TRANSLATION REGULATION OF UV-LIGHT-INDUCED TRANSCRIPTION
FACTOR NF-KAPPA-B AND ONCOGENE COX-2

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

Csaba F. László
March 2009

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This dissertation titled
TRANSLATION REGULATION OF UV-LIGHT-INDUCED TRANSCRIPTION
FACTOR NF-KAPPA-B AND ONCOGENE COX-2

by

CSABA F. LÁSZLÓ

has been approved for
the Department of Chemistry and Biochemistry
and the College of Arts and Sciences by

Shiyong Wu
Associate Professor of Chemistry and Biochemistry

______________________________

Benjamin M. Ogles
Dean, College of Arts and Sciences
ABSTRACT

LÁSZLÓ, CSABA F., Ph.D., March 2009, Chemistry and Biochemistry

Translation Regulation of UV-Light-Induced Transcription Factor NF-κB and Oncogene COX-2 (97 pp.)

Director of Dissertation: Shiyong Wu

NF-κB plays an important role in ultraviolet light-induced skin tumorigenesis. Activation of NF-κB by UV-irradiation is composed of two phases. The early-phase culminates with maximal levels of DNA binding ability at 4 hours post-irradiation and is dependent on translational inhibition. The late-phase activation of NF-κB occurs between 16 and 48 hours post-irradiation and the mechanism is not clear due to the fact that NF-κB was activated even in the presence of high level of its inhibitor protein, IκBα. Here, we provide evidence that in the late-phase of UV-induced NF-κB activation, IκBα depletion is the combined result of regulation at both transcriptional and translational levels. Neither ubiquitination nor proteasomal degradation have detectable attributions to IκBα breakdown. We also demonstrate that UV only induces phosphorylation of p65 at Ser 276, while TNFα induced phosphorylation at both Ser 276 and 536 sites of p65. Based upon our results, we propose a novel mechanism for translation-regulated IκBα depletion and MSK-mediated NF-κB activation at 24 hours post UV-irradiation.

Besides NF-κB activation, ultraviolet light also induces a prolonged expression of COX-2. While transcriptional regulation of COX-2 expression is intensively studied, the role of translational regulation of COX-2 synthesis upon UV-irradiation is not yet clear.
Here, we show that phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) plays an important role in the regulation of COX-2 expression after UV-irradiation. Our data shows that UV light induces COX-2 expression in wild-type mouse embryo fibroblasts (MEF\(^{S/S}\)) and that the inducibility is reduced in MEF\(^{A/A}\) cells in which the phosphorylation site, Ser-51 in the eIF2α, is replaced with a nonphosphorylatable Ala (S51A). UV light-induced transcription of COX-2 is delayed in MEF\(^{A/A}\) cells, which correlates with NF-κB activation, as previously reported. Our data also shows that translational efficiency of COX-2 is higher in MEF\(^{A/A}\) cells than in MEF\(^{S/S}\) cells, but not at the late stage of UV-irradiation. This may be due to the translational regulation of COX-2 binding protein TIAR expression, which is reduced in MEF\(^{S/S}\) cells but not in MEF\(^{A/A}\) cells at 24 hours post-UV. In addition, our data indicates that newly synthesized COX-2 protein is more stable in MEF\(^{A/A}\) cells than in MEF\(^{S/S}\) cells. These results suggest that translation initiation plays a role in the complex and dynamic regulation of COX-2 expression. Based on our results, and the use of Ingenuity Pathway Analysis™, we propose a novel eIF2α phosphorylation-centered network for the regulation of COX-2 expression after UV-irradiation.

Approved: _____________________________________________________________

Shiyong Wu

Associate Professor of Chemistry and Biochemistry
ACKNOWLEDGMENTS

First, I would like to thank my advisor and mentor, Dr. Shiyong Wu, for his guidance and patience regarding both professional and personal matters. I will always cherish the memories of our debates from which there was so much to learn. Thank you for leading by example, making it easy to look up to you and thank you for bestowing the passion for research upon me.

I would like to thank my committee members Dr. Douglas Goetz, Dr. Glen P. Jackson, Dr. Susan C. Evans and Dr. Shiyong Wu for the time and energy they spent on my dissertation and their valuable suggestions regarding my projects.

I would also like to thank all the past and present research group members for their help, support and interesting discussions: K. Suzanne George, Peter Goughnour, Jian Wang, Wei Lu, O. Luke Carpenter, Lei Wang, Wei Liu, Dr. Walid Elyassaki, Dr. Todd Parker. Special thanks to Suzanne Parker for kindly starting me on the amazing journey of the molecular biology laboratory techniques.

Thanks are also due to the staff of the Chemistry and Biochemistry department and Edison Biotechnology Institute for assisting me in numerous ways during my stay at Ohio University: Carrie Linscott, Carolyn Khurshid, Rollie Merriman, Paul Schmittauer, Robert Dotson and Lori Abdella.

Furthermore I would like to thank Drs. David and Valerie Young for their kind support and friendship.
Very special thank you to the late Dr. Almasi Miklos, without whom I would not be where I am today, his memory will guide me through my career.

Last but not least I would like to thank my wife and my family and friends from home who supported me throughout the years and never failed to encourage me and sacrificed much so I could unwaveringly stay on my chosen path.

THANK YOU ALL!
I dedicate this work to my beloved wife,

Ünige Laskay

and my dear parents,

Ferenc and Emilia László
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<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>AP2</td>
<td>activating protein 2</td>
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<tr>
<td>Bcl-3</td>
<td>B-cell lymphoma 3-encoded protein</td>
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<tr>
<td>BiP</td>
<td>immunoglobulin heavy chain-binding protein</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<td>CKII</td>
<td>casein kinase 2</td>
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<tr>
<td>C/EBP</td>
<td>CAAT/enhancer-binding protein</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>eIF2</td>
<td>eukaryotic initiation factor 2</td>
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<td>eukaryotic initiation factor 2 alpha kinase</td>
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LPS        lipopolysaccharide
MAPK       mitogen-activated protein kinase
MEF        mouse embryonic fibroblast
MSK        mitogen- and stress-activated protein kinase-1
NES        nuclear export signal
NFIL6      C/EBPβ
NF-κB      nuclear factor κB
NLS        nuclear localizing signal
PKA        protein kinase A
PKCα       protein kinase C, alpha
PKR        dsRNA induced protein kinase
PERK       RNA dependent protein kinase-like ER kinase
RT-PCR      reverse transcription polymerase chain reaction
ROS        reactive oxygen species
SP1        specificity protein 1
TAD        transactivation domain
TCF4       transcription factor 4
TIAR       T-cell restricted intracellular antigen 1-like protein
TNF        tumor necrosis factor
UPR        unfolded protein response
UV         ultraviolet light
CHAPTER 1: INTRODUCTION

Objective: The goal of this study is to determine the role of translation in the UV induced activation of NF-κB and COX-2, as well as to reveal mechanistical details and novel factors or interactions that pertain to these activation pathways.

Ultraviolet (UV) is a part of the light spectrum to which we are daily exposed. There are three types of UV radiation: UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). Earth's atmospheric composition (ozone, water vapors, O₂, N₂, CO₂, dust) has the protective ability to absorb the higher energy components of UV thus allowing only UV-A and UV-B to pass to Earth's surface. Besides the many positive life giving properties (e.g. vitamin D production), prolonged exposure to UV light also has many disruptive effects at the cellular level of a living organism. One of the diseases for which UV is the major culprit is skin cancer.

Non-melanoma skin cancer (NMSC) makes up for almost 50% of cancers diagnosed each year in the United States (American Cancer Society). Clinical studies have shown that the use of sunscreens has a limited effect on preventing this type of cancer, leading us to the conclusion that other approaches are needed in tackling the disease.

Biochemists and molecular biologists have been trying to elucidate the molecular pathways of the cell, the building block of all living organisms. All cellular activity originates at the level of the DNA, the “great codex” from which all information is translated into living matter. The process of DNA transcription is facilitated and
regulated by transcription factor proteins that through binding to a DNA sequence up or down regulate the transcription of certain genes.

If transcription is the process that deciphers the genealogical code, it is translation that delivers the “goods” through protein synthesis. While transcriptional regulation of gene expression is intensively studied, the role of translational regulation on protein synthesis following UV-irradiation is not yet clear.

It has been determined that the transcription factor NF-κB has a role in the development of skin carcinomas. The well-known involvement of NF-κB in the inflammatory process and the recently proposed link between cancer and inflammation extends the importance of UV-NF-κB pathways to other types of cancers [1]. However, NF-κBs role in the immune response, the context where it was first discovered, provides it significance in numerous immunity related diseases.

COX-2 activity, on the other hand, has been linked to tumor formation because of its overexpression in numerous malignancies [2] and because COX inhibitors like non-steroidal anti-inflammatory drugs (NSAID) inhibit tumor formation and metastasis [3].

Considering all of the above, it is imperative for us to understand the mechanisms of translation regulation and clarify the cellular pathways surrounding UV-induced NF-κB and COX-2 in order to open a gate of possibility for targeted drug design. The focus of the study is directed towards mapping out these signal transduction pathways and the discovery of novel interactions among involved factors.
CHAPTER 2: TRANSLATION REGULATION; AN ALTERNATE NF-κB ACTIVATION PATHWAY

2.1 Summary

Activation of the transcription factor NF-κB is a highly regulated multi-level process. The critical step during activation is the release from its inhibitor IκB, which, like any other protein, is under the direct influence of translation regulation. Herein we summarize in detail the current understanding of the impact of translational regulation on NF-κB activation. We also show that the classical NF-κB activation pathways occur simultaneously with, and are complemented by, translational down-regulation of the inhibitor molecule IκB, the importance of one or the other being shifted in accordance with the type and magnitude of the stressing agent or stimuli.

2.2 History

In 1986 David Baltimore’s laboratory discovered a nuclear protein in mature B cells that binds to a 10 nucleotide stretch of double-stranded DNA in the κ immunoglobulin light chain enhancer (GGGACTTTCC) [4]. It was soon proven that this nuclear factor had a role in the mediated expression of the κ light-chain and that it’s localization in the nuclei is associated with different cellular stimuli [5]. Further studies show that NF-κB is involved in the regulation of the expressions of many genes that are

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mostly related to the immune and inflammatory response, along with genes determining developmental processes, cellular growth, and apoptosis [6, 7].

2.3 NF-κB family members

The mammalian NF-κB family is composed of five members: p65 (RelA), RelB, NF-κB1 (p50 and its precursor p105), c-Rel, and NF-κB2 (p52 and its precursor p100) (Fig. 2.1) [8, 9]. They all have in common a 300 amino acid Rel homology domain (RHD) located close to the N terminus of the protein [10]. However, while p65 and p50 were found to be present in the majority of cells, the other three members (RelB, cRel and p52) were suggested to be only expressed in lymphoid cells [11]. The RHD contains sequences that are accountable for the homo- or hetero-dimerization of the family members. Of the five members, only three, p65, RelB and c-Rel, contain a trans-activation domain (TAD) which is needed to promote transcription by facilitating the employment of activators and banishment of repressors [12]. Subsequently, homodimers of the other two members, p52 and p50 are unable to activate transcription. Instead, they attenuate expression of target genes.
2.4 The role of IκB in regulation of NF-κB activation

The activity of NF-κB is regulated at multiple levels. The best known regulatory step is the cytoplasmic to nuclear transport of activated NF-κB p65:p50 heterodimer [14, 15]. Without stimulation, cytoplasmic compartmentalization of NF-κB in cells is due to binding through the RHD to a member from the family of proteins called inhibitor of NF-κB (IκB) (Fig. 2.2). The IκB family consists of IκBα, IκBβ, IκBε, IκBγ, BCL-3 and the two NF-κB precursors p100 and p105 [16, 17]. IκBα and IκBβ achieve the cytoplasmic localization by masking the nuclear localization sequence (NLS) of amino acids on the NF-κB p65 subunit [18-20]. Failure to mask the NLS of the p65 subunit in addition to the existence of a nuclear export sequence (NES) on IκBα and p65 results in the constant shuttling of IκBα:p65:p50 complexes between the cytoplasm and nucleus. On the other
hand, IκBβ:p65:p50 complexes are restricted to the extra nuclear compartment, this phenomena adding to the complexity of NF-κB regulation.

Figure 2.2 IκB protein family members [13].

2.5 The role of kinases in regulation of NF-κB activation

After removing IκB, a second level of regulation is conferred mainly by stimulus-induced phosphorylation of NF-κB [21]. A protein kinase A (PKA) phosphorylation site was identified on both p65 and c-Rel at Ser 276, located 25 amino acids from the NLS, inside the Rel homology domain (RHD) [22]. Over-expression of PKA leads to a higher DNA-binding activity of NF-κB. This is mainly due to the fact that phosphorylated Ser 276 inhibits intermolecular association with inhibitors, thus facilitating nuclearization and DNA binding [21, 23]. The same phosphorylation also promotes interaction with coactivator CREB binding protein (CBP/p300) [22]. A similar mechanism of NF-κB activation was identified during TNFα stimulation when p65 phosphorylation occurred at Ser 529 mediated by casein kinase II (CKII) [24, 25]. Also, during TNFα stimulation
another activating phosphorylation occurs at Ser 536 by IKK [26]. It is worthy to note that the same catalytic activity of IKK is required for IκB phosphorylation followed by ubiquitination and NF-κB activation by direct phosphorylation; a fact that adds to the complexity of IKK mediated NF-κB activation [27]. The activity of stimulated NF-κB is down-regulated by a feedback pathway through the newly synthesized IκBα, one of the first genes activated by NF-κB. IκBα enters the nucleus, binds to NF-κB and exports it to the cytosol [28, 29].

2.6 The classical NF-κB activation mechanism

Upon extra- or intracellular stimulation, the IκBs are phosphorylated by an IκB kinase (IKK), ubiquitin targeted and undergo proteosomal degradation thus automatically exposing the NLS necessary for NF-κB nuclear localization [30, 31]. IKK is a 700 kDa protein complex consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ or NEMO -NF-κB essential modulator-) [32-34]. Activation of the catalytic subunits takes place by phosphorylation followed by intra- and intermolecular trans-autophosphorylation, releasing their kinase domains (Fig. 2.3).
A host of NF-κB inducers have been recognized so far, they consist of but are not limited to: proinflammatory cytokines, tumor necrosis factor (TNFα), interleukin 1 (IL-1), double-stranded RNA (dsRNA), viruses and a variety of cell stressors like UV, reactive oxygen species (ROS) and genotoxic agents [10, 35]. Some of NF-κB activators, such as cytokines and TNFα, achieve activation through the classical activation mechanism (Fig. 2.4).
Figure 2.4 Classical NF-κB activation pathway.
2.7 The impact of translation initiation on NF-κB activation

An entirely different approach to NF-κB activation is provided by translational regulation via the eukaryotic initiation factor 2 (eIF2). During the initiation step of translation, eIF2 forms a complex with GTP and Met-tRNA forming a ternary complex, which associated with the small ribosomal unit contributes to the selection of the start codon. The release from the ribosome is achieved at the expense of hydrolyzation of GTP to GDP. In order to restart the initiation cycle the guanine exchange factor eIF2B refreshes the eIF2-GDP to eIF2-GTP [36]. The phosphorylation on Ser 51 of the α subunit of eIF2 (eIF2α) stabilizes the eIF2-GDP-eIF2B initiation-complex preventing GDP-GTP exchange, thus halting the translational initiation process [37, 38]. The eIF2α phosphorylation inhibits initiation of protein synthesis at a general level, allowing only the selective translation of some proteins that are required for mounting a stress response [39, 40]. It has been shown that UV, ROS, heat shock and hypoxia regulate NF-κB through a much more branched and complex cellular pathways [41-44]. The common feature of these general inducers is that they bring about translation inhibition as a defense cellular response through their noxious effects.

Key players in translational regulation are a host of serine-threonine kinases that can phosphorylate the Ser 51 of eIF2α. Four eIF2α kinases (EIF2AKs) have been identified. While each of the EIF2AKs has its own specific inducers, some stimulus such as UV and hypoxia also activate one or more of the kinases (Fig. 2.5).
EIF2AK1, known as the heme-regulated inhibitor kinase (HRI), is a critical component during erythroid maturation that regulates the stoichiometric ratio of hemoglobin components: α-globin, β-globin and heme [45]. Two separate heme binding sites were identified in HRI [46]. HRI is activated by heme deficiency in multi-stages through series of auto-phosphorylations [47]. The phosphorylation of HRI first stabilizes its monomer that lacks eIF2α kinase activity but has first heme-binding site occupied. Further phosphorylation of HRI induces the dimerization and confers heme sensitivity [48]. During high heme concentrations, heme binds to the second binding site inhibiting HRI kinase activity thus allowing for protein and implicitly hemoglobin translation [49].
In the situation of insufficient heme accumulation, the second heme-binding site remains unoccupied which leads to the induction of HRI kinase activity and inhibition of translation by eIF2α phosphorylation [50]. While heme deficiency leads to activation of NF-κB, there is no direct evidence yet to show that the translation inhibition is involved in the activation signaling pathways. Besides heme deficiency, other NF-κB activators such as arsenite-induced oxidative stress and heat shock were also found to activate HRI [51].

EIF2AK2, known as the interferon-induced double-stranded (ds) RNA-dependent protein kinase (PKR), plays a critical role in anti-viral defense [52]. The binding of the dsRNA exposes an ATP binding site inducing dimerization and subsequent auto-phosphorylation leading to an active form of PKR [53-55]. A variety of stimuli, like growth factors and cytokines, activate PKR independently of dsRNA through PKR-associated activator proteins [56, 57]. Initially, PKR was suggested to directly phosphorylate IκB [58], but further studies show that PKR actually binds to IKK or acts upstream facilitating IKK to phosphorylate IκBα at serines 32 and 36 [59-62]. While the roles of PKR and its catalytic activity in NF-κB activation remain controversial [59-62], several PKR activators, such as dsRNA and interferon gamma (IFNγ) have been shown to induce NF-κB activation.

EIF2AK3, also known as the PKR like endoplasmic reticulum (ER) related kinase (PERK), is an ER membrane localized kinase [63-65]. Its inactive monomer state is stabilized by an ER chaperone Immunoglobulin (Ig) heavy chain binding protein (BiP). Under ER-stress, BiP releases PERK, which undergoes dimerization, trans-
phosphorylation and sequentially activation [64-67]. Outside or inside perturbations negatively affect the protein-folding process in the ER resulting in an accumulation of malfolded proteins, which triggers the unfolded protein response (UPR) While UPR transcriptionally activates the expression of ER chaperone to facilitate the folding process, it translationally inhibits general protein synthesis through phosphorylating eIF2α to reduce the accumulation of newly synthesized proteins in the ER [68]. The converging point between the accumulation of unfolded proteins and global translation inhibition by eIF2α phosphorylation was determined to be PERK [63, 69]. The PERK-mediated eIF2α phosphorylation and translation inhibition is directly involved in ER-stress-mediated NF-κB activation upon various stimuli, such as hypoxia, UV and thapsigargin [42, 43, 70-73].

EIF2AK4 is also known as the amino acid starvation dependent general control of amino acid biosynthesis kinase (GCN2) [74, 75]. It is an amino acid abundance controlled eIF2α kinase, which is activated during amino acid starvation. Its specific role is to halt protein translation while activating the translation of factors that are needed in amino acid synthesis [65, 76]. The activation mechanism involves a histidyl-tRNA synthase (HisRS) homologous sequence where the excess of uncharged tRNAs bind during amino acid deprivation [77]. A C-terminal RNA binding region is also required for its dimerization, activation and association with ribosome [78, 79]. The GCN2-mediated eIF2α phosphorylation and translation inhibition was shown to be directly involved in amino acid starvation induced NF-κB activation [70]. Besides nutritional stresses, the HisRS similar sequence also allows for activation by other stresses, such as
UV and proteosome inhibition [76, 80]. While there is no evidence yet to show that GCN2 is directly involved in NF-κB activation upon proteosome inhibition, it has been demonstrated that GCN2 mediates UV-induced NF-κB activation [81].

2.8 Regulation of IκB turnover

NF-κB is stranded in the cytoplasm bound by its inhibitor protein IκB. Even though both IκBα and IκBβ are able to inhibit NF-κB, it is IκBα that bears the major role in regulating its activation [82]. NF-κB is a transcription factor that has a fast response time in order to react promptly to cellular stress. In order to achieve this fast activation, the IκB levels are tightly and rapidly regulated. While activation of receptor signaling cascades, such as through TNFα and interleukin-1 (IL-1), often leads to phosphorylation, ubiquitination and proteolysis of IκB, whereas the more general cellular stimulus, such as UV and hypoxia, also possess the ability to induce translational inhibition of IκB synthesis.

IκB regulation occurs through two mechanisms: a signal-dependent and a signal-independent (basal degradation) process [83]. IκB turnover is tightly linked to its structural domains. The centrally located ankyrin repeats are necessary for NF-κB binding and the two terminal regions are implicated in the degradation of IκB. The N-terminal sequence contains two IKK phosphorylation sites, Ser 32 and 36 [84-87] and two ubiquitination sites, Lys 21 and 22 [88, 89]. The phosphorylation and ubiquitination of these sites promote IκB degradation in the 26S proteosome. The C-terminal region
contains a PEST domain (Pro, Glu, Asp, Ser and Thr rich regions), which is associated in general with high turnover proteins [90]. The PEST site in addition to multiple casein kinase II (CKII) phosphorylation sites on the C-terminal region are needed for both signal induced degradation [87, 91, 92] and basal turnover of IκB [21, 85, 93, 94].

The rate of degradation of IκB is also very much influenced by its association with NF-κB. Free IκB has a 30-40 minute half-life but the NF-κB associated one has a 5-fold longer degradation time [95-98]. Free IκB constitutes only a 15% fraction of the total cellular IκB [95] and is a weak substrate for IKK phosphorylation [99]. The basal turnover of free IκB requires the CKII phosphorylation sites, while the signal dependent degradation is induced by IKK phosphorylation. For the NF-κB associated IκB, the basal turnover is also regulated by CKII phosphorylation, while the signal-induced degradation is regulated by both CKII and IKK phosphorylation [83, 98, 100-102] (Table 2.1).

Table 2.1 Role of kinases in regulation of IκB turnover.

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<th>Basal Turnover</th>
<th>Signal induced degradation</th>
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</thead>
<tbody>
<tr>
<td>Free IκB</td>
<td>CKII</td>
<td>IKK</td>
</tr>
<tr>
<td>Bound IκB</td>
<td>CKII</td>
<td>IKK + CKII</td>
</tr>
</tbody>
</table>

While IKK and CKII regulate the removal rate of IκB, the EIF2AKs determine the synthetic rate of IκB. The phosphorylation of eIF2α by EIF2AK leads to inhibition of global protein synthesis, including IκB. Since IκB has a relatively high basal turnover
rate, the inhibition of new IκB synthesis results in a rapid depletion of IκB and shifting the dynamic balance from NF-κB associated IκB towards free NF-κB and IκB (Fig. 2.6).

*Figure 2.6 Model for translation regulation of NF-κB activation (CellDesigner®).*
2.9 Targeting NF-κB for therapeutic development

NF-κB plays an important role in regulation of the process of innate and adaptive immune responses. Its ability to activate transcription of genes encoding cytokines (e.g. TNFα, IL-1, IL-2, IL-6), chemokines, adhesion molecules (e.g. ICAM, VCAM, E-selectin), inducible enzymes (e.g. iNOS, COX-2) and antimicrobial peptides (β defensine) gives it a central role in the overall process of immune response [82]. NF-κB also regulates genes outside the immune system presumably having an anti-apoptotic effect that would give an opportunity to the cell to repair DNA damage. Deregulation of these genes may lead to many diseases, such as cancer, atherosclerosis, arthritis and AIDS, among others [6]. NF-κB has been a target for development of therapeutics for many diseases [103]. Since eIF2α phosphorylation also impacts NF-κB activation, compounds that affect eIF2α phosphorylation through the aforementioned kinases will be potential therapeutics for treatment of various diseases. Indeed, several ER-stress inducing drugs are already in the spotlight for their ability to induce apoptosis in malignant cells. The chemotherapeutic agents doxorubicin and cisplatin, although known to mainly target DNA, were also shown to induce ER-stress and activate PERK [104-107]. Interferon and TNF-α are both antiviral proteins that have been used in combination with chemo- and radiation therapy and that possess the ability to activate PKR [108, 109]. In addition, the potential for successful use of proteasome inhibitors for cancer treatment may be granted by the ability of these compounds to induce apoptosis through the blocking of protein degradation, which implicitly leads to ER-stress. The anti-multiple myeloma drug Velcade (PS-341) for example, which is a proteasome
inhibitor, inhibits IκB degradation. In fact, Velcade also disrupts protein folding in the ER resulting in ER-stress [110-112]. In summary, elucidating the role of EIF2AK in mediation of NF-κB activation may lead us to a better understanding of the mechanisms of current NF-κB targeted drugs and development of new therapeutics to treat diseases related to deregulation of NF-κB.
3.1 Summary

NF-κB plays an important role in UV-induced skin tumorigenesis. Activation of NF-κB by UV-irradiation is composed of two phases. The early-phase culminates with maximal levels of DNA binding ability at 4 hours post-irradiation and is dependent on translational inhibition. The late-phase activation of NF-κB occurs between 16 and 48 hours post-irradiation, however the mechanism is not clear due to the fact that NF-κB was activated in the presence of high level of IκBα. Here, we provide evidence that without translational inhibition, the transcription of IκBα was induced by UV-irradiation. In the late-phase of UV-induced NF-κB activation, the IκBα depletion was the combined result of regulation at both transcriptional and translational levels. Neither ubiquitination nor proteasomal degradation had detectable attributions to IκBα breakdown. We also demonstrated that UV only induced phosphorylation of p65(S276), while TNFα induced phosphorylation at both Ser 276 and 536 sites of p65. Based upon our results, we propose a novel mechanism for translation-regulated IκBα depletion and MSK-mediated NF-κB activation at 24 hours post UV-irradiation.

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3.2 Introduction

Ultraviolet (UV) light activates nuclear factor-kappa B (NF-κB) [113-116], which plays an important role in skin tumorigenesis and cancer therapy [117, 118]. NF-κB activation upon UV-irradiation is composed of two phases. The early-phase of activation culminates with maximal levels of DNA binding ability at 4 hours post-irradiation. The UV-induced early-phase activation of NF-κB is dependent on translational inhibition [119, 120] and is independent of induced activation of IκB kinase (IKK) alpha [32, 114]. UV induces activation of the eukaryotic initiation factor 2 (eIF2) kinases, PERK and GCN2, which phosphorylate the alpha subunit of eIF2 (eIF2α) at Ser51 [119, 120]. The phosphorylation of eIF2α leads to IκBα depletion and NF-κB activation through inhibition of IκBα synthesis [121].

The UV-induced late-phase activation of NF-κB occurs between 16 and 48 hours post-irradiation [115, 116]. The mechanism for the UV-induced late-phase activation of NF-κB is suggested to be dependent on DNA-damage signaling, IKK activation and ubiquitin-mediated IκB degradation [113, 114]. However, our previous results demonstrate that NF-κB is activated in the presence of high level of IκBα in mouse embryonic fibroblast (MEF) cells with a Ser51→Ala mutation at the phosphorylation site in the eIF2α (MEF^A/A) [119]. Here, we elucidated that IκBα depletion in the late-phase of UV-irradiation was a result from a combined regulation at both transcription and translation levels. Neither the ubiquitination nor the proteasomal cleavages have detectable attributions to the late-phase UV-induced IκBα depletion. We also provide
evidence that the UV-induced late-phase activation of NF-κB was mediated through PKA/MSK pathway and was independent of IκBα depletion.

3.3 Materials and methods

Cell culture. Wild-type mouse embryo fibroblasts (MEF\textsuperscript{SS}) and mutated ones (MEF\textsuperscript{AA}), where Ser 51 on the alpha subunit of the eukaryotic initiation factor (eIF2α) is mutated to a non-phosphorylatable Ala, were grown in 10% FBS enriched DMEM media (Cellgro) containing MEM essential and non-essential amino acids (Invitrogen). The cells were incubated at 37 °C and 10% CO\textsubscript{2}.

UV Irradiation. UV was generated from a 15 W UVC lamp (UVP). The intensity of UV was standardized by a UV meter (UVP) set at 3 W/m\textsuperscript{2}. The media was withdrawn during the irradiation. After UV light irradiation, fresh medium was added to each plate.

Protein extraction. Nuclear and cytosolic proteins were extracted from the cells using NucBuster Protein Extraction Kit according to manufacture’s procedure (EMD Chemicals). Protein concentrations were measured with a Bio-Rad protein DC assay kit (Bio-Rad Laboratories). Total proteins were extracted from the cells using Nonidet P-40 lysis buffer (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl, 0.1% SDS). The cell debris was removed by centrifugation.

Analysis of total IκBα. Equal amounts of protein samples were subjected to SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were probed with either an anti-IκBα antibody or a mouse monoclonal anti-β-actin antibody (Sigma). A rabbit polyclonal anti-IκB antibody (sc-371; Santa Cruz Biotechnology) was used for
mouse IκBα. After extensively washing with Tris-buffered saline plus Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were detected by using a SuperSignal™ chemiluminescent kit (Pierce).

Assay for p65 phosphorylation. Equal amounts of nuclear and cytosolic proteins were loaded on a 10% SDS-PAGE, followed by electroblotting onto nitrocellulose membrane. The membrane was then probed with anti-p65 (06-418, Upstate Biotechnology), phospho-p65(536) (3033, Cell Signaling) or phospho-p65(276) (3037, Cell Signaling) antibodies followed by HRP-conjugated, secondary antibodies. To show the equal loading of the protein, the membrane was stripped using western blot stripping solution (Pierce) and the β-actin was probed with anti-β-actin antibody (Sigma).

Quantitative real-time PCR. Total RNA was extracted from UV treated MEFs by RNeasy Mini Kit (Qiagen). Complementary DNA was prepared from 1 μg of RNase free DNase treated RNA, using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR reactions were prepared with iQ SYBR Green Super Mix (Bio-Rad) and 0.2 μM concentration of the following primers:

IκBα: 5’: TTGGTCAGGTGAAGGGAGAC; 3’: AGGTCTGCCTCAAGACTGCT.
β-actin: 5’: TATGGAATCCTGTGGCATCC; 3’: GTACTTGCGCTCAGGAGGAG.

The reaction was performed on an iCycler (Bio-Rad) starting with incubation at 95°C for 2.5 minutes followed by 40 cycles of 60 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C. Data was analyzed with the comparative delta Ct method. Relative
amounts of IκBα transcripts were normalized to the levels of β-actin mRNA in each sample.

3.4 Results

3.4.1 Phosphorylation of eIF2α prevents UV-induced transcriptional activation of IκBα

The early-phase NF-κB activation within 12 hours post-UV is due to the translational inhibition of IκBα synthesis [81, 119]. However, the regulation of late-phase NF-κB activation at 24 hours post-UV irradiation remains unclear. Our previous data showed that the level of IκBα stayed high in MEF^{A/A} cells at 24 hours post-UV treatment, when NF-κB is fully activated [119]. However, the mechanism for maintaining higher level of IκBα via translational regulation is not clear. To access the regulatory mechanism of IκBα at 24 hours post-UV, we first determined transcription and translation of IκBα in the irradiated MEF^{S/S} and MEF^{A/A} cells. Quantitative real-time RT-PCR was used to determine the levels of mRNA in UV treated and non-treated cells. At 24 hours post-UV treatment, the mRNA levels of IκBα were not significantly changed in MEF^{S/S} cells, but increased 5-fold in MEF^{A/A} cells (Fig. 3.1, Panel A). The total amount of IκBα protein was decreased in MEF^{S/S} cells and remained high in MEF^{A/A} cells (Fig. 3.1, Panel B), which agrees with our previous observation [119]. These results suggest that phosphorylation of eIF2α prevents transcriptional activation of IκBα expression, while it inhibits translation in the late period post UV-irradiation, thus
reduction of IκBα in the late-phase UV induced NF-κB activation is the combined result of regulation at both transcriptional and translational levels.

Figure 3.1 Inability to block translation allows for transcriptional activation of IκBα. (A) Quantitative real-time PCR of IκBα mRNA expression in non-treated and UV-irradiated MEFs. Relative amounts of IκBα transcripts were normalized to the levels of β-actin housekeeping gene in each sample. (B) Western blot analysis of total IκBα at 24h post UV-C irradiation (30 J/m²) of MEFS/S and MEF/A cells. The intensities of the bands were quantified by ImageJ (v 1.31, NIH). Expression levels were normalized by the levels of β-actin and shown as a percentage of IκBα expression at 0 h after induction.
3.4.2 UV-induced IκBα reduction is ubiquitination and proteasomal degradation independent

Earlier reports indicated that UV activates IKK during the late-phase of NF-κB activation [113]. On the other hand, IKK phosphorylates IκBα and promotes its depletion through ubiquitination and proteasomal degradation [122, 123]. Given that translational inhibition also plays a role in the regulation of IκBα at 24h after UV-irradiation (Fig. 3.1), we determined the contributions of ubiquitination and proteasomal degradation for maintaining the level of IκBα upon UV-irradiation. In the present studies a membrane-permeable proteasome inhibitor MG132 (Sigma-Aldrich) and an IκBα ubiquitin ligase inhibitor Ro106-9920 (Sigma-Aldrich) were used to block UV-induced IκBα depletion. Surprisingly, both inhibitors failed to influence IκBα levels in MEF^{S/S} and MEF^{A/A} cells at 24 hours post-UV-irradiation (Fig. 3.2, Panels A and B). Although Ser 32 and 36 phosphorylated IκBα was assayed, due to the lack of phosphorylation or perhaps due to the transient nature of phosphorylated IκBs, they were not detected. Nevertheless, TNFα-induced IκBα was totally rescued by Ro106-9920 (10 μM) in MEF^{S/S} (Fig. 3.2, Panel C, Lane 4). The results suggest that neither ubiquitination nor proteasomal degradation have detectable attributions to the late-phase UV-induced IκBα depletion. These results also suggest that IKK may not be involved during this period in the UV-induced activation of NF-κB.
Figure 3.2 The ubiquitination and proteasome inhibitors failed in rescuing UV-induced IκBα depletion. Total amounts of IκB and β-actin in UV-irradiated or TNFα-treated MEF_{SS} and MEF_{AA} cells were determined by western blot analysis. The cells were treated or not treated with UV-irradiation (30 J/m², Panel A and B) for 24 hours or TNFα (10 ng/mL, Panel C) for 1 hour in the presence or absence of MG-132 (5 mM, Panel A) or Ro-106 (5 or 10 μM, Panel B and C) as indicated. The intensities of the bands were quantified by ImageJ (v 1.31, NIH). The expression levels of IκB were normalized by the expression levels of β-actin and expressed as a percentage of IκB expression at 0 h post-UV light irradiation.
3.4.3 UV-induced NF-κB activation is via an IκBα independent pathway

From amongst the multiple NF-κB subunits it is mainly RelA(p65) that is activated by UVC-irradiation [118, 119]. We previously showed that the 65 kDa NF-κB (p65) is activated in the presence of high level of IκBα in MEF^{A/A} cells upon UV-irradiation [119]. Now we showed that UV-induced IκBα depletion is independent of ubiquitination and proteasomal cleavages. To further determine the pathway(s) that is(are) involved in the UV-induced late-phase NF-κB activation, we analyzed UV-induced translocation of p65 in the nucleus of UV-treated and non-treated MEF^{S/S} and MEF^{A/A} cells. At 24 hours post-UV-irradiation nuclear p65 levels increased 3.3-fold and 3.7-fold in MEF^{S/S} and MEF^{A/A} cells, respectively (Fig. 3.3, Panel A; Table 3.1), which agrees with our previous observation [119]. This result confirms that UV-induced late-phase activation of NF-κB is independent of translational inhibition of IκBα. The UV-induced reduction of IκBα correlates but does not cause NF-κB activation in MEF^{S/S} cells.

To assess whether IKK is involved in UV-induced late-phase activation of NF-κB, we analyzed phosphorylation of p65 at serine 536, which is known to be achieved through the IKK signaling pathway [124, 125]. The cells were UV-irradiated and the phosphorylation of p65 at serine 536 was monitored by immunoblot analysis of nuclear proteins using specific antibody against phosphorylated p65(S536). At 24 hours post-UV-irradiation, the phosphorylation of p65(S536) is not changed (Fig. 3.3, Panel A, Lane 1 vs. 2; Table 3.1) in wild-type MEF^{S/S} cells, while it is dramatically diminished in the mutated MEF^{A/A} cells (Fig. 3.3, Panel A, Lane 3 vs. 4; Table 3.1). The results suggest
that UV-induced late-phase activation of NF-κB is independent of IKK at this level of regulation.

After ruling out the IKK signaling pathway, we determined whether the NF-κB activation occurs via PKA and MSK-mediated signaling. Both PKA and MSK phosphorylate p65 at Ser 276, which is located in the Rel homology domain (RHD) [23]. Specific antibody against phosphorylated p65(S276) was used to probe the phosphorylation of nuclear p65 in MEFSS and MEFAA cells at 24 hours post-UV-irradiation. While not detected in untreated cells, the phosphorylation of p65(S276) was significantly increased in both cell lines after UV-irradiation (Fig. 3.3, Panel A, Lane 1 vs. 2 and 3 vs. 4; Table 3.1). These results indicate that UV-induced late-phase activation of NF-κB is correlated with phosphorylation of p65(S276).

To determine the relationship of p65 phosphorylation and nuclear translocation, we measured the p65 and phospho-p65 in the cytosol. The total amount of p65 and phospho-p65 was not changed in the cytosol after UV-irradiation (Fig. 3.3, Panel B). However, while the phospho-p65(S276) was not detected, the p65(S536) was highly phosphorylated in the cytosol (Fig. 3.3, Panel B). These results suggest that p65(S276) is phosphorylated directly in the nucleus. Since the p65(S276) is phosphorylated by PKA in the cytosol [12, 22, 126] and MSK in the nucleus [127], the results also suggest that UV-induced NF-κB activation occurs via MSK-mediated phosphorylation of p65(S276). To further elucidate whether the phosphorylation pattern of nuclear p65 is unique for UV-irradiation, we measured the phosphorylation of nuclear p65(S536) and p65(S276) in the same cell lines after TNFα treatment. The results demonstrate that TNFα induces
phosphorylation of p65 at both S536 and S276 sites in MEF^{S/S} cells (Fig. 3.3, Panel C, Lane 1 vs. 2), which agrees with previous reports stating that TNFα activates both IKK and PKA/MSK signaling pathways [128]. These results suggest that UV-induced late-phase activation of NF-κB is truly independent of IKK signaling pathway.
Figure 3.3 The localization and phosphorylation of NF-κB at 24 h post-UV-irradiation. The amounts of nuclear (Panel A and C) or cytosolic (Panel B) p65, p65-P(536), p65-P(276) and β-actin in UV-irradiated or TNFα-treated MEF<sub>S/S</sub> and MEF<sub>A/A</sub> cells were determined by western blot analysis. Proteins analyzed in Panel A and B were run on the same gel but the nuclear proteins from panel A required a longer exposure to film which
is representative of the fact that only a small fraction of the total available NF-κB is needed in the nucleus for activation of transcription. The cells were treated or not treated with UV-irradiation (30 J/m², Panel A and B) for 24 hours or TNFα (10 ng/mL, Panel C) for 1 hour.

Table 3.1 Quantitative estimation of p65 and phospho-p65 in the nucleus.

<table>
<thead>
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<th>MEF(^{S/S})</th>
<th>MEF(^{A/A})</th>
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<tr>
<td>UVC(J/m(^2))</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>p65</td>
<td>1.0</td>
<td>3.3±0.8</td>
</tr>
<tr>
<td>p65-P(536)</td>
<td>1.0</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>p65-P(276)</td>
<td>-</td>
<td>++</td>
</tr>
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</table>

Band intensities of the nuclear p65, p65-P(536), p65-P(276) and β-actin (Fig. 3.1, Panel A) were quantified by ImageJ (v 1.31, NIH). The expression levels were normalized by the expression levels of β-actin and expressed as a percentage of their respective expressions at 0 h post-UV light irradiation. (-) Undetected. (++) Detected. Numbers represent the averages and standard deviations of a triplicate experiment.

3.5 Discussion

Activation of NF-κB is a highly regulated multi-level process. NF-κB forms a complex with IκB, which masks NF-κB’s nuclear localization sequence and retains it in the cytoplasm of cells. One well-established mechanism for NF-κB activation is that IKK is activated and phosphorylates IκB at the N-terminal serines (serine 32 and 36 for IκB\(\alpha\)), which leads to the dissociation of IκB from NF-κB and is rapidly degraded through the proteasomal pathway. The free NF-κB then translocates to the nucleus and activates the target genes [99, 129, 130]. Although the most important regulatory step is
the cytoplasmic to nuclear transport [14, 15], the trans-activation potential needed to promote transcription is conferred by additional posttranslational modifications, which mainly consist of serine phosphorylations in the trans-activation domain and RHD [131].

The UV-induced activation of NF-κB occurs through two pathways: a membrane dependent pathway in the early-phase, and a nuclear DNA damage dependent pathway in the late-phase of activation [113]. We previously provided evidence that UV does not reduce the half-life of IκBα within 12 hours post-irradiation; instead, UV inhibits nascent protein synthesis, which leads to a reduction in the net amount of IκBα due to the short half-life of IκBα [113]. We also demonstrated that expression of a non-phosphorylatable mutant of eIF2α (S51A) protects the UV-induced reduction of IκBα, which indicated that the reduction of IκBα is translationally regulated. As a result, we concluded that for the early period, UV light induction of NF-κB is owed to translational inhibition of IκBα synthesis through eIF2α Ser-51 phosphorylation. However, due to the coexistence of high IκBα levels and still activated NF-κB in MEF<sup>A/A</sup> cells at 24 h post irradiation, the results were inconclusive for the late period activation. To further determine the mechanism for UV-induced late-phase activation of NF-κB, we first determine transcriptional and translational regulation of IκBα expression upon UV-irradiation. Our results showed that mRNA levels of IκBα increased 5-fold in MEF<sup>A/A</sup> cells (Fig. 3.1, Panel A), while remained the same in MEF<sup>S/S</sup> cells. This is the first time to demonstrate that phosphorylation of eIF2α prevents transcriptional activation of IκBα expression in the late-phase of UV-irradiation. While the transcripts of IκBα were increased, the total amount of IκBα protein is unchanged in MEF<sup>A/A</sup> cells. This is probably due to the fact
that translation efficiency is partially reduced in the UV-irradiated MEF\textsuperscript{A/A} cells at 24 hours post-irradiation as we previously reported [72, 119]. Indeed, the amount of newly synthesized I\(\kappa\)B\(\alpha\) protein remained the same in MEF\textsuperscript{A/A} cells after UV-irradiation [119]. These results demonstrated that I\(\kappa\)B synthesis after UV-irradiation is regulated at both transcriptional and translational levels.

After analyzing the synthesis of I\(\kappa\)B\(\alpha\), we determined the role of ubiquitination and proteasomal degradation for maintaining the level of I\(\kappa\)B\(\alpha\) upon UV-irradiation. Our results demonstrated that neither ubiquitination nor proteasomal degradation have detectable attributions to the late-phase UV-induced I\(\kappa\)B\(\alpha\) depletion (Fig. 3.2, Panels A and B). To further determine whether UV activates IKK at 24 hours post-irradiation, we measured p65 phosphorylation at serine 536, which is a direct target for activated IKK [23, 26]. Our results indicated that UV did not induce phosphorylation at this level in MEF\textsuperscript{S/S} cells (Fig. 3.3, Panel A, Lane 1 vs. 2; Table 3.1). Since the downstream effects of IKK are undetected at both the proteasomal degradation pathway or at the NF-\(\kappa\)B Ser 536 phosphorylation, we concluded that IKK activity is not significant during the late-phase of UV-irradiation. Interestingly, the phosphorylation of p65(S536) was dramatically diminished in the mutated MEF\textsuperscript{A/A} cells upon UV-irradiation (Fig. 3.3, Panel A, Lane 3 vs. 4; Table 3.1). To verify whether the impact of translational regulation on p65(S536) phosphorylation is unique for UV-irradiation, we analyzed TNF\(\alpha\)-induced p65(S536) phosphorylation in the same cells. Our results showed that p65(S536) phosphorylation was induced by TNF\(\alpha\) in MEF\textsuperscript{S/S} cells, but reduced in MEF\textsuperscript{A/A} cells. These results demonstrate for the first time that translation regulates the
phosphorylation of p65(S536). Another common post-translation modification site on p65 is at serine 276, which is phosphorylated by PKA in the cytosol [12, 22, 126] and MSK in the nucleus [127]. The phosphorylation of p65(S276) was dramatically increased in the nucleus in both cell lines after UV-irradiation (Fig. 3.3, Panel A, Lane 1 vs. 2 and 3 vs. 4; Table 3.1), while it was not significantly changed in cytosol (Fig. 3.3, Panel B, Lane 1 vs. 2 and 3 vs. 4). Since IκBα is required to be degraded for PKA activation [23] and we detected no p65(S276) phosphorylation in the cytosol, our results suggest that UV-induced late-phase activation of NF-κB could be mediated by the MSK signaling pathway.

In summary, IκBα depletion during the late-phase NF-κB activation by UV-irradiation is the combined result of regulation at both transcriptional and translational levels. Neither ubiquitination nor proteasomal degradation have detectable attributions to the IκBα depletion. The UV-induced late-phase activation of NF-κB is not a result of lower IκBα levels, but this depletion correlates with the pattern of inducing a most probably MSK-mediated p65(S276) phosphorylation. Based on our results, we proposed a novel mechanism for UV-induced late-phase activation of NF-κB (Fig. 3.4).
Figure 3.4 Proposed model for UV light-induced late-phase activation of NF-κB.
CHAPTER 4: THE ROLE OF TRANSLATIONAL REGULATION IN ULTRAVIOLET LIGHT-INDUCED CYCLOOXYGENASE-2 EXPRESSION

4.1 Summary

Ultraviolet light (UV) induces a prolonged expression of COX-2. While transcriptional regulation of COX-2 expression is intensively studied, the role of translational regulation of COX-2 synthesis upon UV-irradiation is not yet clear. Here, we show that the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) plays an important role in the regulation of COX-2 expression after UV-irradiation. Our data shows that UV light induces COX-2 expression in wild-type mouse embryo fibroblasts (MEFSS) and that the inducibility is reduced in MEFAA cells in which the phosphorylation site, Ser-51 in the eIF2α, is replaced with a nonphosphorylatable Ala (S51A). UV light-induced transcription of COX-2 is delayed in MEFAA cells, which correlates with NF-κB activation as we previously reported (Wu et. al, J. Biol. Chem., 2004). We also prove that translational efficiency of COX-2 is higher in MEFAA cells than in MEFSS cells, but not at the late stage of UV-irradiation. This may be due to the translational regulation of COX-2 binding protein TIAR expression, which is reduced in MEFSS cells but not in MEFAA cells at 24 hours post-UV. In addition, our data indicates that newly synthesized COX-2 protein is more stable in MEFAA cells than in MEFSS cells. These results suggest that translation initiation plays a role in a complex and

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dynamic regulation of COX-2 expression. Based on our results and the use of Ingenuity Pathway Analysis™, we propose a novel eIF2α phosphorylation-centered network for the regulation of COX-2 expression after UV-irradiation.

4.2 Introduction

Cyclooxygenase enzymes (COX) catalyze the rate-limiting step in the production of prostaglandins (PG) from arachioic acid (Fig. 4.1). COX-1 and COX-2, the two isoforms of cyclo-oxygenase, perform different cellular actions. COX-1 is a constitutively active housekeeping enzyme expressed at low levels in most tissues and acts as a housekeeping regulator of gastric and renal homeostasis [132]. COX-2 is the inducible isoform activated, amongst others, by inflammatory cytokines, oncogenes, growth factors and UV radiation [133].

COX-2 expression is intricately regulated through multiple signaling pathways. Several transcription factor binding sites (CREB, C/EBP, TCF4, NFIL6, AP2, SP1 and NF-κB) in the COX-2 promoter region have been identified [134]. The most well studied activation mechanism of UV-induced COX-2 transcriptional activation is the p38MAPK, which activates the CREB/ATF1 pathway [135]. Our previous report indicated that UV induces NF-κB activation via ER-stress signaling pathway [42, 72], which is also known to activate COX-2 [136]. However the role of NF-κB in the UV-induced transcriptional activation of COX-2 is still controversial [137].

Besides transcriptional regulation, COX-2 expression is also translationally regulated by the binding of a host of RNA-binding proteins to the AU-rich element
(ARE) in the 3’-untranslated region (3’-UTR) of the COX-2 mRNA [138-140]. These proteins control COX-2 expression through influencing its mRNA stability, and activate or inhibit translation of the bound mRNA molecule [141],[142]. Our and other’s previous reports also indicated that the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) upon UV activates NF-κB through translation inhibition of IκB alpha synthesis [39, 42, 143]. In this report, we show a mechanism of translational regulation of UV-induced COX-2 expression both at global levels and that of RNA binding proteins’ as well.

![Figure 4.1 Role of COX enzymes.](image)

4.3 Materials and methods

Cell culture - Wild type mouse embryo fibroblasts (MEF^{S/S}) and mutated ones (MEF^{A/A}), in which Ser 51 on the alpha subunit of the eukaryotic initiation factor (eIF2α) is mutated to a non-phosphorylatable Ala, were grown in 10% FBS enriched DMEM
media (Cellgro) containing MEM essential and non-essential amino acids (Invitrogen). The cells were incubated at 37°C.

_UV Light irradiation_ - UVC light was generated from a 15W ultraviolet C light source (UVP). The intensity of ultraviolet C light was standardized by using a UV light meter (UVP) set at 3 W/m². The media was withdrawn during irradiation.

_Protein extraction_ - The irradiated cells were harvested at the indicated time-points post-irradiation using Nonidet P-40 lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl, 0.1% SDS) for total protein extraction. Cytoplasmic and nuclear proteins were extracted using NucBuster Protein Extraction Kit from Novagen (EMD Biosciences). Both extraction reagents were supplemented with protease inhibitors of Cocktail Set 3 from Calbiochem (EMD Biosciences). Protein concentrations were measured with a Bio-Rad protein DC assay kit (Bio-Rad).

_Western analysis_ - Equal amounts of protein samples were subjected to SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were probed with rabbit polyclonal antibodies anti-COX-2 (sc-1747R, Santa Cruz), anti-COX-1 (sc-7950, Santa Cruz), goat polyclonal anti-TIAR (sc-1749, Santa Cruz), anti-hnRNP C1/C2 (sc-10037, Santa Cruz), mouse monoclonal anti HuR (sc-5261; Santa Cruz) and anti-β-actin antibodies (Sigma). After extensively washing with Tris-buffered saline plus Tween 20 (TBS-T), the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins were detected by using a SuperSignal™ chemiluminescent kit (Pierce).
**Total protein synthesis assay.** The UV light-irradiated MEFs were pulse-labeled with \[^{35}\text{S}]\text{-Met/Cys} as described above. The \[^{35}\text{S}]-incorporation was analyzed by SDS-PAGE by loading equal amounts of cell lysate. The gel was stained with Coomassie Blue R-250 for total protein detection and treated with En3Hance (PerkinElmer Life Sciences). The gel was then dried for autoradiography.

**Assay for COX-2 synthesis and turnover.** Cells were UV light-irradiated (30 J/m\(^2\)). At the indicated time points after irradiation, the cells were incubated with methionine/cysteine-free minimal essential medium (Cellgro) for 15 min and then pulse-labeled with Redivue pro mix \[^{35}\text{S}]\text{-Met/Cys} (100 \mu\text{Ci/mL}) (Amersham Biosciences) for 30 minutes in Met/Cys-free minimal essential medium (Cellgro). After washing with phosphate buffer saline (PBS), the cells were harvested or continuously incubated in fresh complete medium for 2 hours before harvesting. The cell extracts were prepared in radioimmunoprecipitation (RIPA) buffer (Tris-HCl: 50 mM, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA). The protein concentration was measured with a Bio-Rad protein DC assay kit. COX-2 was then immunoprecipitated from equal amounts of proteins using anti-COX-2 antibody (sc-1747R, Santa Cruz) and protein A-agarose (Vector). The immunoprecipitates were subjected to SDS-PAGE. The gel was stained with 0.2% Coomasie Blue R-250, treated with En3Hance (PerkinElmer Life Sciences) and vacuum dried. The amounts of \[^{35}\text{S}]-labeled COX-2 were then analyzed by autoradiography.

**Assay for COX-2 stability.** Cells were UV light-irradiated (30 J/m\(^2\)). At the indicated time points after irradiation, the cells were harvested or treated with
cyclohexamide (100 μg/mL) for one hour before harvesting. Total amount of COX-2 was determined by western blot analysis.

Quantitative real-time PCR. Total RNA was extracted from UVC-treated cells by RNeasy Mini Kit (Qiagen). Complementary DNA was prepared from 1 μg of RNase free DNase treated RNA, using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR reactions were prepared with iQ SYBR Green Super Mix (Bio-Rad) and 0.2 μM concentration of the following primers:

COX-2: 5’: GCTGTACAAGCAGTGGCAAA; 3’: CCCCAAAGATAGCATCTGGA.
β-actin: 5’: TATGGAATCCTGTGGCATCC; 3’: GTACTTGCCTCAGGAGGAG.

The reaction was performed on iCycler (Bio-Rad) starting with incubation at 95°C for 2.5 minutes followed by 40 cycles of 60 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°. Data was analyzed with the comparative delta Ct method. Relative amounts of COX-2 mRNA were normalized to the levels of β-actin mRNA in each sample.

Computational analysis of the COX-2 activation pathway – The COX-2 regulatory pathways were identified based on previously published research from within the Ingenuity Pathways Analysis library of Ingenuity Pathways Analysis 6 (Ingenuity® Systems) (Appendix A).

4.4 Results

4.4.1 Inducibility of COX-2 upon UV is eIF2α phosphorylation dependent.

COX-2 expression is induced by NF-κB activation under various stimuli [144]. Since NF-κB activation upon UV-irradiation is regulated by translational inhibition of
IκB synthesis, we have assessed the role of translation on the UV-induced COX-2 expression. UV-irradiation inhibits translation through activation of PERK and GCN2, which phosphorylate eIF2α [39, 42]. We first examined whether eIF2α phosphorylation plays a role in UV-induced expression of COX-2. MEF cells with wild-type eIF2α (MEF<sup>S/S</sup>) or with a Ser-51→Ala mutation at the phosphorylation site in eIF2α (MEF<sup>A/A</sup>) were used in the experiments [42, 72]. Western blot analysis demonstrated that COX-2 expression was increased 1.5 to 2.1-fold from 4 to 12 hours (Fig. 4.2, Panel A, Lanes 2-4 vs. 1; Panel B) in MEF<sup>S/S</sup> cells, whereas COX-2 increased only 0.4 to 0.8-fold in the same time period in MEF<sup>A/A</sup> cells after UV-irradiation (Fig. 4.2, Panel A, Lanes 6-9; Panel B). The COX-2 levels were reduced in both cell lines at 24 hours post-UV (Fig. 4.2, Panel A, Lanes 5 and 10; Panel B). To determine whether elimination of eIF2α phosphorylation specifically impacts COX-2 expression, we analyzed COX-1 expression in UV-treated MEF<sup>S/S</sup> and MEF<sup>A/A</sup> cells. Our data showed that COX-1 expression levels are the same in both cell lines before and after UV-irradiation (Fig. 4.2). These results suggest that eIF2α phosphorylation may play a dual-role in regulation of COX-2 expression upon UV-irradiation. While translational inhibition leads to the activation of NF-κB and transcriptional activation of COX-2 expression in MEF<sup>S/S</sup> cells after UV-irradiation, maintaining a high level of active eIF2 increases translational efficiency of COX-2 in MEF<sup>A/A</sup> cells with or without UV-irradiation.
Figure 4.2 Effect of translation on UV-induced COX-2 expression. (A) Western blot analysis of COX-2 and COX-1 at the indicated time points post UV-C irradiation (30 J/m²) of MEF<sup>S/S</sup> and MEF<sup>A/A</sup> cells. Equal loading was ensured by β-actin detection. (B) The relative intensities of COX-2 protein bands were quantified by ImageJ (v.1.31, NIH). Results represent the means ± SEM for three independent experiments and levels are expressed relative to the COX-2 levels at 0 h post UV for both cell-lines.
In order to show the global protein synthesis levels for the wild-type and mutated cells during UV irradiation we first determined the total incorporation of $^{35}$S-Met/Cys (Fig. 4.3). As expected the Ser 51 to Ala mutation reverses the down-regulation of protein synthesis induced by UV.

*Figure 4.3* eIF2α Ser51 phosphorylation inhibits global protein translation. MEF$^{+/+}$ and MEF$^{+/−}$ cells were irradiated with 30 J/m² of UV-C then they were pulse-labeled with $[^{35}$S]-Met/Cys at the indicated time points post irradiation. Newly synthesized proteins were detected by autoradiography (shown in left panel), and total amount of proteins were visualized by Coomassie Blue R-250 staining (right panel).
Table 4.1 The translation efficiency and stability of COX-2 after UV-irradiation.

<table>
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<tr>
<th>Cell Lines</th>
<th>MEF^{SS}</th>
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<td>0 4 24</td>
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<td>Translation Efficiency (TE)*</td>
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<td>1 2.31±0.24 0.78±0.28</td>
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<tr>
<td>Stability (Stab) *</td>
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<tr>
<td>Stab/TE **</td>
<td>1 0.24 0.60</td>
<td>1 0.31 1.09</td>
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</table>

* Numbers represent the averages and standard deviations of a duplicate experiment.
** Numbers represent the ratios of the averages of the two experiments.

To elucidate the mechanism of translational regulation of COX-2 expression, we analyzed the kinetics of COX-2 synthesis and degradation using $^{35}$S-Met/Cys metabolic pulse labeling and pulse-chase methods. Our data shows that the COX-2 synthesis and protein stability was higher in MEF^{AA} cells than in MEF^{SS} cells without UV-irradiation (Fig. 4.4, Panels A and B, Lane 4 vs. 1), which explains the higher background expression of COX-2 in MEF^{AA} cells (Fig. 4.2, Lane 6 vs. 1). The COX-2 synthesis in both cell lines was increased at 4 hours (Fig. 4.4, Panels A, Lane 2, 5 vs. 1, 4) and then decrease at 24 hours (Fig. 4.4, Panels A, Lane 3, 6 vs. 1, 4) post-UV-irradiation. The stabilities of newly synthesized COX-2 were similar (Fig. 4.4, B1/A1 vs. B4/A4; Table 4.1) and were decreased at 4 hours in both cell lines after UV-irradiation (Fig. 4.4, B2/A2 vs. B1/A1 and B5/A5 vs. B4/A4; Table 4.1). However, at 24 hours post-UV-irradiation, while the stability of newly synthesized COX-2 was decreased in MEF^{SS} cells (Fig. 4.4,
A3/B3 vs. A1/B1), it was slightly increased in MEF^{A/A} cells (Fig. 4.4, B6/A6 vs. B4/A4; Table 4.1). These results suggest that eIF2α phosphorylation destabilized newly synthesized COX-2 in the late-stage of UV-irradiation.

**Figure 4.4** Translational efficiency and stability of COX-2 after UV-irradiation. (A) Metabolic labeling and IP analysis of newly synthesized COX-2 in UV irradiated MEF^{S/S} and MEF^{A/A} cells. (B) Pulse-chase analysis of COX-2. Cells were metabolically pulse labeled with [\textsuperscript{35}S]-Met/Cys, followed by a 2 h chase period. COX-2 was then immunoprecipitated from equal amounts of proteins and the [\textsuperscript{35}S] labeled COX-2 was detected by autoradiography.
To further determine the contribution of protein synthesis and degradation in regulation of COX-2 level after UV-irradiation, we analyzed the effect of cyclohexamine (CHX), a translation inhibitor, on UV-induced COX-2 expression. Surprisingly, our data shows that CHX did not affect COX-2 levels in MEF<sup>S/S</sup> cells (Fig. 4.5), while it significantly decreased them in MEF<sup>A/A</sup> cells after UV-irradiation (Fig. 4.5, Lanes 11, 12, vs. 8, 9).

**Figure 4.5** Total COX-2 protein levels in UV irradiated (30 J/m<sup>2</sup>) and CHX (100 μg/mL, 1 h) treated MEF<sup>S/S</sup> and MEF<sup>A/A</sup> cells. The maintenance of steady levels of COX-2 in MEF<sup>S/S</sup> cells was not due to the failure of CHX in inhibition of protein synthesis since IκBα levels were reduced in both cell lines in the same samples (data not shown). The results suggest that a combination of variation in protein synthesis and degradation pattern is accountable for the steadily increasing COX-2 levels detected in both cell lines after UV-irradiation.

4.4.2 **Translation inhibition activates COX-2 transcription**

UV-irradiation inhibits protein synthesis through phosphorylation of eIF2α [72]. However our results indicated that newly synthesized COX-2 was increased in both cell
lines at 4 hours post-UV. To determine whether the increased expression of COX-2 is due to transcriptional activation of COX-2 expression, we assayed COX-2 mRNA levels with quantitative real time PCR in the total RNA of UV irradiated MEF\textsuperscript{S/S} and MEF\textsuperscript{A/A} cells. The acquired data shows that transcription levels of COX-2 increased more than 6-fold at 4 hours and 13-fold at 24 hours post-UV in the MEF\textsuperscript{S/S} cells, but showed only a modest increase at 4 hours and a 5-fold increase in MEF\textsuperscript{A/A} cells at 24 hours (Fig. 4.6). The increase of transcription of COX-2 (Fig. 4.6) correlates with the activation of NF-κB in the two cell lines after UV-irradiation \cite{41}. The results suggest that the translational regulation of NF-κB activation plays a role in UV-induced expression of COX-2.

*Figure 4.6 UV-induced eIF2α phosphorylation up-regulates COX-2 transcription. Quantitative real-time PCR of COX-2 mRNA expression at 0, 4 and 24 hours after UV treatment of MEF cells. Relative amounts of COX-2 transcripts from a triplicate experiment were normalized in each sample to the levels of β-actin housekeeping gene and expressed relative to the expression immediately after UV-irradiation.*
4.4.3 The phosphorylation of eIF2α affects the expression and activation of COX-2 mRNA-binding proteins

The UV-induced translational inhibition was 100% and 50% countered in MEF^{AA} cells at 4 and 24 hours post-UV respectively [42]. However, our data indicated that COX-2 protein synthesis was increased (Fig. 4.4, Panel A, Lane 5 vs. 4) while the transcript levels were not significantly changed in the cells (Fig. 4.6) at 4 hours post-UV. In contrast, COX-2 protein synthesis was decreased significantly (Fig. 4.4, Panel A, Lane 6 vs. 4), while transcript levels were increased 5-fold at 24 h (Fig. 4.6). These results suggest that the translation efficiency of COX-2 mRNA is regulated not only by eIF2α phosphorylation after UV-irradiation. To further elucidate the mechanism of translational regulation of COX-2 expression, we analyzed the extent of effect of UV-induced eIF2α phosphorylation on the expression and localization of two COX-2 mRNA-binding proteins, HuR and TIAR. HuR is known to increase and TIAR to reduce translation efficiency of the bound mRNA. Both proteins are predominantly nuclear proteins but exert their roles of binding to the RNA in the cytoplasm [145].

Western blot analysis shows that cytoplasmic levels of HuR/TIAR were increased in both MEF^{SS} and MEF^{AA/A} cells at 4 hours post-UV (Fig. 4.7, Panel A, Lane 2, 5 vs. 1, 4). Since HuR and TIAR have the opposite effects in regulation of COX-2 translation, the result suggests that they likely do not play dominant roles in regulation of COX-2 expression in the early stage of UV-irradiation. Interestingly, at 24 hours post-UV, while cytoplasmic HuR was decreased in both cell lines (Fig. 4.7, Panel A, Lanes 3 and 6),
TIAR was slightly increased above the base level in MEF<sup>S/S</sup> cells (Fig. 4.7, Panel A, Lanes 3 vs. 1), and was significantly increased in MEF<sup>A/A</sup> cells (Fig. 4.7, Panel A, Lanes 6 vs. 4). These results imply that translation of COX-2 could be less efficient in the late stage of UV-irradiation due to a relatively lower HuR/TIAR ratio (Fig. 4.7, Panel A, Lanes 3, 6 vs. 1, 4). The results also suggest that the low efficiency of COX-2 translation (Fig. 4.4 Panel A) in the presence of higher levels of mRNA (Fig. 4.6) at 24 hours post-UV could be a combined effect of HuR/TIAR ratio and eIF2α phosphorylation.

To further analyze the impact of eIF2α phosphorylation on regulation of HuR and TIAR expression and activation, we determined the amounts of the proteins in nucleus. Our data indicated that HuR levels were not reduced in either MEF<sup>S/S</sup> or MEF<sup>A/A</sup> cells after UV-irradiation (Fig. 4.7, Panel B), which suggests that HuR expression was not correlated to eIF2α phosphorylation. In contrast to HuR, TIAR expression was not increased at 4 hours post-UV (Fig. 4.7, Panel B, Lanes 2, 5 vs. 1, 4), while the activity was increased (Fig. 4.7, Panel A, Lane 2, 5 vs. 1, 4). Interestingly, TIAR expression was significantly decreased in MEF<sup>S/S</sup> cells but stayed the same in MEF<sup>A/A</sup> cells at 24 hours post-UV (Fig. 4.7, Panel B, Lane 3, 6 vs. 2, 5), while activity is decreased in MEF<sup>S/S</sup> cells but increase in MEF<sup>A/A</sup> cells (Fig. 4.7, Panel A, Lane 3, 6 vs. 2, 5). These results suggest that while HuR expression was not altered, TIAR expression was impacted by translation inhibition, especially in the late stage of UV-irradiation.
A. Cytosolic:

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B. Nucleic:

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Figure 4.7 Effect of UV on post-transcriptional regulators of Cox-2. Cytoplasmic (Panel A) and nuclear (Panel B) lysates of UV irradiated MEF<sup>S/S</sup> and MEF<sup>A/A</sup> cells were subjected to Western analysis of HuR and TIAR. For detection of correct loading and subcellular contamination, expression levels of β-actin and nuclear marker hnRNP C1/C2 were also monitored.
4.5 Discussion

High expression levels of COX-2 in cancer cells indicate its significance in carcinogenesis [2, 146]. COX-inhibitors, such as non-steroidal anti-inflammatory drugs (NSAID), inhibit tumor formation and metastasis [3, 147]. It was reported that LPS, TNFα, IL-1, IL-6, IFN and UV all could induce COX-2 expression through various, multiple and sometimes overlapping signaling pathways. LPS induces the TRAF6-NF-κB, the ERK-MSK-CREB [148] and the p38MAPK-ERK-C/EBP pathways [149]. IL-1 and IFN-gamma signal through the cAMP-PKA-CREB cascade [150-152], while TNFα-induced COX-2 expression was achieved by activating ERK and NF-κB [153]. UV-irradiation induces COX-2 expression [135, 154, 155]. It was previously suggested that UV induces COX-2 through the p38MAPK activated CREB/ATF1 transcriptionally regulated pathway [135, 137]. However, the COX-2 promoter region also contains the binding sites of various other transcription factors, including C/EBP, TCF4, NFIL6, AP2, SP1 and NF-κB [134, 156]. We previously reported that UV activates NF-κB by inducing phosphorylation of Ser 276 [157], which is the targeting site of p38MAPK activated MSK-1 [158-160]. The role of NF-κB during UV-induced COX-2 expression is also evident because ER stress was suggested to be a COX-2 inducer [136] and UV is a well-known activator of ER stress [72, 161, 162]. There is evidence that p38 MAPK and NF-κB induce COX-2 through different signaling pathways [163]. While NF-κB induces activation only at the transcriptional level, p38MAPK activates COX-2 transcription through CREB/ATF1 and also takes part in the regulation of the COX-2 mRNA stability as well as translation efficiency [144]. Message stability and translational efficiency are
regulated through the employment of RNA-binding proteins that attach to the AU rich element (ARE) of the 3’UTR [138, 164].

Previous studies demonstrate that the phosphorylation of eIF2α plays a role in the early stage of UV-induced NF-κB activation [39, 42]. We now have systematically analyzed the extent of the effect of eIF2α phosphorylation on COX-2 expression upon UV-irradiation. Our results demonstrate that eIF2α phosphorylation does not only regulate the global synthesis of COX-2 but also impacts the regulators and stabilities of COX-2 at both transcription and translation levels. The influence of eIF2α phosphorylation upon COX-2 expression was studied by using MEF\textsuperscript{S/S} and MEF\textsuperscript{A/A} cells. Our data show that the expression of COX-2 was induced much more in MEF\textsuperscript{S/S} cells than in MEF\textsuperscript{A/A} cells after UV-irradiation (Fig. 4.2). The reduced inducibility of COX-2 in MEF\textsuperscript{A/A} cells could be due to the higher level of background expression (Figs. 4.2 and 3.4). While COX-2 protein synthesis rates were more than doubled at 4 hours post-UV in both cell lines (Fig. 4.4, Panel A and Table 4.1), the stabilities of the newly synthesized COX-2 were reduced approximately 70% (Fig. 4.4, Panel B/A; Table 4.1, bottom row). The newly synthesized protein in MEF\textsuperscript{A/A} cells appeared to have a lower impact on the increase of total amount of COX-2, probably due to the background COX-2 levels that were approximately 5 times higher in MEF\textsuperscript{A/A} cells than in MEF\textsuperscript{S/S} cells (Fig. 4.2, Panel A, Lane 6 vs. 1). Indeed, inhibition of protein synthesis by CHX affected more the total amount of COX-2 in MEF\textsuperscript{A/A} cells than in MEF\textsuperscript{S/S} cells (Fig. 4.5), which correlated with the higher synthesis rate of newly synthesized COX-2 in MEF\textsuperscript{A/A} cells (Fig. 4.4, Panel A). Interestingly, inhibition of protein synthesis did not appear to affect the total amount
of COX-2 in MEF^{S/S} cells and it only reduced them to background level in MEF^{A/A} cells (Fig. 4.5). In addition, after inhibiting new protein synthesis, the levels of COX-2 in both cell lines after UV-irradiation were similar to the background level of COX-2 in MEF^{A/A} cells. The results suggest that a steady amount of “matured” COX-2 may be more stable than the newly synthesized one. It will be interesting to further investigate whether COX-2 is stabilized after its association with the cell membrane.

While the protein synthetic rate is expected to be higher in MEF^{A/A} cells at 0 and 4 hours post-UV, it was unexpected that the translation of COX-2 was significantly inhibited in MEF^{A/A} cells at 24 hours post-UV since our previous results demonstrated that translation was protected in these cells due to the non-phosphorylatable mutation of eIF2\(\alpha\) [42]. To further determine the mechanism of UV-induced expression of COX-2, we analyzed mRNA levels of COX-2 using the real time quantitative PCR (RT-qPCR). We found that transcriptional activation of COX-2 is significantly reduced in MEF^{A/A} cells compared to the wild type cells (Fig. 4.6). The transcript levels can be associated with NF-\(\kappa\)B activation patterns in the two cell-lines after UV-irradiation. The high mRNA levels at 24 h post irradiation shown by the RT-PCR in the MEF^{A/A} cells appears to be the result of the action of NF-\(\kappa\)B which we showed to be active during this period despite the inability of UV to block translation [41, 42]. Since the COX-2 transcript levels and translation rates upon UV-irradiation were not correlated to each other, we analyzed the levels of two COX-2 mRNA-binding proteins, HuR and TIAR, in the two cell lines after UV treatment. HuR stimulates COX-2 mRNA translation, while TIAR inhibits it [165]. Our data demonstrate that HuR expression could bypass UV-induced
and eIF2α phosphorylation-mediated translation inhibition (Fig. 4.7), whereas TIAR expression was significantly inhibited at 24 hours post-UV (Fig. 4.7, Panel B, Lane 3). The differential expression of HuR and TIAR altered the ratio of activated HuR/TIAR (Fig. 4.7, Panel A) and impacted the translation efficiency of COX-2 after UV-irradiation (Fig. 4.4, Panel A).

In summary, our results indicate that the UV-induced eIF2α phosphorylation-mediated translation inhibition plays a role in regulation of COX-2 expression via a complex mechanism at both transcriptional and translational levels. Using Ingenuity Pathways Analysis™ (Ingenuity Systems, Inc), we generated a description of a novel mechanism for the regulation of UV-induced expression of COX-2 expression (Fig. 4.8). We propose that activation of eIF2α kinases leads to translational inhibition of IκB synthesis and activation of NF-κB, which in turn induces COX-2 transcription in the early stage of UV-irradiation. eIF2α phosphorylation has a two tier effect. On one hand it reduces the translation efficiency of COX-2 mRNA at both early and late stages of UV-irradiation through its global translational inhibition and on the other hand it reduces the expression of COX-2 mRNA binding protein TIAR, thus promoting the translation of its target in the late stages of UV-irradiation.
Figure 4.8 Proposed model for UV induced COX-2 regulation. The molecular networks were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems).
REFERENCES


APPENDIX A: INGENUITY PATHWAYS ANALYSIS (INGENUITY® SYSTEMS)

Ingenuity Systems provides a powerful tool for proteomic/genomic studies and functional cellular pathway analyses. IPA allows for the detection and characterization of the subject proteins/genes contained in the Ingenuity Pathways Knowledge Base. This knowledge base is developed from a large database of previously published data with the use of Natural Language Processing (NLP) of the full text articles. Networks of these focus proteins/genes can then be algorithmically generated based on their connectivity and overlaid onto a global molecular network.

A graphical representation of the molecular relationships between proteins/genes can be constructed. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as line. All lines are supported by at least one reference from the literature, from a textbook, or from information of canonical pathways stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the protein. Lines are displayed with various labels that describe the nature of the relationship between the nodes (e.g., P for phosphorylation, T for transcription).

Ingenuity headquarters are in Redwood City, California. All the information presented here regarding IPA can be found on the company web site:

www.ingenuity.com
APPENDIX B: PATHWAY STUDIO 6 (ARIADNE GENOMICS®)

Pathway Studio 6 developed by Ariadne Genomics (Rockville, MD) is a similar product to IPA. Its core component MedScan is a text mining tool that extracts data from the abstracts of previously published articles from PubMed and builds a database called ResNet. Although the Natural Language Processing system is automatically used only on abstracts, the user can import data into the database from full text version articles as well. The main advantage of Pathway Studio over IPA consists of the ability to use MedScan independently as a data mining tool directly on articles in various electronic formats. The acquired knowledge is finally represented as graphical representations of molecular networks by the graphical interface component of Pathway Studio.

Ariadne headquarters are located in the Maryland I-270 Technology Corridor. All the information presented here regarding Pathway Studio can be found on the company web site: www.ariadnegenomics.com
CellDesigner is an open source diagram editor developed by the Systems Biology Institute (Tokyo, Japan). It can be used for building drawing based biochemical networks represented as a process diagram, with the possibility of running functional simulations. The software is compatible with Systems Biology Markup Language (SBML), a computer readable language that can represent molecular pathway models (www.celldesigner.org).