-NAME 1 h prior to UVB radiation, BAY11-7085 and JSH-23 were added immediate after UVB. Cells were collect at 6 h post UVB irradiation. Total RNA was extracted for the cells and mRNA level of IKKα was determined by qPCR. Error bar represents standard deviation of three sets of independent experiments. *, p < 0.05 versus control group.

**NF-κB Activation Protects Cell Death upon UVB Radiation**

Since both cNOS and NF-κB have dual roles in regulation of apoptosis and cell survival (107,158-160), both the short-term (4-6 h) and long-term (6 days) effects of L-NAME, BAY11-7085 and JSH-23 were examined on cell survival and recovery after UVB radiation using apoptotic and clonogenic assays respectively.

For short-term effect of 50 mJ/cm² UVB radiation, the irradiation alone decreased the cell survival rate to approximately 85% and 71% at 4 h and 6 h post-irradiation respectively (Fig. 4.14A, Lanes 4 and 7). While the drug alone did not have statistically significant effect on cell survival (Fig. 4.14A, Lanes 2, 3 vs. 1), inhibition of NF-κB activity by BAY11-7085 and JSH-23 further decreased cell survival rate from 71% to approximately 66% at 6 h post-UVB (Fig. 4.14A, Lanes 8-9). Interestingly, while the
continuous treatment of L-NAME increased the cell survival rate from 81% to 85% at 4 h but had not statistically significant effect at 6 h post-UVB (Fig. 4.14B, Lane 5 vs. 3 and Lane 8 vs. 6), the acute treatment of L-NAME increased cell survival rate to approximately from 81% to 88% at 4 h and 69% to 74% at 6 h post-UVB (Fig. 4.14B, Lane 4 vs. 3 and Lane 7 vs. 6). Again, the treatment of drug alone had no significant effect on the cell survival rate (Fig. 4.14B, Lane 2 vs. 1).
Figure 4.14. UVB-induced cell apoptosis assay with or without drug treatments. HaCaT cells were exposed to UVB-irradiation with different drug treatments, and were collected 4 h and 6 h post-UVB. Annexin V / PI apoptosis detection kit was used to detect cell apoptosis. The numbers of cells positive for ANX5, PI and both ANX5/PI were counted. Cell survival rate (R) was calculated as: R=\([1\times10^5 - \text{numbers of positive stained cells}] / 1\times10^5\). The error bars present the standard deviations of three sets of independent experiments. (A) HaCaT cells were exposed to UVB-irradiation with and without JSH-23 or BAY11-7085 treatment. (B) Cells were exposed to UVB-irradiation and treated with acute or continuous L-NAME treatment. *, \(p<0.05\) versus corresponded control; **, \(p<0.05\) versus corresponded UVB group.
For long-term effect of L-NAME and NF-κB inhibitors on cells, a lower dose (8 mJ/cm²) of UVB was used and clonogenic assay was performed. UVB radiation alone reduced the colony formation to 29-37% (Fig. 4.15). The 6 h and 24 h treatment of JSH-23 or L-NAME alone did not show a statistically significant effect on colony formation, but the 144 h (6 days) treatment of either JSH-23 or L-NAME alone reduced colony formation (Fig. 4.15A, B). With UVB irradiation, the treatment of JSH-23 further reduced colony formation from 30% to 16-9% depending on the length of drug treatment (Fig. 4.15A, Lanes 6-8 vs. 5). However, the treatment of L-NAME increased colony formation from 30% to 54-33% depending on the length of drug treatment (Fig. 4.15B, Lanes 6-8 vs. 5). Further analysis revealed that the double treatment with JSH-23 and L-NAME had not statistically significant effect on colony formation after UVB irradiation (Fig. 4.15C, Lane 5 vs. 1), indicating that JSH-23 and L-NAME could cancel each other’s effect on UVB-reduced colony formation.
Figure 4.15. Clonogenic assay of UVB-irradiated HaCaT cells. Cells with or without drug treatment were exposed to 8 mJ/cm² UVB irradiation, and 5x10³ cells were plated and cultured in 6-well plates for 6 days. Cells were then fixed with cold methanol and stained by 1% crystal violet in 25% methanol. The error bars present the standard deviations of three sets of independent experiments. (A) Cells were treated with L-NAME for 1 h before UVB irradiation, and removed L-NAME at 6, 24, 144 h after UVB irradiation. (B) JSH-23 were added immediate after UVB irradiation and removed at 6, 24, 144 h after UVB irradiation. (C) L-NAME and JSH-23 were added together for 6 h treatment after UVB irradiation. *, p<0.05 versus corresponded control; **, p<0.05 versus corresponded UVB group; ^, p<0.05 lane 5 versus lane 3.
Discussion

Previous studies indicated that UVC-induced eIF2α phosphorylation played an important role in regulation of NF-κB activation in the early phase (within 12 h) of radiation (34,35,40,161). Previous studies from our lab also showed that UVB radiation induced an immediate activation of cNOS, which mediated the activation of eIF2α kinases PERK and GCN2 (106,107). In this study, it has been demonstrated that cNOS activation contributed to the activation of NF-κB post-UVB irradiation. Inhibition of cNOS with a continuous treatment of L-NAME led to a partial inhibition of IκB reduction (Fig. 4.1) and NF-κB activation (Figs. 4.3-4.6) through translational regulation pathway (Fig 4.2). The role of cNOS in regulation of UVB-induced NF-κB activation was confirmed by an NF-κB luciferase reporter assay indicating that L-NAME could inhibit the UVB-induced luciferase expression; and the inducibility of luciferase expression by UVB was diminished in cNOS null HEK293 cells (Figs. 4.5, 4.10). In addition, it appears that both eNOS and nNOS are involved in the regulation of UVB-induced NF-κB activation because siRNA knockdown nNOS or eNOS can partially inhibit IκB reduction and NF-κB nuclear translocation post-UVB irradiation (Figs. 4.7-4.9). However, not like the cNOS null HEK293 cells, the effects of siRNAs on IκB expression and NF-κB activation were limited even with double cNOS/nNOS knockdown, which suggest that cNOS activity might be more critical than its quantity in regulation of NF-κB activation after UVB irradiation; and that knockdown one NOS may lead to the activation of other NOSs as previously reported (162).
The translational inhibition of IκB synthesis as well as ubiquitin and proteasome-mediated IκB degradation coordinately regulate the IκB reduction after UVC irradiation (34,35). The data showed that inhibition of the ubiquitin and proteasome pathway by MG132 and Ro106 could restore the IκB level after UVB irradiation (Fig. 4.11, Lanes 3-4), indicating that the pathway plays a critical role in regulation of UVB-induced IκB reduction. The data also showed that the inhibition of IκB phosphorylation by BAY11-7085 or cNOS activation by L-NAME, but not the inhibition of NF-κB activity by JSH-23, increased IκB level after UVB irradiation (Fig. 4.11, Lanes 5, 11 and 8 vs. 2). Interestingly, the protective effect of L-NAME was additive to the effect of MG132 or Ro106 (Fig. 4.11, Lanes 11-13 vs. 5-7). These results agreed with the previously report suggesting that cNOS-mediated IκB reduction after UVB irradiation is independent of ubiquitin and proteasome pathway (106). IKKα phosphorylates IκB and promotes its degradation via ubiquitin and proteasome pathway (14,154-156). Previous studies suggested that only background activity but not activation of IKKα is required for UVC-induced reduction of IκB (31,33,34). The data indicated that while the protein status of IKKα was not statistically significantly changed after UVB irradiation (Fig. 4.12, Lane 5 vs. 1), inhibition of IκB phosphorylation, NF-κB activity or cNOS could significantly reduce the protein level of IKKα after UVB irradiation (Fig. 4.12, Lanes 6-9). On the other hand, the mRNA status of IKKα was substantially reduced upon UVB irradiation and none of the drug treatment resulted in a notable change of the mRNA level of IKKα (Fig. 4.13). Since the only common function of the treatments was to inhibit NF-κB
activation after UVB irradiation, these results suggest that NF-κB activation post-UVB stabilizes IKKα.

Both cNOS and NF-κB play dual roles in regulation of apoptosis (158-160,163-166). To better understand the roles of cNOS and NF-κB in regulation of cell fate after UVB irradiation, we determined the effects of JSH-23 and L-NAME on cell death and recovery after UVB irradiation. The data indicated that inhibition of NF-κB activity had an opposite effect than the inhibition of cNOS on UVB-induced cell death (Fig. 4.14A vs. B), even thought L-NAME could inhibit the activation of NF-κB (Figs. 4.3-4.6). Similar results were observed from clonogenic assays (Fig. 4.15). One possible reason is that L-NAME inhibits the activity of NF-κB via inhibiting cNOS, which contributes to the production of ONOO⁻ after UVB irradiation. Thus when L-NAME inhibited NF-κB, it also reduced the production of ONOO⁻, which promotes cell death (165,167,168). An elevation of ONOO⁻ can lead to the oxidation of cholesterol (169), which plays a critical role in regulating UVB-induced apoptosis via induction of lipid rafts clustering and Fas aggregation (170,171); and that NF-κB activation can induce iNOS expression (172) and an elevated NO⁻ production inhibits caspase 3 activation in late stage of UVB irradiation (173). Based on these reports and the above data, it is proposed that the early activation of cNOS promotes apoptosis via induction of ONOO⁻ elevation, and inhibits apoptosis via activation of NF-κB followed by induced expression of iNOS and escalated NO⁻ production. This hypothesis is further supported by the data that the acute treatment of L-NAME (not inhibited NF-κB) was slightly better than the continuous treatment of L-NAME (inhibited NF-κB) in protection of UVB-induced cell death (Fig. 4.14B, Lanes 4,
7 vs. 5, 8); and that JSH-23 and L-NAME could cancel each other’s effect on UVB-reduced colony formation (Fig. 4.15C, Lane 5 vs. 1).

Conclusion

To conclude, a signaling pathway is proposed as shown in Fig. 4.16. UVB irradiation activates cNOS, which leads to the phosphorylation of eIF2α and translational inhibition of IκB synthesis. With the intact degradation pathway of IκB, the translation inhibition of IκB reduced IκB protein level thus activates NF-κB, which protects IKKα from UVB-induced reduction. The activated NF-κB also protects cells from UVB-induced apoptosis; yet, this anti-apoptotic function can be neutralized by the pro-apoptotic effect of ONOO⁻.

Figure 4.16. Proposed model for signaling pathways involved in activation of NF-κB in the early phase upon UVB radiation.
CARNOSOL IN PROTECTING UVB-INDUCED SKIN DAMAGE

Introduction

Carnosol is a natural compound extracted from rosemary and sage, which are common ingredients used in traditional Mediterranean cuisine (174). Mediterranean diet and herbs have been associated with a decreased risk of cardiovascular and diabetic diseases for decades (175,176). Most recently, the identification and characterization of the anti-cancer properties of these herbs has received intensive interest (177,178). Among all the compounds extracted from these herbs, carnosol, first isolated from sage in 1941, has been demonstrated to be beneficial to health for its anti-inflammation, anti-oxidation and anti-cancer properties (179-181). Because of its structure similarity to sex hormones, research has been done on the effect of carnosol mainly on breast and prostate cancers (Fig 5.1) (182-186).

![Chemical structure of carnosol](image)

*Figure 5.1. The chemical structure of carnosol.*

These previous reports successfully demonstrated that carnosol could physically bind to estrogen and androgen receptors, and thus modulates the receptor function to
inhibit cancer growth (183,184). However, very little is known on the effect of carnosol on skin cancer. UV radiation is a well-known carcinogen for skin cancer development. Overexposure to ultraviolet B light (UVB) radiation leads to various skin cancers, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and cutaneous malignant melanoma (1,2). One of the possible carcinogen mechanisms is that UVB radiation causes DNA damage through the induction of free radicals and reactive oxygen species (ROS) (107). As carnosol is known to be an anti-oxidant with its phenol structure, there is reason to believe that carnosol may be able to reduce the UV-induced ROS and thus protect skin cell from UV-induced damage, and may potentially be able to reduce UV-induced skin cancer.

In this study, the data demonstrated that carnosol could reduce the UVB-induced intracellular ROS level, which consequently lead to DNA damage protection and cell death reduction. In the meanwhile, combined treatment of carnosol with UV radiation could reduce the growth of skin cancer cells in a synergistic effect.

Material and Methods

Cell Culture

Human keratinocyte HaCaT cells (kindly provided by Dr. Nihal Ahmad, University of Wisconsin-Madison) and mouse embryonic fibroblast (MEF) cells were grown in Dulbecco’s Minimal Essential Medium (Cellgro) supplemented with 10% fetal bovine serum and penicillin/streptomycin, at 37°C with 5% CO2.
**UVB Radiation**

UVB was generated from a Bench XX-Series UV Lamp (UVP Inc.) equipped with two 15-watt UVB tubes (UVP Inc.). The intensity of UVB was calibrated by a UVP model UVX digital radiometer (UVP Inc.) after the lamps warmed up for 5 min. The dose rate for 8 mJ/cm² or 50 mJ/cm² of UVB radiation was 0.8 or 3.8 mW/s respectively. Medium was removed before exposing the cells to UVB. After UVB radiation, fresh medium was added to the culture plates with or without drugs, and the cells were continue incubating at 37 ºC with 5% CO₂ until further analysis.

**Drug Treatment**

Carnosol (Cayman) was added to cells at indicated concentration at 1 h before exposing the cells to UVB radiation. After radiation, cells were continuously incubated with or without carnosol in the medium at 37 ºC with 5% CO₂ until further analysis.

**ROS Measurement**

CM-H₂DCFDA (Invitrogen) was used to measure the total ROS level in cells. CM-H₂DCFDA was dissolved in DMSO to a stock solution of 500 μM and diluted in PBS to final concentration of 5 μM. CM-H₂DCFDA was added into cells 1 h prior to UVB exposure and the fluorescence of the dye was measured every 20 minutes or read at indicated time point after UVB radiation using luminometer (Molecular devices Spectra Max M2).
**Nitric Oxide and Peroxynitrite Measurement**

The level of NO$^+$ was measured by 5 mM diaminofluorescein diacetate (DAF-2DA) (ex490/em520 nm) and ONOO$^-$ was measured by 5 mM diaminorhodamine (DAR) (ex488/em515 nm). 1x10^5 HaCaT cells were seeded in 96-well plate one day before measurement. At indicated time point after UVB radiation and treatment with or without carnosol, medium was replaced with 200 µL reaction buffer, containing 10 µL L-arginine (1 mM) and 0.1 µL dye. The cells were then incubated in dark for 2 h at room temperature and the fluorescence was measured by luminometer (Molecular devices Spectra Max M2). The reading was then normalized by AlamarBlue cell viability assay (Life technology). 10% of the Alamar Blue reagent was added to cells with medium followed by 2 h incubation at 37°C in dark then read the fluorescent at ex570/em590 nm using luminometer (Molecular devices Spectra Max M2).

**Western Blot Analysis**

Cells were lysed with Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl pH 8.0, 0.1% SDS) with proteinase inhibitor mixture (Complete™, Roche Molecular Biochemicals) at indicated time point. Cell lysate was incubated on ice for 15 min and then centrifuged at 14,000 rpm at 4 °C for 15 min. Protein concentration was measured by Protein DC Assay kit (Bio-Rad Laboratories). Equal amounts of protein were subjected on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then blocked in 5% milk in Tris buffered saline plus Tween 20 (TBST) for 45 min and probed with anti-γH2AX (Cell Signaling),
anti-pCHK (Cell Signaling), anti-p276-NF-κB (Santa Cruz), anti-NF-κB p65 (Santa Cruz), anti-IκB (Santa Cruz), or anti-β-actin (Santa Cruz) at 4 °C overnight. After washing with TBST, the membrane was incubated with corresponding HRP-conjugated anti-rabbit or anti-mouse antibody for 45 min at room temperature. Membrane was then washed three times in TBST followed by two times in TBS, and developed in West Pico Supersignal chemiluminescent substrate (Pierce).

**Electrophoretic Mobility Shift Assay**

A 22-bp synthetic oligonucleotide (5’-AGTTGAGGGGACTTTCCCAGGC-3’) containing the specific NF-κB-binding site was annealed and labeled with \( \gamma^{32}P \)-ATP using T4 polynucleotide kinase. A DNA-binding reaction mixture of total 20 µL containing poly(dI:dC), labeled probe, binding buffer (10 mM pH 8.0 Tris HCl, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton-X 100, 12.5% Glycerol and 0.2 mM DTT) and 10 µg of cell nuclear extract was incubated at room temperature for 30 min and loaded onto a 5% nondenaturing polyacrylamide gel for electrophoresis. The gel was run in 0.5 X TBE buffer at 120 V, transferred to a double layer of Whatman paper and dried on a gel dryer for 45-60 min at 76 °C. The dried gel was used to expose an autoradiography film (Denville) at -80 °C and the NF-κB bound \( ^{32}P \)-labeled DNA was detected.

**Cell Survival Analysis**

Total cell number of \( 1 \times 10^5 \) was used for each analysis using flow cytometer. Cell survival rate (R) was calculated as: \( R = [1 \times 10^5 - \text{number of positive stained cells}] / 1 \times 10^5 \).
Fluorescein isothiocyanate (FITC) conjugated-annexin V (ANX5)/propidium iodide (PI) apoptosis detection kit (BD Biosciences) were used to stain the cells through the determination of the loss of membrane phospholipid symmetry and membrane integrity. Briefly, the cells were harvested by 0.25% trypsin digestion, combined with the cells floating in the medium and washed twice with cold PBS. Cells were then suspended in ANX5 binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). The cell suspension was mixed with 5 µL ANX5-FITC and 5 µL PI. The cell mixture was incubated for 15 min in dark at room temperature and the ANX5/PI double-stained cells were analyzed using a FACSort Flow Cytometer (BD Science) equipped with CellQuest software (BD Science).

**Clonogenic Assay**

Immediate after treatment, cells were harvested with 0.25% trypsin-EDTA and counted, and then 5x10³ cells were plated in 6-well plates. After 6 days, cells were fixed with cold methanol for 10 min at 20 °C and then stained by 1% crystal violet in 25% methanol for 10 min. Cells were then rinsed with distilled water and colonies with a size greater than 0.4 mm were counted by Kodak IS in vivo F system equipped with Kodak Molecular Imaging Software (Eastman Kodak).

**Statistics**

Student’s t-test was used for all the statistical data analysis and p<0.05 is considered as statically significant.
Results

_Carnosol Reduces UVB-induced ROS in Human Keratinocytes_

To determine the role of carnosol in UVB-induced intracellular ROS elevation, the general oxidative stress level was measured in the HaCaT cells using CM-H$_2$DCFDA dye upon UVB radiation with or without carnosol treatment. Firstly, the cellular ROS level was measured at 6 h post UVB radiation (50 mJ/cm$^2$) with a dose-dependent carnosol manner from 0.1 µM to 30 µM. Treatment without UVB radiation but with carnosol alone hardly had any effect on the ROS level. With UVB radiation, on the other hand, the ROS level increased about 2 fold. With 0.1 µM or 0.5 µM carnosol treatment, there was no significant decrease of the ROS level, while with 10 µM carnosol treatment, the ROS level decreased to about 1.5 fold, with a further decrease to about 1.3 fold with 20 or 30 µM carnosol treatment (Fig 5.2). Since 30 µM carnosol showed no further deduction on ROS level compared to 20 µM, 20 µM carnosol treatment was used for further analysis.
Figure 5.2. Dose dependent inhibition of intracellular ROS level by carnosol upon UVB radiation. HaCaT cells were seeded in 96 well plate and incubated with CM-H$_2$DCFDA dye one hour prior to UVB exposure, with various doses of carnosol treatment. ROS was measured at 6 h post UVB radiation using ex490/em520 nm. The data represents three to five sets of independent experiments. *, $p<0.05$ versus corresponded control group, **, $p<0.05$ versus UVB alone.

Next, the cellular ROS level in a time dependent manner upon UVB radiation was measured. The ROS level at 20 min interval for up to 12 h was recorded post UVB radiation. While there is no significant change in ROS level with carnosol treatment alone, from as early as 20 min, carnosol showed the ability to decrease the ROS level, and maintained the deduction of up to 10 h post UVB radiation (Fig 5.3A). While take a closer reading at every 6 h, carnosol decreased the ROS level for about 20% right after the UVB radiation, and had about 30% decrease compared to UVB radiation alone at 240, 480, and 720 min after radiation (Fig 5.3B).
Figure 5.3. Carnosol reduces intracellular ROS level after UVB radiation. HaCaT cells were seeded in 96 well plate and CM-H$_2$DCFDA dye was used to measure intracellular ROS level. Carnosol (20µM) was added to cells 1 h prior to UVB exposure, and ROS level was measured at 20 min intervals post UVB. (A) Intracellular ROS level at 20 min intervals with or without carnosol treatment. (B) Statistical analysis of intracellular ROS level at 240, 480, 720 min post UVB radiation with or without carnosol (20 µM) treatment. *, $p<0.05$ versus corresponded control group; **, $p<0.05$ versus corresponded UV group.
Previous research showed that both NO and ONOO\textsuperscript{\cdot} would be induced by UVB radiation (147,187). By using DAF-2DA (5 mM) and DAR (5 mM), the effect of carnosol on intracellular NO and ONOO\textsuperscript{\cdot} level were also measured, in a time dependent manner upon UVB radiation. As previously showed, both NO and ONOO\textsuperscript{\cdot} level would be increased upon UV radiation. With carnosol treatment, the data showed that while carnosol had little effect on the NO or ONOO\textsuperscript{\cdot} levels of cells without radiation, it can reduce both NO and ONOO\textsuperscript{\cdot} level on average of about 40% from 2 h to 24 h post UVB radiation (Fig. 5.4).
Figure 5.4. Quantitative analysis of NO$^+$ and ONOO$^-$ after UVB radiation. HaCaT cells were irradiated with UVB radiation in the presence or absence of carnosol (20 µM). At indicated time point, cells were stained with DAF-2DA or DAR. The fluorescent intensity was then measured and normalized to cell viability. (A) Quantitative measurement of NO$^+$. (B) Quantitative measurement of ONOO$^-$. *, $p<0.05$ versus corresponded group with carnosol treatment upon UVB radiation.

Carnosol Protects UVB-induced DNA Damage

The free radicals induced by UVB radiation is known to be able to cause indirect DNA damage in skin cells, and the accumulation of DNA damage will cause cancer development (188-190). As carnosol could reduce the UVB-induced ROS elevation, if
carnosol could protect cells from UVB-caused DNA damage was investigated next. Both human keratinocyte (HaCaT) and mouse embryonic fibroblast (MEF) cells were used here, and the protein levels of phospho-H2AX (γH2AX) and phospho-Chk were used as DNA damage marker. In HaCaT cells, at early phase (15 min and 1 h) post UVB radiation, the phosphorylation level of both H2AX and Chk was significantly reduced by carnosol treatment (Fig 5.5A). Similar results were observed in MEF cells as both the phosphorylation level of H2AX and Chk reduced at early stage post UVB radiation (Fig 5.5B). These data indicated that carnosol could reduce UVB-induced DNA damage, possibly through the reduction of ROS level.

Figure 5.5. Carnosol partially protects UVB-induced DNA damage. (A) HaCaT cells and (B) MEF cells were exposed to 50 mJ/cm² UVB radiation in the presence or absence of carnosol. Cells were lysed at indicated time point and protein levels of phosphorylated H2AX (γH2AX) and phosphorylated Chk were measured. The data represents three sets of independent experiments.
As carnosol reduced intracellular ROS level and protected cells from UVB-induced DNA damage, if it could protect cell death upon UVB radiation was further investigated. Both time and dose dependent assays were performed using HaCaT cells. In dose dependent assays (1-30 µM), there was no significant cell survival change with carnosol treatment alone (Fig. 5.6A, Lanes 2-5 vs. 1). With UVB radiation, carnosol had no statistically significant effect at 1 or 10 µM (Fig. 5.6A Lanes 7,8 vs. 6); while at 20 µM carnosol treatment, cell survival rate was increased from 20% of UVB radiation alone to 30% (Fig. 5.6A Lane 9 vs. 5), with no further increasing at 30 µM carnosol treatment while there was limited toxicity of carnosol to the cells without UVB radiation (Fig. 5.6A Lane 10 vs. 5). For the time dependent cell survival rate assay, at 6 h post UVB radiation, there was no significant with or without 20 µM carnosol treatment (Fig. 5.6B Lane 4 vs. 3); while at 12 h post UVB radiation, cell survival rate increased from 30% to 40% (Fig. 5.6B Lane 6 vs. 5), and at 24 h post UVB radiation, cell survival rate increased from 7% to 15% (Fig. 5.6B Lane 8 vs. 7), which indicated that the protection of cell lasted at least up to 24 h post UVB radiation (Fig. 5.6B). Similar results were also observed in MEF cells, the cell survival rate was increased from about 88% to 97% at 12 h (Fig. 5.6C Lane 4 vs. 3), 80% to 90% at 24 h (Fig. 5.6C Lane 6 vs. 5), 62% to 70% at 36 h (Fig. 5.6C Lane 8 vs. 7), 43% to 58% at 48 h respectively (Fig. 5.6C Lane 10 vs. 9). Taken together, the data suggest that carnosol could protect UVB-induced cell death, possibly mediated by protection of DNA damage and reduction of elevated ROS level.
Figure 5.6. Carnosol increases cell survival rate upon UVB radiation.
Cells were exposed to UVB radiation with different dose of carnosol treatment. At indicated time point, cells were collected and stained by Annexin V/PI to detect cell death. For every 1X10⁵ cells counted, cell survival rate (R) was calculated as: R=[1x10⁵ – number of positive stained cells]/1x10⁵. (A) HaCaT cell survival rate with different concentrations of carnosol treatment at 12 h post UVB radiation. (B) Time dependent assay in the presence or absence of carnosol treatment in HaCaT cells. (C) MEF cells treated with 20 µM carnosol at indicated time point. The error bars present the standard deviation of three sets of independent experiments. *, p<0.05 versus control group, **, p<0.05 versus corresponded UV group.
Carnosol Inhibits UVB-induced NF-κB Activity

On the other hand, the protective effect of carnosol on cell death is not as significant as it is predicted to be, which suggests that there may be other possible signaling pathways manipulated by carnosol. As ROS may mediate the induction NF-κB activity in UVB radiation, and carnosol reduces UVB-induced ROS level in cells, if carnosol will also affect the activity of NF-κB was studied. The effect of carnosol on IκB protein level in a time and dose dependent manner was firstly determined. The data showed that IκB level was decreased upon UVB radiation alone (Fig. 5.7A Lanes 9, 5 vs. 1), and carnosol could partially protect IκB level at 2, 4 h post UVB radiation in a dose dependent manner (1, 10, 20 µM) (Fig. 5.7A Lanes 6-8 vs. 5; Lanes 10-12 vs. 9) and the protection exists at least until 6 h (Fig. 5.7B) in HaCaT cell. Similar results were also observed in MEF cells with the partial recovery of IκB level when the cells were treated with carnosol (20 µM) (Fig 5.7C). As the phosphorylation of NF-κB usually indicates its activity, the phosphorylation of NF-κB at Serine 276 site was detected, correspondingly the data also showed that carnosol could reduce the phosphorylation of NF-κB at S276 at 20 µM concentration (Fig 5.8). To further confirm the NF-κB activity, EMSA assay was performed to test the binding activity of NF-κB to its DNA target. Upon UVB radiation, NF-κB activity induced by 20% at 2 h (Fig. 5.9 Lane 5 vs. 1) and peaked at about 2.6 fold at 4 and 6 h (Fig. 5.9 Lanes 6,7 vs. 1). With the treatment of carnosol (20 µM), there was no statistically significant change at 2 h (Fig. 5.9 Lane 8 vs.5), and the NF-κB activity induction could be significantly reduced at 4 and 6 h by reducing to 2.2 and 1.5
fold respectively (Fig. 5.9 Lanes 9, 10 vs. 5.6). Altogether, the data suggest that carnosol could inhibit NF-κB activity, possible through the reduction of ROS level. Since NF-κB is known to be anti-apoptosis, inhibiting its activity may lead to the induction of cell death.

**Figure 5.7.** Carnosol inhibited UVB-induced NF-κB activation. Cells were exposed to UVB radiation in the presence or absence of carnosol. Cells were lysed at indicated time point and protein levels were measured by western blot analysis. (A) Western blot for IκB protein level at 2, 4 h post UVB radiation with indicated concentration of carnosol in HaCaT cells. (B) Western blot for IκB protein level in HaCaT cells at 6 h post UVB radiation at 10 or 20 µM carnosol treatment. (C) Western blot analysis for IκB protein level in MEF cells at 6 h post UVB radiation in the presence or absence of carnosol.
Figure 5.8. Carnosol inhibited UVB-induced NF-κB phosphorylation. HaCaT cells were collected at 6 h after UVB radiation treated with 0, 10 or 20 μM carnosol. Western blot analysis was performed for S276 site phosphorylation of NF-κB.
Figure 5.9. Electromobility shift assay for NF-κB binding activity. (A) HaCaT cells were collected at 2, 4, 6 h post UVB radiation in the presence or absence of carnosol treatment (20 μM). (B) Statistical analysis of the EMSA assay using two to three sets of independent experiments. *, p<0.05 versus control group; **, p<0.05 versus UV group at indicated time point.
Carnosol Inhibits Cancer Cell Progression with a Synergetic Effect upon UVB Radiation

Since carnosol protects cell death upon UVB radiation, it would also be interesting to learn how it affects skin cancer cells. Clonogenic assay was performed to study the progression rate of A431 cancer cell upon UVB radiation with or without carnosol treatment. With carnosol treatment alone, the colony formation rate decreased to about 65% (Fig. 5.10 Lane 2 vs. 1), while UVB radiation alone decreased the colony formation to about 43% (Fig. 5.10 Lane 3 vs. 1). With combined treatment of carnosol and UVB radiation, the colony formation was further decreased to about 17%. The data indicated that double treatment with carnosol and UVB radiation together could lead to a synergistic effect on reduction of cancer cell survival (Fig. 5.10).

![Graph showing relative colony formation](image)

**Figure 5.10.** Carnosol decreases A431 cancer cell colony formation. Squamous carcinoma cell A431 was used for the clonogenic assay. Cells were exposed to 8 mJ/cm² UVB exposure in the presence or absence of carnosol. 5x10³ cells were plated into six well plate immediately after treatment and incubated for 8 days. The error bars present the standard deviation of three sets of independent experiments. *, p<0.05 versus control group; **, p<0.05 versus UV group at indicated time point.
Discussion

UV radiation is known to cause the induction of ROS, which causes oxidative stress and DNA damage in cells (187,190). The accumulation of DNA damage in cells will consequently lead to cancer development. Phenolic compounds are known to be able to reduce the ROS in cells as antioxidants (191-193). In this study, the data revealed that carnosol, a natural compound with a phenol structure, could reduce the cellular NO, ONOO-, and total ROS level in irradiated human skin cells (Figs. 5.2-5.4). Moreover, this reduction of free radicals could lead to the partial protection of UVB-induced DNA damage (Fig. 5.5). The result suggested that carnosol, by protecting DNA damage of irradiated cells may have the potential to reduce the carcinogenesis caused by over exposure to UV light. In the meanwhile, UVB radiation is also known to induce NF-κB activity via ROS, which protects cell from apoptosis (194) (195). By inhibiting ROS, carnosol inhibits the UV-induced NF-κB activity at the same time (Figs. 5.7-5.9). Therefore, although inhibit ROS and protect DNA damage in the early phase upon UVB radiation, inhibiting NF-κB at the same time led to a minor protection in cell death (Fig. 5.6). Because of its structure similarity to sex hormones, carnosol has been shown to have the characteristic in inhibiting the growth and survival rate in prostate, breast cancer, etc., in previous research (183,184). Carnosol has also been shown to have protective role in DMBA or TPA-induced skin carcinogenesis in mouse models (196). The data demonstrated here for the first time that In skin cancer cells, carnosol had a synergetic effect when combined with UVB radiation, lead to a further decreasing in cancer cell progression rate (Fig. 5.10). This effect could be due the imbalance of ROS
in cancer cells and NF-κB signaling pathways inhibition. Based on the results discussed above, a model was proposed in Fig. 5.11.

Conclusion

To conclude, the data demonstrated that carnosol could reduce the UVB-induced ROS elevation in cells. The reduction of intracellular ROS could also lead to the protection of DNA damage caused by UVB exposure. On the other hand, by reducing ROS level, carnosol also partially reduce the UVB-induced NF-κB activation. In addition to protect normal cell apoptosis caused by UVB radiation, carnosol has also been shown to have synergetic effect on reducing skin cancer progression together with UVB radiation.

*Figure 5.11.* Model for the effect of carnosol upon UVB radiation.
CHAPTER 6 : FUTURE DIRECTION

Increasing evidence showed that overexposure to UVB radiation is carcinogenic and UV-damaged skin had an increased chance of developing one of the forms of skin cancers, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and cutaneous malignant melanoma (1,2). Recent studies indicated that routinely using sunscreen could reduce UV-induced melanoma, the most deadly form of skin cancer, in half (197). The question is why these sunscreens cannot do better since even a low SPF 15 sunscreen can block 93% of UV rays and a low dose exposure of UV will not cause skin cancer. Sunscreens mainly contain two kinds of chemicals to reduce the effect of UV exposure. One (such as zinc oxide) is used to reflect UV ray away and the other one (such as PABA) is used to absorb UV. The problem is underlined in the mechanism of action of para-aminobenzoic acid (PABA). After absorbing UV, the energy for the absorbed UV ray can break down PABA to form free radicals, which can potentially cause DNA damage and membrane lipid oxidation (189) (198). DNA damage has been known to cause DNA mutations and thus cancer development. In chapter 5, we have demonstrated that carnosol can not only partially reduce UVB-induced ROS elevation and thus protect UV-induced DNA damage in normal skin cells, but also prohibit skin cancer cell progression with or without UV radiation, and thus inhibit skin cancer progression. Based on these results, we hypothesize that carnosol can be a potential therapeutic for chemoprevention and treatment of UVB-induced skin cancers via inhibition of UVB-induced skin cell transformation by reducing ROS-mediated DNA damage, and suppression of skin cancer progression by inhibiting membrane receptors-
mediated cell growth signaling pathways. The following three specific aims are proposed for the future work.

Specific Aim 1: Determine the chemopreventive effect of carnosol on UVB-induced transformation of normal skin cells. The working hypothesis for aim 1 is that carnosol can prohibit the skin cancer development due to overexposure of UVB radiation, thus it has the potential to be an effective chemopreventive agent. In this aim, UVB-induced transformation of normal skin cells will be studied. The signaling pathways that involved in cell transformation and manipulated by carnosol will also be examined.

Specific Aim 2: Determine the chemotherapeutic effect of carnosol on skin cancer cells. The working hypothesis for aim 2 is that carnosol can reduce skin cancer cell progression by itself and it has synergistic effect on reducing cancer progression with UVB radiation. Under this specific aim, the physiological and pathological impacts of carnosol on skin cancer cells received or not received UVB radiation will be studied.

Specific Aim 3: Determine the therapeutic effect of carnosol on UVB-induced cancer development and progression using animal models. Mice models will be used to verify the effects of carnosol on cell lines in this specific aim. SKH-1 hairless mice will be exposed to UVB radiation repeatedly for skin cancer development, while carnosol will be applied to the mice skin to study its effect. The cancer development and progression level will be determined in both mice skins with or without carnosol treatment.
Specific Aim 1: Determine the Chemopreventive Effect of Carnosol on UVB-induced Transformation of Normal Skin Cells

In specific Aim 1, the effect of carnosol on the transformation of normal skin cell to cancer cell will be investigated. Based on the results discussed above, carnosol could reduce the UVB-induced ROS level and partially protect the DNA damage, yet the detailed mechanism of how ROS was reduced and how the DNA-damaged was protected still remained unknown. In addition, the signaling pathways involved in carnosol treatment will also be explored.

Determine the Effect of Carnosol on NOX Family Members

The NOX activity assay after UVB radiation, with or without carnosol treatment will be performed using lucigenin enhance chemiluminescence assay. If there would be any chance in the NOX activity, then the protein level of each NOX family members will be studied to determine which one/s may play the critical role in ROS production.

Determine the Effect of Carnosol on NOS Family Members

As previously reported, the uncoupling of eNOS, the dimerization ratio of nNOS, and the induction of iNOS would be increased in the early phase (<6 h) post UVB radiation. Therefore, if and how carnosol would effect the dimerization and protein expression of these NOS proteins will further be determined. For the cells treated with or without carnosol, non-reducing gel electrophoresis will be used to determine the dimer
and monomer of NOS, and the expression level of iNOS will be determined by standard western blot analysis.

**Determine if the DNA Protection by Carnosol is Free Radical Dependent**

DNA damage caused by UVB radiation could be either (1) directly DNA damage or (2) ROS-induced DNA damage. Although carnosol can reduce UV-induced intracellular ROS level, we cannot rule out the possibility that carnosol could reduce the non-ROS caused DNA damage, therefore, we will determine here in which way carnosol protects UVB-induced DNA damage.

Cyclobutane pyrimidine dimer (CPD) will be formed by UVB-induced direct DNA damage. The CPD dimers will be detected with anti-thymine dimer antibody (Abcam) using immunofluorescent assays. Cells will be treated with UVB radiation with or without carnosol treatment, fixed and stained by primary anti-thymine dimer and secondary fluorescent antibody. In addition, the effect of carnosol on oxidative DNA damage will also be studied. Oxidative DNA damage will be detected by immunostaining of 8-hydroxydeoxyguanosine (8OhdG), which is a classical oxidative DNA damage marker due to hydroxyl radical attack. Cells will be treated as mentioned above and cellular 8OhdG level will be detected using anti-8OhdG antibody (Abcam).

**Determine the Apoptotic Downstream Signaling of NF-κB**

As shown in Chapter 5, the UVB-induced phosphorylation of NF-κB will be inhibited by carnosol, and carnosol can also protect UVB-induced cell death. The
apoptotic downstream pathways, including the expression level of Bcl-2, Bcl-xL, and BAD, will be studied.

_Determine the Effect of Carnosol on Cancer Carcinogenesis_

Skin cell carcinogenesis will be accomplished by performing soft-agar assay for anchorage independent cell growth. HaCaT cells will be treated with UVB exposure 5-6 times with three-day interval, with or without carnosol. Then the cells will be seeded onto soft agar at the end of treatment. By counting the colonies formed in the soft agar, the effect of carnosol on cell carcinogenesis can be determined. Alternatively, a couple of cancer transformation markers, such as the mRNA and protein level for keratin 5, CD133, CD24, CD44, etc., could also be tested.

Specific Aim 2: Determine the Chemotherapeutic Effect of Carnosol on Non-melanoma Skin Cancer Cells

As shown in Chapter 5, carnosol could inhibit skin cancer cell growth and sensitize them to UVB radiation. In this specific aim, the mechanisms of how and why normal skin cells and cancer skin cells respond differently to carnosol will be studied, the consequently physiological outcomes of carnosol on skin cancer cells will be investigated as well.
Determine the Effect of Carnosol on Skin Cancer Cell Membrane Receptors

Compared to normal cells, skin cancer, especially non-melanoma skin cancers, express more purinergic receptors while less apoptotic receptors (e.g. TRAIL receptors) on their cell membrane (199-201). Previous report demonstrated that carnosol bound to estrogen and/or androgen receptors in breast and prostate cancers, and as the data showed that carnosol could sensitize SCC cell line A431 to UVB radiation, there is possibility that carnosol could affect the purinergic or apoptotic receptors on the cell membranes. Therefore, in here, if and how carnosol would affect these membrane receptors would be studied, as well as the physiological effect of carnosol SCC cells.

Based on previous report, non-melanoma skin cancers had a higher expression level of purinergic receptors, and these receptors are critical to regulate cell proliferation and survival signaling pathways (199,202,203). The binding assay of carnosol on these receptors will be performed to see if carnosol could affect the activity of these purinergic receptors. In addition, the protein level, and their downstream pathway markers, such as the phosphorylation ERK and cAMP production level will also be measured.

Determine the Binding of Carnosol on TRAIL Receptors upon UVB Radiation

TRAIL receptors have been shown to be critical to the survival of both melanoma and non-melanoma skin cancer cells (5,200,201). Moreover, NF-κB is known to be one of the downstream substrate of TRAIL receptor (204,205) and carnosol could reduce the activation of NF-κB. Therefore, the effect of carnosol on the TRAIL receptors will be
studied to determine if carnosol also inhibit NF-κB through the receptors. Similar to the tests of purinergic receptors, the binding affinity of carnosol to these receptors, as well as their expression level, will be explored.

_Determine the Effect of Carnosol on Cancer Proliferation._

To investigate the effect of carnosol on cancer cells, human squamous cancer cell A431 will be used. A431 cells will be treated with or without carnosol, combined with UVB radiation, and then cell proliferation rate and cell cycle distribution will be determined by MTT assay and flow cytometry.

_Determine the Effect of Carnosol on Cancer Metastasis_

To further study the effect of carnosol on cancer cells, the metastasis status of the cells treated with or without carnosol along with UVB radiation will be studied. The cell surface expression of common ECM protein markers, such as E-cadherin, vimentin and fibronectin, will be evaluated using flow cytometric analysis. To further confirm the outcome of protein expression change, cell invasion using transwell invasion assay will also be studied.

Specific Aim 3: Determine the Role of carnosol on UVB-induced Cancer Development and Progression Using Animal Models

In specific Aim 3, the cancer chemopreventive potential of carnosol against UVB-induced skin cancer formation, progression and metastasis using mouse model will be
analyzed. SKH-1 hairless mouse will be used, to determine whether apply of carnosol will affect the development of squamous cancer, and the progression and metastasis of cancer.

_Determination if Carnosol Prevents UVB-induced Skin Cancer Development_

In this sub-aim, whether carnosol can reduce or prevent photocarcinogenesis will be determined. SKH-1 hairless mice, a well-accepted mouse model for UVB-induced skin cancer research, will be used in these studies (206,207). The effect of topical treatment of carnosol on cell apoptosis in skin sections prepared at 6 and 24 h post-UVB will first be determined. The extent of the effect of these topical treatments of the same drugs on skin edema, skin hyperplasia and epidermal ornithine decarboxylase induction will also be studied. Furthermore, the extent of effect of the topic treatment of carnosol on tumor formation and progression will also be investigated.

_Determination if Carnosol Suppresses Skin Cancer Progression_

In this sub-aim, whether carnosol can reduce melanoma metastasis and progression with or without UVB irradiation will be determined. SKH-1 hairless mice will be expose repeatedly to UVB radiation until there is skin cancer formation. Then carnosol will be applied onto the tumor region, and the level of cancer progression will be determined by the tumor volume and immunochemistry staining using markers such as HOXB7 and Ki-67, and metastasis will be determined at various organs such as liver,
colon, intestine, stomach, etc. (208,209). By comparing the tumor size and metastasis status, the effect of carnosol on the progression of skin cancer can be determined.
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